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THE EFFECT OF THIOL-COMBINING AGENTS ON POLYPEPTIDE SYNTHESIS IN CELL-FREE SYSTEM FROM *ESCHERICHIA COLI*

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Abstract—Among the thiol-combining reagents studied 2,3-dicyano-1,4-dithiaanthraquinone, quajazulene, *p*-bromobenzylisothiocyanate, ethylester of 2-isothiocyano-3-methylbutanoic acid, *N*-trichloromethylthiotetrahydrophthalimide and *N*,*N*-dimethyl-*N*′-phenyl-(*N*′-fluorodichloromethylthio) sulfamide exhibited a marked inhibitory effect on poly(U)-lysyl polyphenylalanine synthesis. These compounds inactivated the S-100 postribosomal supernatant fraction. A distinct inhibitory effect on ribosomes was found with two isothiocyanates, namely, ethylester of 2-isothiocyano-3-methylbutanoic acid and in particular *p*-bromobenzylisothiocyanate. The latter, however, did not influence the ribosomal peptidyl transferase activity measured in the presence of puromycin. All the compounds studied acted as inhibitors of the elongation factor EF-G. Particularly effective in this respect was 2,3-dicyano-1,4-dithiaanthraquinone. Several of the compounds investigated represent new thiol-combining reagents differing by their chemical reactivity with R-SH compounds as well as by other physico-chemical properties.

RIBOSOMES and the factors from the S-100 supernatant fraction required for protein synthesis contain essential SH-groups. Ribosomes treated with sulfhydryl reagents lose their activity.[1,2] The action of highly reactive SH-reagents, such as, *p*-chloromercuribenzoate, *N*-ethylmaleimide as well as 5,5′-dithiobis-(2-nitrobenzoic acid) results in a rapid dissociation of the *E. coli* ribosomes to 30 S and 50 subunits.[3–6] Since such a dissociation may be prevented by the addition of thiols,[2,3] it is assumed that SH-groups are required for an association. Besides these SH-groups one must take into account also other SH-groups the blocking of which leads to a loss of the protein-synthesizing activity of ribosomes. This is borne out by the findings that the action of lower concentrations of SH-reagents results in a loss of ribosomal activity without causing their dissociation.[7] Earlier experiments with puromycin have indicated that the preservation of SH-groups is probably not important for the peptide bond formation.[8]

Among the protein factors that are indispensable for bacterial protein synthesis the initiation factor IF-2 can be inhibited by the sulfhydryl reagent *N*-ethylmaleimide and can be protected against its inhibitory effect by the addition of GTP.[9] The inhibition of the elongation factor EF-G by sulfhydryl reagents has been known for some

Abbreviations used: Poly(A), polyadenylic acid; Poly(U), polyuridylic acid; DTT, dithiothreitol, Phe-tRNA, phenylalanyl-tRNA.

time.[10] Recently it was found that the elongation factors EF-Tu and EF-Ts also contain essential sulfhydryl groups which are sensitive to *N*-ethylmaleimide and some information about the role of these groups in partial reactions of the two named factors has been obtained.[11,12] *N*-Tosyl-L-phenylalanyl-chloromethylketone was found to be an inhibitor of protein synthesis which selectively inhibits EF-T (i.e. the complex EF-Tu and EF-Ts) from *E. coli* and also the similar S_1S_3-factor from *Bacillus stearothermophilus*. This inhibitor did not influence the activity of other components of protein-synthesizing systems (aminoacyl-tRNA synthetases, ribosomes, EF-G and S_2 respectively[13,14]). The effect of *N*-tosyl-L-phenylalanyl-chloromethylketone is due to the interference of the inhibitor with the SH-group(s) of the EF-T factor.[15]

