DIFFERENTIAL INACTIVATION OF SOLUBLE RETICULOCYTE TRANSFER FACTORS WITH N-ETHYLMALEIMIDE*

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Sulfhydryl compounds have been found to be important for peptide synthesis in cell-free systems derived from several sources (Simpson, 1962). The need for reduced glutathione (GSH) in the reticulocyte transfer system was demonstrated by Bishop et al. (1960); it was found to protect or activate one or more transfer enzymes (Bishop and Schweet, 1961a). In a subsequent report Arlinghaus et al. (1964) indicated that the enzyme, "peptide synthetase", required for peptide bond formation (related to Fraction II below) was subject to inactivation in the absence of GSH, but that their "binding enzyme" (related to Fraction I below) was not sensitive to the omission of GSH, as measured by the poly U-directed, GTP-dependent enzymatic binding of phenylalanyl-sRNA to ribosomes. Sutter and Moldave (1966) have reported a similar requirement for sulfhydryl compounds in the activation of "transferase II" from rat liver.

In the present investigation, the sulfhydryl alkylating reagent, N-ethylmaleimide (NEM), was found to inactivate one or more of the reticulocyte transfer factors of Fraction II required for the synthesis of polyphenylalanine. NEM was found to have little or no effect on GTP-dependent enzymatic binding under the conditions used. NEM-inactivated Fraction II was not reactivated by excess GSH. It is suggested that NEM may be used to study GTP-dependent enzymatic binding without peptide formation in relatively crude systems of the type described below.

Materials and Methods: N-ethylmaleimide was obtained from the Sigma Chemical Co. Methods and other materials, including rabbit liver phenylalanyl-sRNA, ribosomes and the soluble enzyme fraction, were similar to those previously described (Ravel et al., 1966). The soluble transfer factors were separated into two synergystic fractions required for peptide bond formation by elution

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from calcium phosphate gel using a modified form of the procedure developed by Bishop and Schweet (1961b). Eluate 2 will be referred to below as Fraction II and contains one or more factors related to the "peptide synthetase" reaction (Arlinghaus et al., 1964). Eluate 4 will be referred to below as Fraction I and contains one or more factors related to the "binding enzyme" reaction reported

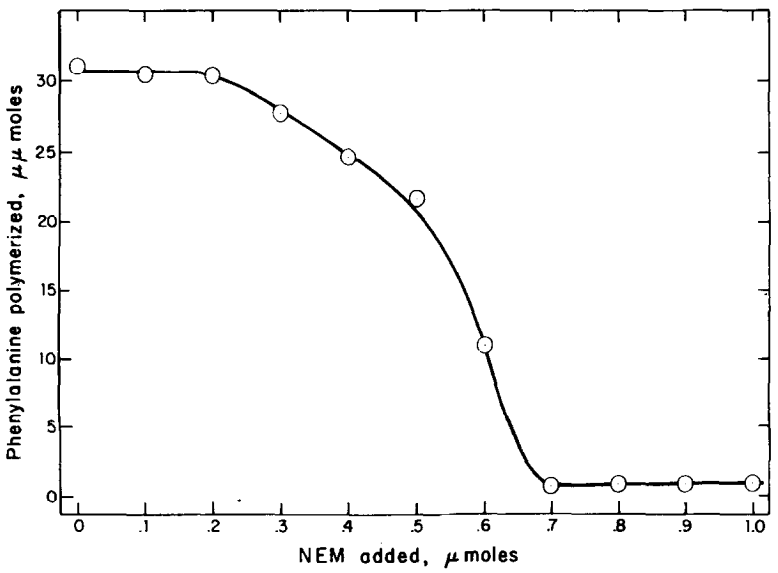

Figure 1: Polymerization of phenylalanine after treatment of enzymes and ribosomes with varying concentrations of NEM. The indicated amounts of NEM were added to 1 mg of ribosomes and 180 μg of protein of Fraction I + II in 0.25 ml of 0.01 M Tris, pH 7.5. After 10 minutes at 0°, the remaining components of the reaction mixture were added to give the following final concentration in a total volume of 0.5 ml: 0.08 M Tris, pH 7.5, 0.07 M KCl, 0.008 M $MgCl_2$, 0.2 mM GTP, 100 μg of poly U, and 210 μg of charged rabbit liver s-RNA containing 70 μμmoles of C^{14}-phenylalanine. The reaction mixture was incubated at 37° for 5 minutes, then the reaction was stopped by the addition of 5% TCA and the amount of labeled phenylalanine polymerized into hot TCA insoluble material was determined as previously described.

