*A REACTION ASSOCIATED WITH NONENZYMATIC BINDING IN THE RETICULOCYTE TRANSFER SYSTEM**

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Work with the cell-free reticulocyte system, undertaken to study limiting factors related to the initial formation of an active complex between mRNA, ribosomes, and aminoacyl-sRNA, led to the conclusion that NaF could be used to differentially inhibit the initiation of peptides. NaF was found to inhibit *in vitro* globin biosynthesis by interfering with the initiation of new globin chains on ribosomes under conditions in which the inhibitor had little or no detectable effect on the extension of nascent peptide chains that remain attached to ribosomes during their isolation.[1] Further investigation[2] using the reticulocyte transfer system indicated that NaF inhibited the nonenzymatic formation of a complex between phenylalanyl-sRNA, poly U, and ribosomes (nonenzymatic binding). Resistance to NaF inhibition of the poly U-directed transfer of phenylalanine from phenylalanyl-sRNA into polyphenylalanine could be established by preincubating ribosomes, poly U, and phenylalanyl-sRNA. Preincubation of any two of these components failed to establish resistance. The development of resistance was not dependent on added enzyme or guanosine 5′-triphosphate (GTP), but appeared to parallel nonenzymatic binding in experiments in which time, $MgCl_2$ concentration, and temperature of preincubation were varied. The close parallel between the conditions required for nonenzymatic binding and the development of resistance to inhibition by NaF indicated that NaF might inhibit the initiation of new peptide chains by interfering with the presumably nonenzymatic formation of a complex between aminoacyl-sRNA, mRNA, and ribosomes. In this case, the development of resistance to NaF during preincubation might be equivalent to the formation of such a complex; in other words, to nonenzymatic binding. Although the development of resistance during preincubation was shown to require magnesium ions, maximum resistance to NaF could be obtained at relatively low magnesium concentration (8 mM) that, in itself, resulted in only limited nonenzymatic binding. These results prompted us to examine ways of distinguishing between nonenzymatic binding and a previously unrecognized reaction or function related to chain initiation.

We wish to report that we are now able to distinguish between nonenzymatic binding and the development of resistance to inhibition by NaF, and that the development of resistance is apparently dependent upon a soluble factor and an N-ethylmaleimide-sensitive factor or site present in deoxycholate-washed ribosomes. It is suggested that a reaction associated with the initial formation of a complex between mRNA, ribosomes, aminoacyl-sRNA, and one or more soluble enzymes may be involved. The possible relation between the reticulocyte and bacterial chain-initiation factors[3–6] is briefly discussed.

Materials and Methods.—Preparation of rabbit liver sRNA: Frozen livers, from young rabbits that had been fasted for 36 hr, Type 1, were obtained from Pel-Freeze Biologicals, Inc., Rogers, Arkansas, and stored at −90°C until used. The livers (300 gm) were allowed to thaw slightly in

800 ml of 0.1 *M* Tris, pH 7.5, containing 0.003 *M* $MgCl_2$ and 0.024 *M* KCl, and then were homogenized in a Waring Blendor at 0°. An equal volume of freshly distilled 90% phenol was added to the supernatant solution obtained from centrifugation of the homogenate at 14,600 × *g* for 10 min. The mixture was shaken at room temperature for 1 hr, and then was cooled to 10° and centrifuged at 27,000 × *g* for 10 min. One-tenth vol of 1.0 *M* K acetate, PH 5.0, was added to the aqueous phase obtained from centrifugation, followed by the addition of 2 vol of cold 95% ethanol. The mixture was kept at −10° for 2–6 hr, and the precipitated RNA was recovered by centrifugation at 14,600 × *g* for 20 min. This precipitated RNA was extracted three times at 0° with a total of 30 ml of 1.0 *M* NaCl, each extraction being followed by centrifugation at 27,000 × *g* for 10 min. The material insoluble in 1.0 *M* NaCl, primarily ribosomal RNA, was discarded. Two vol of 95% ethanol were added to the combined 1.0 *M* NaCl extracts and the precipitated sRNA was separated as described above. This sRNA was stripped of amino acids by incubation in 9 ml of 0.5 *M* Tris, pH 8.9, for 45 min at 37°, followed by dialysis for 12 hr against 1 liter of glass-distilled water. The average yield was about 45 mg of soluble RNA that appeared homogeneous in the analytical ultracentrifuge. The sRNA exhibited an average acceptor capacity of about 0.8 μmoles of phenylalanine per gram of RNA under the conditions of charging described previously.[2]