The objective of the present study was to investigate the effect of several new types of thiol-combining reagents on the synthesis of the polypeptide chain in a cell-free system from *E. coli*, and in particular on the activity of ribosomes and on the factor EF-G. The reagents to be studied were selected with a view to their differing chemical reactivity to SH-compounds as well as to the type of reaction. The first group consisted, besides *N*-ethylmaleimide which is known to inhibit some partial reactions of protein synthesis, of 2,3-dicyano-1,4-dithiaanthraquinone and γ,γ-bis-4-ethylphenyl-α,β-dibromoisocrotonic acid. Their reaction with R-SH represents an addition to the double-bond $>C{=}C<$ present in their molecules. This reaction was studied on model R-SH compounds (thioglycolate, cysteine and glutathione), and in experiments with proteins and with Ehrlich's ascitic carcinoma cells was proved to be responsible for the cytotoxic effect.[16,17] Another group of substances studied, which differ by their reactivity as well as lipophilicity, are synthetic aralkylisothiocyanates, such as, *p*-bromobenzylisothiocyanate,γ-isothiocyanobutyric acid and ethylester of 2-isothiocyano-3-methylbutanoic acid. Their reactions with R-SH are of the Ad_N type whereby the addition occurs on the carbon of the electrophilic group-NCS.[18] The significance of reactions of such (multitarget) inhibitors with protein SH-groups was revealed by a systematic study of the relationships between the chemical structure, biological activity and mode of action in a series of natural and synthetic isothiocyanates.[19,20] *N*-Tosyl-L-phenylalanyl-chloromethylketone acts as an alkylating agent and in the present study its reactivity to cysteine is characterized. Finally, in the last two substances studied (*N*-trichloromethylthiotetrahydrophthalimide and *N*,*N*-dimethyl-*N'*-phenyl-(*N'*-fluoro-dichloromethylthio) sulfamide a reaction with R-SH is assumed via the sulfur atom in their molecule, thereby producing the corresponding disulfides and amino compounds. Their reaction with the protein SH-groups was studied on D-glyceraldehyde-3-phosphate: NAD-oxidoreductase and on other enzymes.[21]

MATERIALS AND METHODS

2,3-Dicyano-1,4-dithiaanthraquinone and quajazulene were purchased from Merck A. G., *N*,*N*-dimethyl-*N'*-phenyl-(*N'*-fluoro-dichloromethylthio) sulfamide and *N*-trichloromethylthiotetrahydrophthalimide were obtained from Farbenfabrik Bayer, the former being further purified by recrystallization from hot methanol (the insoluble part was removed by filtration and the filtrate precipitated with water). *N*-Ethylmaleimide was obtained from Koch-Light Chem. Corp. γ,γ-Bis-4-ethylphenyl-α,β-dibromoisocrotonic acid was supplied by the Research Institute of Pharmacy

and Biochemistry, Prague. *N*-Tosyl-L-phenylalanyl-chloromethylketone was purchased from Calbiochem. The isothiocyanates were prepared by thiophosgenation of the corresponding amines and prior to use were purified by vacuum distillation. 5,5-Dithiobis-(2-nitrobenzonic acid) was provided by Aldrich Chem. Corp., U.S.A.

Poly(A), poly(U), GTP and ATP were the products of Calbiochem. DTT was obtained from Koch-Light Chem. Corp. and puromycin from the Nutritional Biochemical Corporation.

^{14}C-Phenylalanine (sp. act. 100 mCi/m-mole) and ^{14}C-lysine (sp. act. 85 mCi/m-mole) were the products of the Institute for Research, Production and Application of Radioisotopes, Prague. γ-^{32}P-GTP (used at the sp. act. 1080–560 mCi/m-mole) was from the Radiochemical Centre, Amersham, England.