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by the workers mentioned above. Eluate 3 contains appreciable amounts of the active components of Fractions I and II and will be referred to as Fraction I + II below. The assay systems used to measure enzymatic binding and hot TCA insoluble polyphenylalanine are as previously described (Ravel et al., 1966). The specific components of the systems used are listed in the legends of the figures and Table I. Deoxycholate-washed, NaF treated ribosomes that have very low capacity to support the polymerization of phenylalanine in the absence of poly U were

used in all experiments reported here. Enzymatic binding was measured by a slightly modified form of the membrane filter technique developed by Nirenberg and Leder (1964). The values given for enzymatic binding include enzymatically bound phenylalanyl-sRNA, any polyphenylalanine bound to the ribosomes, and a small amount of phenylalanyl-sRNA that is non-enzymatically bound at the concentration of $MgCl_2$ used.

Results and Discussion: Preincubation of ribosomes and soluble enzyme fractions with NEM blocks the poly U-directed synthesis of polyphenylalanine, as indicated in Figure 1. Ribosomes and Fraction I + II were preincubated in an ice bath for 10 minutes with the indicated amounts of NEM. The remaining components of the poly U-directed transfer system were then added and the mixture was incubated for 5 minutes at 37°. NEM, in amounts greater than about 0.7 μmole per assay tube, caused nearly complete inhibition of polymerization; however the exact amount of NEM required to block polymerization is dependent on the amount of GSH present in the relatively crude enzyme fractions used. In the experiments described below, inactivation was carried out using 2.0 μmoles of NEM per assay tube.

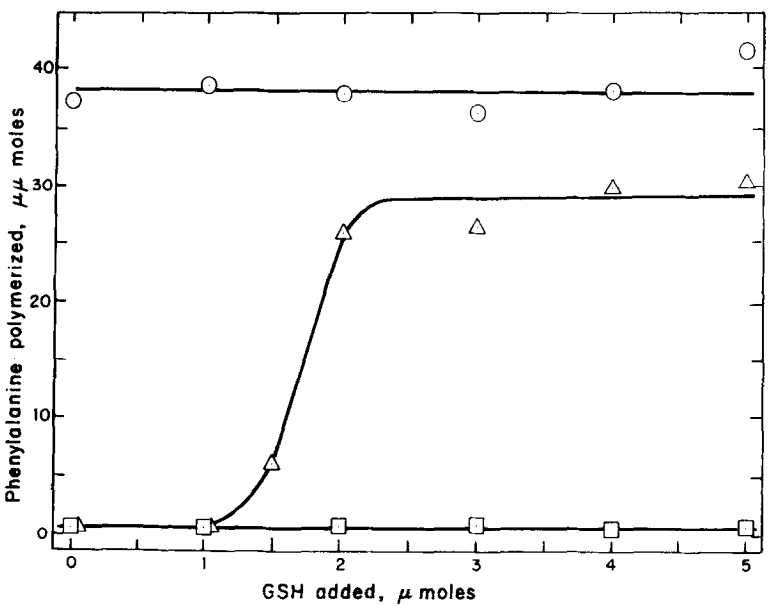

Figure 2: The effects of GSH on the NEM treated and untreated polymerizing system. Ribosomes or ribosomes and Fraction I + II were treated with 2.0 μmoles of NEM as indicated in the legend of Figure 1. After NEM treatment, GSH and then the other components of the system, and Fraction I + II when indicated, were added. Polymerization was then determined as described above.

○—○—○, no NEM added to the system
△—△—△, NEM treated ribosomes and untreated Fraction I + II
□—□—□, NEM treated ribosomes and NEM treated Fraction I + II

NEM-inactivated soluble fraction was not reactivated by GSH under the conditions described here, as shown by the data presented in Figure 2 for the case in which both ribosomes and soluble fraction were treated with NEM. These data are not in agreement with the earlier conclusions of Bishop and Schweet (1961a) who reported that NEM-inactivated enzyme could be reactivated by GSH. We are acquainted with the difficulties of removing one or more components of transfer Fraction II from preparations of ribosomes and have frequently observed an apparent reactivation by GSH in cases in which ribosomes were not treated with NEM. In light of these results and in consideration of the stability of alkylated products generally formed by the reaction of NEM with biological materials, it seems likely that the results reported by Bishop and Schweet may not have been due to reactivation of an NEM-inactivated enzyme, but rather to the protection or activation by GSH of the polymerizing enzymes present in their ribosome preparations. In experiments reported below, we have added 5.0 μmoles of GSH to remove excess NEM and to protect sensitive soluble components.