Assay conditions: Unless otherwise stated, each phenylalanine polymerization assay mixture contained the following components in a final volume of 0.5 ml: 0.06 *M* Tris, pH 7.5; 0.07 *M* KCl; 0.008 *M* $MgCl_2$; 0.2 mM GTP; 0.01 *M* reduced glutathione (or, if stated, 0.005 *M* dithiothreitol); 100 μg of poly U; 250 μg of ribosomes; 110 μg of enzyme fraction I + II; and 70 μμmoles of C^{14}-phenylalanyl-sRNA (75–100 μg of sRNA from rabbit liver, 2000 cpm). Incubations were for 5 min at 37°. NaF at a concentration of 0.02 *M* was present where indicated during incubation only.

The components of preincubation mixtures were the same as those for incubation mixtures except that preincubation mixtures did not contain GTP or enzyme fractions I and II and preincubations were carried out in a final volume of 0.25 ml.

The standard assay for nonenzymatic binding was the same as for polymerization except that GTP and enzyme fractions I and II were not included, and the $MgCl_2$ concentration was as indicated in the legends to the figures.

The standard condition for *N*-ethylmaleimide (NEM) treatment of ribosomes was 0.05 *M* NEM, for 10 min at 0°. The ribosome concentration during treatment was 5 mg/ml, unless otherwise stated. After treatment, excess NEM was reacted with reduced glutathione (or dithiothreitol where indicated) at a 2 to 1 molar excess for 5 min at 0°. Other supplies, conditions, and procedures were as previously described.[2]

Results.—Differential loss of the ability of ribosomes to support polymerization and to develop resistance to inhibition by NaF: Initial efforts to demonstrate the involvement of a soluble factor in the development of resistance to inhibition of polymerization by NaF were based on procedures designed to remove a hypothetical factor from reticulocyte ribosomes during successive cycles of suspension and sedimentation by centrifugation. To date, various ribosome-washing procedures of this type have failed to provide evidence of a soluble factor related to the development of

TABLE 1

EFFECT OF NEM TREATMENT OF RIBOSOMES ON THEIR ABILITY TO DEVELOP RESISTANCE TO NaF INHIBITION OF POLYMERIZATION

Conditions	Polymerization (μμmoles phenylalanine) −NaF	+NaF	Per cent resistance
Untreated ribosomes			
No preincubation	45	8	18
Preincubation	44	45	102
NEM-treated ribosomes			
No preincubation	43	3	7
Preincubation	41	12	29

All conditions for NEM treatment, preincubation (8 min), and incubations are as described in the *Materials and Methods* section.

resistance. However, polymerization on ribosomes treated with relatively high concentrations of *N*-ethylmaleimide was found to be sensitive to NaF even after preincubation under conditions that led to nonenzymatic binding. The data presented in Table 1 reflect the difference in the ability of ribosomes treated with 0.05 *M* NEM for ten minutes at 0° and untreated ribosomes to establish resistance to inhibition of polymerization by NaF. Under the conditions used, NaF causes an 80–90 per cent inhibition of polymerization with both NEM-treated and untreated ribosomes. Ribosomes treated with NEM under these conditions retain their ability to support polymerization in the absence of NaF.