All components of the protein synthesizing system were prepared from *E. coli* cells, strain B. The ribosomes, washed with a buffer containing 0·5 M NH_4Cl, were prepared as described elsewhere.[22] The S-100 fraction was prepared by the method described in[23] and the factor EF-G by the method of Nishizuka and Lipmann.[24] The unfractioned tRNA was charged with phenylalanine.[25] ^{14}C-Polylysyl-tRNA was isolated from the ribosomal system in which poly(A)-directed synthesis *de novo* took place.[26]

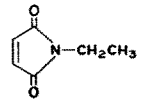
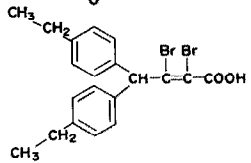
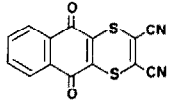
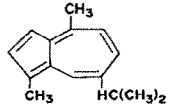
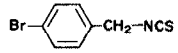
The composition of the protein synthesizing mixtures and a description of the assays of polyphenylalanine synthesis, EF-G-dependent GTP hydrolysis, and the transfer of the peptide residue from polylysyl-tRNA to puromycin as well as the conditions of the action of the inhibitors are stated in context with the pertinent experiment in the "Results".

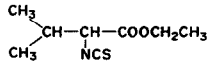
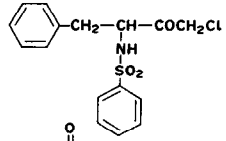
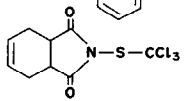
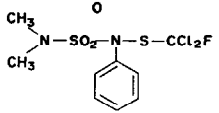
Determination of reactivity to cysteine. In this study we determined the reaction rate constants k (M^{-1} sec^{-1}) for the reaction of γ-isothiocyanobutyric acid, of ethylester of 2-isothiocyano-3-methylbutanoic acid and of *N*-tosyl-L-phenylalanylchloromethylketone with cysteine at 25°C. In the case of isothiocyanates the reaction kinetics were followed spectrophotometrically.[18] In the reaction of *N*-tosyl-L-phenylalanyl-chloromethylketone neither the reactants nor the reaction product exhibited any characteristic absorption of u.v. light (values of molar absorption $\log \epsilon < 4$). For this reason 5,5-dithiobis-(2-nitrobenzonic acid) was used as the agent for observing the reaction kinetics with cysteine. The reaction took place at 25° in a mixture containing 0·2 M Tris, pH 7·2, 0·1 mM to 0·5 mM cysteine and 0·2 mM *N*-tosyl-L-phenylalanylchloromethylketone. At 5 min intervals 1 ml aliquots were taken from the mixture, 0·1 ml of 5,5-dithiobis-(2-nitrobenzonic acid) was added and after incubation for 10 min at 25° 4 ml of methanol were added. the concentration of the 2-nitro-5-mercaptobenzonic acid formed was determined by spectrophotometry at 412 nm. By plotting the logarithm of the concentration of the product against time a dependency was obtained which was used for calculating the reaction rate constant.[15] Due to the low reactivity of *N*-tosyl-L-phenylalanyl-chloromethylketone to cysteine the values of the actual reaction rate constants, determined with the initial concentrations of cysteine 0·22 mM, and 0·5 mM did not differ.

RESULTS AND DISCUSSION

Reactivity to cysteine. The last column in Table 1 presents a survey of the SH-reagents studied, denoting the actual reaction rate constants k (M^{-1} sec^{-1}) for the

Table 1. Effect of inhibitors on the activity of ribosomes and of S-100 fraction in poly(U)-directed polyphenylalanine synthesis in per cent of untreated controls fractions

Compound (0·5 mM)	Formula	Inhibition of poly(Phe) synthesis (%) Pretreatment of S-100 fraction	Pretreatment of ribosomes	Reactivity to cysteine k (M^{-1} sec^{-1})
N-Ethylmaleimide		100	11	$3{\cdot}05 \times 10^4$
γ,γ-Bis-4-ethylphenyl-α,β-dibromisocrotonic acid		7	0	< 1·00
2,3-Dicyano-1,4 dithiaanthraquinone		72	0	3·96
Quajazulene		37	—	1·36
p-Brombenzylisothiocyanate		100	90	$3{\cdot}31 \times 10^2$
γ-Isothiocyanobutyric acid		16	12	3·44

Ethylester of 2-isothiocyano-3-methylbutanoic acid		34	37	2·55
N-Tosyl-L-phenylalanyl-chloromethylketone*		77	0	3·06
N-Trichlormethylthio-tetrahydrophthalimide		49	0	—
N,*N*-Dimethyl-*N'*-phenyl-(*N'*-fluordichlormethylthio) sulfamide		100	12	$2{\cdot}17 \times 10^1$

* The data of the inhibitory effect are taken from Ref. 13.