The amount of polymerization obtained with NEM treated ribosomes and untreated enzyme fraction was approximately 80% of that obtained when neither ribosomes nor enzymes were treated. This result indicated that the capacity of the ribosomes to support the poly U-directed synthesis of polyphenylalanine was not greatly altered by NEM, at least as measured under the conditions used here for the transfer system. This is an interesting and somewhat surprising result, in that reticulocyte ribosomal protein has been found to contain about 1.1% half cystine plus cysteine (Ts'o et al., 1958).

Arlinghaus et al. (1964) reported that GSH was required for the "peptide synthetase reaction" but that the "binding enzyme" could be prepared without GSH and that enzymatic binding was not dependent on this compound. The data presented in Table I support these earlier findings in that one or more components of Fraction II that are required for polymerization are sensitive to inactivation by NEM, but that this reagent does not inactivate the components of Fraction I required for the GTP-dependent enzymatic binding of phenylalanyl-sRNA to ribosomes.

Fraction I contained the soluble components required for poly U-directed, GTP-dependent enzymatic binding of phenylalanyl-sRNA to ribosomes (Arlinghaus et al., 1964; Ravel et al., 1966), but it was contaminated by components of Fraction II required for peptide bond formation so that 12.0 μμmoles of phenylalanine were incorporated into hot TCA insoluble polyphenylalanine when only untreated Fraction I was added to the system as a source of transfer factors. Fraction II used alone as a source of soluble enzyme produces little enzymatically bound phenylalanyl-sRNA or polyphenylalanine, but is synergystic with Fraction

Table I

The Effect of NEM Treatment of Fraction I and Fraction II on Enzymatic Binding and on Polymerization

Fractions Added		μμmoles of Phenylalanine	
NEM treated	Untreated	Bound	Polymerized
-	-	0.9	0.4
-	I	28.1	12.0
-	II	4.4	5.3
-	I and II	36.2	38.9
I	-	13.1	0.2
I*	-	2.4	0.3
II	-	2.0	0.2
I and II	-	10.1	0.4
II	I	18.6	12.4
I	II	24.6	28.3
I*	II*	2.7	0.7

* GTP omitted from the assay

Ribosomes (1 mg) and 40 μg of Fraction I protein and/or 340 μg of Fraction II protein were treated with 2.0 μmoles of NEM where indicated. GSH (5 μmoles) and the other components of the system (Figure 1) including untreated Fraction I or II, as indicated, were then added. Polymerization or binding was measured after a 5-minute incubation at 37°.

I so that 38.9 μμmoles of phenylalanine were polymerized when both fractions were used. NEM treatment of Fraction I reduced its capacity for peptide synthesis to a very low level, as reflected by the decrease in hot TCA insoluble material formed, but ability to carry out GTP-dependent enzymatic binding was retained. Phenylalanine, with no more than trace amounts of diphenylalanine and longer phenylalanine peptides, was recovered and identified from the enzymatically bound product obtained with NEM treated Fraction I. Fraction II had activity for polymerization when it was combined with NEM treated or untreated Fraction I, however the activity of Fraction II for peptide synthesis was destroyed by treatment with NEM. These results are interpreted as indicating a NEM-sensitive soluble component present in Fraction II that is required for peptide synthesis. We have reached a similar conclusion from data obtained using purified preparations from chromatography of Fractions I and II on DEAE-cellulose.

Nishizuka and Lipmann (1966) have found that the GTPase activity as well as polymerization observed in the E. coli transfer system is sensitive to the sulfhydryl reactive compounds, p-chloromercuribenzosulfonate and iodosobenzoate. The GTPase studied by these workers is thought to be related to the requirement for GTP in peptide synthesis when it is properly coupled to the ribosomal system. Preliminary studies with the reticulocyte system indicate that much of the GTPase activity of Fraction I, measured in the absence of ribosomes, phenylalanyl-sRNA and poly U, can be inactivated by NEM but that the enhanced GTPase activity found under conditions that lead to enzymatic binding - in other words, in the presence of ribosomes, poly U and phenylalanyl-sRNA - does not appear to be destroyed by treatment with NEM. The GTP-dependent enzymatic binding observed in the reticulocyte system is thought to be the first enzymatic step in protein synthesis (Schweet and Heintz, 1966).

In work with a relatively crude transfer system derived from E. coli, we find that NEM will inhibit polymerization with little or no effect on non-enzymatic binding. We have been unable to demonstrate GTP-dependent enzymatic binding in the NEM treated E. coli system. Sutter and Moldave (1966) report the activation of rat liver "transferase II" by sulfhydryl compounds, but find that GTP is involved in an interaction between ribosomes and transferase II that does not require transferase I or aminoacyl-sRNA.

These seemingly contradictory reports on the transfer enzymes and the role of GTP in systems from different organisms and laboratories emphasize the need for further investigation of the mechanism of peptide bond formation.

Acknowledgments

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