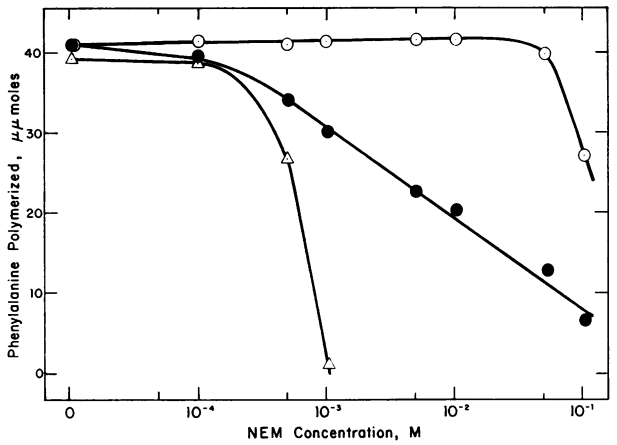

FIG. 1.—The effect of NEM treatment of ribosomes on polymerization, NaF resistance, and enzyme fraction II activity. For polymerization and resistance assays, 250 μg of ribosomes were treated with 0.05 *M* NEM for 10 min at 0° in a volume of 0.05 ml. The inactivation of excess NEM, preincubation (8 min), and incubation were carried out as described in *Materials and Methods*. To measure NaF resistance, NaF was included in the final incubation at 0.02 *M*. For the measurement of fraction II activity, 250 μg of ribosomes and 110 μg of fraction I + II were treated with 0.05 *M* NEM for 10 min at 0° in a volume of 0.15 ml. The inactivation of excess NEM and incubation were carried out as described in *Materials and Methods*, except that no additional enzyme fractions were added to the incubation mixture. ⊙—⊙, Polymerization; ●—●, NaF resistance; △—△, fraction II activity.

Nearly complete resistance to NaF inhibition appears to be established when untreated ribosomes are preincubated with poly U and phenylalanyl-sRNA before the addition of NaF and the other components of the system. The development of resistance with untreated ribosomes is dependent upon magnesium ion concentration, temperature, and time of incubation under conditions leading to nonenzymatic binding, as previously described.[2] In contrast to results with untreated ribosomes, NaF inhibited polymerization by 67 per cent with ribosomes previously treated with NEM.

Loss in the capacity of the ribosomes to establish resistance to NaF depends on an apparent differential inactivation, as indicated by the data presented in Figure 1, which depicts three effects of NEM on the peptide-forming system. An NEM-

sensitive soluble factor required for polymerization (presumably the polymerizing enzyme of Arlinghaus, Shaeffer, and Schweet[7]) is inactivated at relatively low concentrations of NEM, as previously reported.[8, 9] Relatively severe treatment of the ribosomes (high concentration of NEM, higher temperature, or longer time) has an inhibitory effect on their capacity to support enzymatic and nonenzymatic binding, as well as polymerization. Recently, Heintz, McAllister, and Schweet[10] have also reported a loss in the capacity of ribosomes treated with NEM to carry out enzymatic and nonenzymatic binding.

Intermediate concentrations of NEM produce a differential loss in the capacity of ribosomes to develop resistance to NaF inhibition. Incubation of ribosomes with NEM at a concentration of 0.05 *M* for ten minutes at 0° produces approximately 67 per cent inhibition of the ability of the ribosomes to develop resistance to NaF, with little or no effect on their ability to support binding and polymerization.

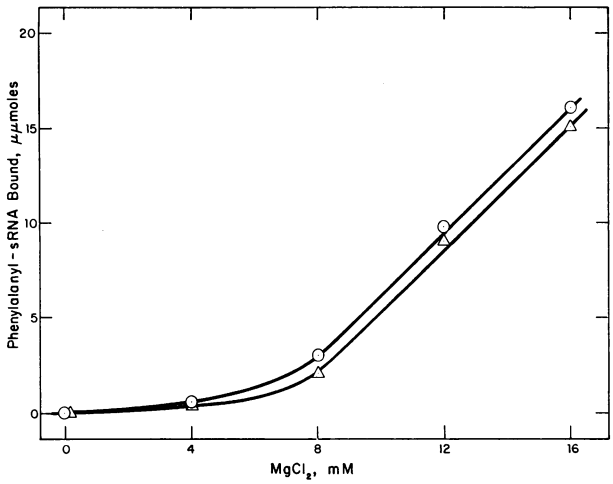

FIG. 2.—The effect of NEM treatment of ribosomes on nonenzymatic binding measured at different magnesium ion concentrations. Ribosomes (10 mg) were treated with 0.05 *M* NEM for 10 min at 0° in 1.0 ml. Untreated ribosomes were allowed to stand under the same conditions without NEM. The unreacted NEM was destroyed with a 2:1 excess of dithiothreitol for 5 min at 0°. One half this amount of dithiothreitol was added to the untreated ribosomes. For each assay, 500 μg of ribosomes were used to measure nonenzymatic binding as described in *Materials and Methods*. ⊙—⊙, Untreated ribosomes; △—△, NEM-treated ribosomes.