The protein-synthesizing reaction mixtures contained in 100 μl the following components: 40 mM Tris–HCl pH 7·4, 10 mM Mg acetate, 160 mM NH_4Cl, 1 mM dithiothreitol, 2 mM GTP, 0·4 mM ATP, 6 μg of poly(U), ribosomes (80 μg of protein), 120 μg of the S-100 fraction and ^{14}C-Phe-tRNA bearing 29 pmoles of phenylalanine. Incubations were carried out at 35° for 15 min and the amount of incorporated phenylalanine was calculated from the radioactivity of the material insoluble in hot trichloroacetic acid.[22] The incorporation of phenylalanine in the incomplete systems (i.e. either ribosomes or S-100 fraction omitted) representing a value lower than 1 pmole was subtracted from all values measured. The amount of polyphenylalanine synthesized in a system whose components were not pretreated with the inhibitor (control sample) approximated 12 pmoles. All values represent an average of parallel determinations.

The preincubations with inhibitors were carried out under the following conditions. The ribosomes or the S-100 fraction (both containing 10 mg of protein per ml) were freed of mercaptoethanol by filtration through a column of Sephadex G-25 equilibrated with 40 mM Tris–HCl pH 7·4, 10 mM Mg acetate and 160 mM NH_4Cl. To 200 μl of the filtrated component 10 μl of a 10 mM solution of the inhibitor in dimethylsulfoxide were added (the final concentration of the inhibitor was 0·5 mM) and the mixture incubated for 15 min at 25°. Then 2 μl of 1 M DTT solution (final concentration of DDT was 1 mM) were added and after another 15 min the thus treated components were used in experiments. The components used in the control sample were treated in the same manner, omitting the inhibitor, and contained the same amount of dimethylsulfoxide.

The k values in the reaction of cysteine with the inhibitors were determined by a procedure described in Methods.

reaction with cysteine (ionized form) at 25° under conditions that ensured a pseudo-monomolecular course of the reactions. The determined values of k varied in the individual compounds within the range of 4 orders.

Effect on the synthesis of polyphenylalanine. The procedure adopted to investigate the effect of the reagents on the poly(U)-directed polyphenylalanine synthesis in a cell-free system of *E. coli* was to add the inhibitors to the S-100 fraction as well as to ribosomes. After 15 min an excess of DTT was added and the thus treated components were added to the complete reaction mixture. The results in Table 1 show that all the reagents studied induced an inhibition of protein synthesis. All the substances investigated had an effect on the S-100 fraction, however, to a varying degree.

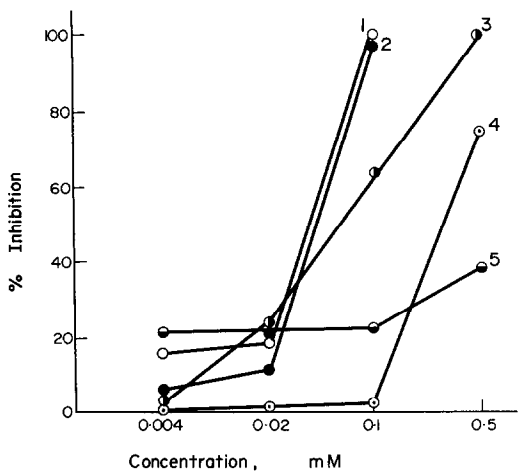


FIG. 1. Effect of inhibitors on the S-100 fraction in poly(U)-directed polyphenylalanine synthesis. 1, *N,N*-dimethyl-*N'*-phenyl-(*N'*-fluoro-dichloromethylthio)/sulfamide; 2, *p*-bromobenzylisothiocyanate; 3, *N*-ethylmaleimide; 4, 2,3-dicyanao-1,4-dithiaanthraquinone; 5, quajazulene. The composition of the reaction mixtures as well as the preincubation of the S-100 fraction with inhibitors was the same as described in Table 1 with the following exception: to 200 μl of the filtered S-100 fraction were added 10 μl of 10 mM, 2 mM, 0·4 mM or 0·08 mM solutions of inhibitors in dimethylsulfoxide, and samples of the S-100 fraction containing the inhibitor were incubated for 30 min at 25°.