Capacity of NEM-treated ribosomes to carry out nonenzymatic binding: Work presented in an earlier communication[2] indicated a close parallel between nonenzymatic binding and the development of resistance to inhibition by NaF. It was concluded that the development of resistance was either dependent upon or identical to the formation of the poly U-directed complex between phenylalanyl-sRNA and washed ribosomes that is referred to as "nonenzymatic binding." The data presented below favor the former interpretation in that nonenzymatic binding can take place in an apparently normal manner on NEM-treated ribosomes that have low capacity to establish resistance to inhibition by NaF. Figure 2 depicts

the relation between nonenzymatic binding and magnesium ion concentration with untreated and NEM-treated ribosomes. The amounts of nonenzymatic binding obtained with varying amounts of untreated and NEM-treated ribosomes incubated with 16 mM magnesium ion have also been found to be very similar.

These data strongly suggest that nonenzymatic binding is not equivalent to the development of resistance in that treated ribosomes retain their capacity to support nonenzymatic binding and polymerization, but have lost much of their capacity to develop resistance. All conditions employed to date for inactivation of ribosomal functions indicate a simultaneous loss in the capacity of the ribosomes to support nonenzymatic binding, enzymatic binding, and polymerization.

Apparent restoration of the ability to develop resistance: A 40–70 per cent saturated ammonium sulfate fraction from the high-speed supernatant obtained after sedimentation of ribosomes from the cell lysate was found to restore complete resistance when preincubated with NEM-treated ribosomes (Table 2). Preparations of either fraction I or II have a generally low but somewhat variable capacity to promote resistance when added at optimal concentration during preincubation. Occasional preparations, particularly of fraction II, show considerably higher activity. An increase in resistance is observed when both fractions I and II are present during preincubation.

Both fractions I and II have previously been shown to contain components required for the synthesis of peptides.[2, 7, 11] Fraction I contains one or more components required for GTP-dependent enzymatic binding, and fraction II contains at least one component deficient in fraction I that is required for the synthesis of peptide bonds. That fractions I and II are each enriched in at least one factor required for polymerization that is deficient in the other and yet that both have low but significant activity in establishing resistance when compared with the 40–70 per cent ammonium sulfate fractions suggest that one or more additional components present in relative abundance in the crude enzyme fraction may be involved in the development of resistance. The involvement of one or more components of the soluble fraction in the development of resistance is shown in another way in the data of Table 3. Conditions have been developed that apparently accomplish differential inactivation of soluble-resistance and polymerization factors during dialysis of the soluble fraction against buffer containing low concentrations of glutathione (GSH). Nearly complete resistance is normally observed after preincubation of untreated ribosomes. However, resistance is not observed if soluble fraction "aged" during dialysis is substituted in the incubation for the regular

TABLE 2

EFFECT OF SOLUBLE FRACTIONS ON NaF RESISTANCE WITH NEM-TREATED RIBOSOMES

Fraction added during preincubation	Polymerization ($\mu\mu$moles phenylalanine) −NaF	+NaF	Per cent resistance
None	39	11	28
AS 40–70	39	41	105
Fraction I	39	14	36
Fraction II	38	18	47
Fraction I and fraction II	37	23	63

NEM-treated ribosomes were preincubated with 0.01 *M* reduced glutathione for 8 min with 400 μg of protein from 40–70% ammonium sulfate fraction, 40 μg of fraction I protein, or 60 μg of fraction II protein, and then incubated with sufficient additional fraction I + II to provide maximum polymerization in the absence of NaF.

TABLE 3

DIFFERENTIAL INACTIVATION OF SOLUBLE RESISTANCE AND POLYMERIZATION FACTORS

Conditions		Polymerization ($\mu\mu$moles phenylalanine)		
Preincubation	Enzyme fraction in final incubation	−NaF	+NaF	Per cent resistance
None	I + II	40	11	26
8 min	I + II	38	39	103
8 min	Aged*	38	8	21

Preincubation was for 8 min. The indicated enzyme fraction was added to the reaction mixtures containing untreated ribosomes, and then polymerization was measured during incubation.
* Aged enzyme was prepared by dialysis of fraction I + II against 100 vol of a buffer solution containing 0.1 *M* Tris, pH 7.5, and 0.001 *M* EDTA for 20 hr at 4°.

soluble fraction that has been stored at −90° in the presence of relatively high concentrations of GSH. The amount of polymerization observed in the absence of NaF with the "aged" soluble fraction is normal, indicating that factors essential for polymerization are not limiting in the system. "Aging" the soluble fraction may affect the sulfhydryl-sensitive factor suggested by the data presented below.

Results from different preparations of "aged" enzyme have proved disturbingly variable and range from those that seem extremely deficient in polymerase activity to those that give complete resistance. A more dependable approach to this problem is clearly needed, preferably through isolation of the factors involved.

Requirement of a sulfhydryl compound for the resistance reaction: It has been possible to carry out nonenzymatic binding, treat the system with NEM under conditions known to inhibit the development of resistance, and then to carry out peptide synthesis during a regular incubation. Experiments of this type have indicated a requirement for sulfhydryl compounds for the development of resistance to inhibition by NaF, as indicated by the data presented in Table 4.

Preincubation of untreated ribosomes without NEM treatment after preincubation produces nearly complete resistance whether dithiothreitol (DTT) is present during the preincubation or not. However, low resistance is observed if DTT is omitted, and the preincubation is followed by treatment with NEM. If DTT is present during preincubation and the preincubation is followed by NEM treatment, nearly complete resistance is observed.

These types of observations, considered together with the other data, lead us to conclude that at least two distinct reactions or stages are involved in the development of resistance. The first is associated with or is equivalent to the formation of the complex formed during nonenzymatic binding. The second stage involves one or more factors present in the soluble fraction and, under the conditions of

TABLE 4

REQUIREMENT FOR A SULFHYDRYL COMPOUND FOR THE DEVELOPMENT OF RESISTANCE

Conditions		Polymerization ($\mu\mu$moles phenylalanine)		
DTT in preincubation	NEM treatment	−NaF	+NaF	Per cent resistance
0	None	41	41	100
0	Yes	40	8	20
Yes	Yes	43	41	95

All preincubations were carried out for 2.5 min with regular ribosomes. Where indicated, DTT was present in the preincubation mixture at a concentration of 5 mM. At the conclusion of the preincubation period, the assay was cooled in ice and NEM was added to give a final concentration of 3.3×10^{-2} *M*. NEM treatment was carried out for 10 min at 0°. Excess NEM was inactivated by the addition of excess DTT and the incubation was continued for an additional 5 min at 0°. The components required for polymerization were added and the mixture was incubated. DTT was present in incubations at a final calculated concentration of 5 mM.

these experiments, is dependent upon DTT. Under conditions in which sulfhydryl compounds are omitted from the preincubation and the system is not treated with NEM after nonenzymatic binding, one or more reactions of the second, sulfhydryl-sensitive stage may occur at a relatively rapid rate during the incubation portion of the experiment.

Discussion.—We interpret the data presented above to reflect a reaction of the reticulocyte transfer system that leads to the stability of the poly U-directed system for polymerization in the presence of NaF, i.e., to NaF resistance. The establishment of resistance to NaF seems to be dependent upon but distinguishable from nonenzymatic binding. It seems to involve ribosomal factors or sites that are sensitive to NEM and at least one soluble factor that is sensitive to "aging." The reaction, as presented above, is dependent upon sulfhydryl compounds; however, it is unclear whether their role is to protect or activate a soluble factor and/or a factor or site present on deoxycholate-washed ribosomes or to enter into the reaction in another way.

It seems clear that the resistance reaction is not one of the previously described steps in the synthesis of peptide bonds in that nonenzymatic binding may be carried out without developing resistance, but that complete NEM-insensitive resistance is developed in the presence of DTT during preincubations not containing GTP. Enzymatic binding and polymerization are highly dependent upon added GTP in this system. Furthermore, the NEM inactivation of the ability of the ribosomes to develop resistance in the absence of added soluble fraction is distinguishable from inactivation of enzymatic binding (relatively insensitive) and polymerization (relatively sensitive). At least one factor with relatively high activity in the crude soluble fraction seems to be involved in the resistance reaction. It is unlikely that the apparent reversal involves a direct reactivation of alkylated derivative formed with NEM, but rather the substitution of an active factor present in the soluble fraction for an NEM-inactivated component. An alternative hypothesis is that inactive ribosomes that are not alkylated by treatment with NEM are activated by soluble factors during preincubation. These activated ribosomes might then exhibit resistance to NaF.