Under the conditions employed in the experiment *p*-bromobenzylisothiocyanate, *N,N*-dimethyl-*N'*-phenyl (*N'*-fluorodichloromethylthio) sulfamide and *N*-ethylmaleimide were the most effective and virtually completely inhibited the activity of the S-100 fraction in polyphenylalanine synthesis. The most reactive among the substances studied was *N*-ethylmaleimide which, however, required a higher concentration to attain a full effect than the former two substances (Fig. 1). This fact indicates that besides chemical reactivity other properties, too, assert themselves in the inhibitors (lipophilicity, steric and other factors). It should be borne in mind also that, in the majority of the inhibitors studied, at a concentration of 0·5 mM the limit of their solubility in the corresponding reaction mixtures had been exceeded. This does not apply to γ,γ-bis-4-ethylphenyl-α,β-dibromoisocrotonic acid and to γ-isothiocyanobutyric acid, the latter being easily soluble in water.

Effect on ribosomes. With regard to the effect on ribosomes, only two of the substances exhibited a more marked action. In the case of *p*-bromobenzylisothiocyanate

TABLE 2. PEPTIDYL TRANSFERASE ACTIVITY OF RIBOSOMES TREATED WITH *p*-BROMOBENZYL-ISOTHIOCYANATE (PUROMYCIN REACTION WITH POLYLYSYL-tRNA)

Ribosomes	Omissions	^{14}C-polylysyl–tRNA precipitated (CnCi)	Δ
Control	—	1·38	0·63
	puromycin	2·01	
*p*BBI-treated	—	1·41	0·58
	puromycin	1·99	

The complete reaction mixture contained in 100 μl: 40 mM Tris–HCl pH 7·4, 10 mM Mg acetate, 160 mM NH_4Cl, 10 μg of poly(A), ribosomes (80 μg of protein), 10 μg of polylysyl-tRNA prepared from ^{14}C-lysine (sp. act. 85 mCi/m-mole) and 0·1 mM puromycin. The reaction was carried out at 0° for 20 min and was terminated by adding cold 5% trichloroacetic acid. The resulting precipitates of ^{14}C-polylysyl-tRNA was collected on nitrocellulose filters, washed with cold 5% trichoracetic acid and the radioactivity on the filters was counted with a window-less methane flow counter.

Δ Represent the difference between the radioactivity bound to tRNA after incubation without puromycin and with puromycin (i.e. the radioactivity released by puromycin). The values given are an average of parallel determinations.

Control and *p*-bromobenzylisothiocyanate-treated ribosomes were obtained by a procedure described in Table 1.

an up to 90 per cent inhibition of ribosomes was achieved. The excess of *p*-bromobenzylisothiocyanate reacted quantitatively with DTT which had been added to terminate the pre-incubation of the ribosomes with the inhibitor, and hence the effect found must be attributed solely to the action of the inhibitor on the ribosomes. On the basis of these results it cannot be excluded that the inhibitor induced even a dissociation of ribosomes. None the less the results included in Table 2 illustrates the preservation of activity of ribosomal peptidyl transferase after the action of *p*-bromobenzylisothiocyanatye and demonstrate also that the formation of the functional 30 S ribosome–50 S ribosome–poly(A)–polylysyl–tRNA complex remains preserved. The transfer of the peptidyl residue from the polylysyl–tRNA to puromycin occurred only in the complete above-mentioned complex[27] and was catalyzed by control as well as by *p*-bromobenzylisothiocyanate-treated ribosomes. It can thus be concluded that *p*-bromobenzylisothiocyanate occupies those SH-groups of ribosomes which do not participate in the transfer of the peptide residue but participate in other reactions enabling the growth of the peptide chain.