The resistance reaction carried out during preincubation seems to bear a similarity to the situation in the fractionated *E. coli* transfer system described by Nishizuka and Lipmann.[12] These investigators have reported that the initial lag for polymerization was overcome by preincubation of all components of their polymerizing system except GTP. Preincubation of the factors or reactants separately or in partial combination did not overcome the lag for polymerization. The length of the induction period was influenced by the quantity of G factor, but not by the amount of T factor present in the system. What seems to be a similar lag was originally reported for the reticulocyte system by Arlinghaus and Schweet.[13]

It is difficult to evaluate the relation between the development of resistance in the reticulocyte system and reports of factors required for efficient polymerization with natural messenger in systems derived from *E. coli*.[3-6] Both the reticulocyte and bacterial reactions are associated with chain initiation. However, the bacterial factor seems to be active only with native messenger RNA or certain synthetic nucleotides beginning with codons at their 5′-end that are specific for formylmet-sRNA. The factors from *E. coli* are not required with other synthetic messengers

such as poly A.[14] The role, if any, of *N*-acylated amino acids in the initiation of polyphenylalanine peptides in the poly U-directed reticulocyte system and in the initiation of polyphenylalanine peptides in the poly U-directed reticulocyte system and in the development of resistance is obscure.

We suggest that the reaction in the reticulocyte system involves the formation of an active complex capable of peptide formation and resistance to NaF. Further, we suggest that the active complex involves at least one enzyme necessary for the transfer of amino acids from aminoacyl-sRNA into peptide linkage. Preliminary results indicate that a high-molecular-weight component of fraction I having binding and specific GTPase activity may be involved.[15]

Summary.—A reaction of the reticulocyte transfer system leading to resistance to inhibition by NaF and associated with chain initiation is described. The reaction is dependent upon one or more soluble factors in added enzyme fractions and upon sulfhydryl compounds. The reaction is dependent upon but distinguishable from nonenzymatic binding. It is not dependent upon added GTP. It is suggested that the formation of an active complex involving ribosomes, messenger RNA, aminoacyl-sRNA, and one or more soluble transfer enzymes is involved.

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[1] Lin, S. Y., R. D. Mosteller, and B. Hardesty, *J. Mol. Biol.*, **21**, 51 (1966).

[2] Ravel, J. M., R. D. Mosteller, and B. Hardesty, these PROCEEDINGS, **56**, 701 (1966).

[3] Eisenstadt, J. M., and G. Brawerman, *Biochemistry*, **5**, 2777 (1966).

[4] Brawerman, G., and J. M. Eisenstadt, *Biochemistry*, **5**, 2784 (1966).

[5] Stanley, W. M., Jr., M. Salas, A. J. Wahba, and S. Ochoa, these PROCEEDINGS, **56**, 290 (1966).

[6] Revel, M., and F. Gros, *Biochem. Biophys. Res. Commun.*, **25**, 124 (1966).

[7] Arlinghaus, R., J. Shaeffer, and R. Schweet, these PROCEEDINGS, **51**, 1291 (1964).

[8] Bishop, J. O., and R. Schweet, *Biochim. Biophys. Acta*, **54**, 617 (1961).

[9] Mosteller, R. D., J. M. Ravel, and B. Hardesty, *Biochem. Biophys. Res. Commun.*, **24**, 714 (1966).

[10] Heintz, R., H. McAllister, and R. Schweet, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), in press.

[11] Bishop, J. O., and R. Schweet, *Biochim. Biophys. Acta*, **49**, 235 (1961).

[12] Nishizuka, Y., and F. Lipmann, these PROCEEDINGS, **55**, 212 (1966).

[13] Arlinghaus, R., and R. Schweet, *Biochem. Biophys. Res. Commun.*, **9**, 482 (1962).

[14] Salas, M., M. Hille, J. Last, A. Wahba, and S. Ochoa, these PROCEEDINGS, **57**, 387 (1967).

[15] McKeehan, W., W. Culp, S. Lin, and B. Hardesty, manuscript in preparation.