Effect on factor EF-G. The elongation factor EF-G is one of the components of the S-100 fraction conditioning the growth of the peptide chain. The results in Table 3 demonstrates that not only *N*-ethylmaleimide but also the other substances studied, in direct relation to their concentrations, reduce its activity. An exceptional effect, comparable to the action of much more specific inhibitors of GTP-ase reaction (fusidic acid, thiostrepton) was observed in 2,3-dicyano-1,4-dithiaanthraquinone which already at a concentration of 40 μM induced more than an 80 per cent reduction in the activity of factor EF-G.

In connection with the results mentioned thus far it should be recalled that up to now the possibility to release SH-group from the addition product protein-SH-isothiocyanate by means of DTT and other mercapto compounds has been studied solely in the case of isothiocyanates. These findings clearly indicated that under the given

TABLE 3. EFFECT OF INHIBITORS ON ELONGATION FACTOR EF-G (EF-G-DEPENDENT GTPASE)

Compound	Concn (mM)	Inhibition (%)
N-Ethylmaleimide	0·5	79
	0·1	42
	0·04	0
2,3-Dicyano-1,4-dithiaanthraquinone	0·5	88
	0·1	90
	0·04	83
Quajazulene	0·5	64
	0·1	18
	0·04	4
p-Bromobenzylisothiocyanate	0·5	45
	0·1	37
	0·04	27
N,*N*-dimethyl-*N'*-phenyl-(*N*-fluorodichloromethylthio) sulfamide	0·5	67
	0·1	51
	0·04	42

The reaction mixtures contained in 100 μl: 40 mM Tris-HCl pH 7·4, 10 mM Mg acetate, 160 NH_4Cl, 1 mM dithiothreitol, ribosome (50 μg of protein), 4 μg of EF-G and 120 pmoles of γ-^{32}P-GTP. The reaction was carried out at 0° for 10 min. The amount of radioactive inorganic phosphate hydrolyzed from GTP was measured by the method of Conway and Lipmann.[24] When omitting EF-G from the reaction mixture, from 1·2 to 1·4 pmoles of inorganic phosphate were released and these values were subtracted to obtain the data shown. The amount of inorganic phosphate determined after the reaction with control EF-G (not treated with the inhibitor) was about 12 pmoles. All values represent an average of parallel determinations.

The factor EF-G was treated with the inhibitor in the same manner as is described in Table 1, except that the column of Sephadex G-25, used for removing SH-compounds, was equilibrated with 10 mM Tris-HCl pH 7·4.

reaction conditions (namely pH 7·4) the protein SH-group could not be regenerated. With regard to the GTP-ase reaction our date demonstrate that the inhibitors used modify the factor EF-G in such a manner that it loses its activity in this reaction. However, it is not clear at which stage the inhibited SH-groups are required. Possibly the inhibited factor fails to interact with GTP or with the ribosome but other mechanisms could also be taken into account.

This study also disclosed new types of SH-reagents that have not yet been used in the chemical modification of proteins with essential SH-groups. In view of their different chemical reactivity, the spatial arrangement of the molecules and their lipophilicity these reagents may serve to increase the range of tools for studying the intimate mechanisms of protein synthesis. The fact that the effectiveness of the inhibitors studied is not simply determined either by the type of reaction or the reaction rate with model SH-compounds suggests that the reaction of the inhibitor with the components of the protein synthesizing apparatus is a rather complex process in which the structure of the protein component at the site of interference plays an important part.

In conclusion we wish to mention that the SH-reagents studied in parallel inhibit the incorporation of the labelled leucine into proteins, and of the labelled adenine into nucleic acids in experiments performed with Ehrlich's ascitic carcinoma cells *in vitro*. This inhibition, however, is due to their primary interference with the energy-

providing reactions. As to the antibacterial effect of *p*-bromobenzylisothiocyanate, which is also being applied in practice, sufficient information is already available elucidating its mode of action.[28,29]

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