Studies on the Inhibition of Protein Synthesis by Selenodiglutathione

By LEENDERT N. VERNIE,* JOHN G. COLLARD,† ANDRIES P. M. EKER,‡
ANK DE WILDT,† and INGRID T. WILDERS*

Divisions of *Biophysics and †Cell Biology, Antoni Van Leeuwenhoek-Huis, The Netherlands Cancer Institute, Amsterdam, and ‡Department of Biochemistry and Biophysics, Delft University of Technology, Delft, The Netherlands

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Amino acid incorporation in a cell-free system derived from rat liver has previously been found to be inhibited by GSSeSG (selenodiglutathione). In the present experiments the effect of GSSeSG on protein synthesis in 3T3-f cells, on growth and protein synthesis in *Escherichia coli*, and on amino acid incorporation in a cell-free system derived from *E. coli*, was studied. GSSeSG inhibits the incorporation of [³H]leucine into protein by 3T3-f cells. This inhibition cannot be reversed by removing GSSeSG and is correlated with the uptake of GSSeSG. Sodium selenite (Na₂SeO₃) and oxidized glutathione had no inhibitory effect in this system. [³H]Uridine or [³H]thymidine incorporation into RNA or DNA was not inhibited, indicating that the primary action of GSSeSG was on protein synthesis. GSSeSG did not influence the growth of *E. coli* in a synthetic medium, although enhanced amino acid incorporation was observed. In the cell-free system derived from *E. coli*, amino acid incorporation was not changed by GSSeSG, indicating that elongation factor G, in contrast to elongation factor 2 of mammalian cell systems, is not blocked by GSSeSG.

Previously we have reported that the reaction product, GSSeSG, derived from Na₂SeO₃ and reduced glutathione, GSH, acted as a very potent inhibitor of amino acid incorporation in a cell-free system derived from rat liver (Vernie *et al.*, 1974). This inhibition resulted from the specific inactivation of elongation factor 2. Neither the binding of aminoacyl-tRNA to the ribosomes by elongation factor 1 nor the peptidyltransferase reaction nor the polyribosomes *per se* appear to be affected (Vernie *et al.*, 1975, 1978).

In the present experiments we have investigated whether GSSeSG also inhibits protein synthesis in cell cultures. In addition the effect of GSSeSG was studied on the bacterial system of *Escherichia coli*, of which protein synthesis, among other things, differs from the mammalian system by using elongation factor G instead of elongation factor 2.

Materials and Methods

Preparation of GSSeSG

GSSeSG was prepared by the reaction of GSH with Na₂SeO₃. The products formed were separated over Dowex 50W (X4) by using the method of Ganther (1971). However, the concentrated GSSeSG

solution was not freeze-dried but passed through a column of Sephadex-G10 made up in twice-distilled water as reported previously (Vernie *et al.*, 1974). GSSeSG was stored in twice-distilled water at -20°C. GS[⁷⁵Se]SeSG was prepared from Na₂[⁷⁵Se]-SeO₃. The amounts of GSSeSG used in the experiments were calculated according to a millimolar absorption coefficient of 1.87 at 263 nm (Ganther, 1971).

Tissue culture

Spontaneously transformed 3T3-f fibroblasts (Van Beek *et al.*, 1973) were seeded in plastic Petri dishes (unless indicated otherwise, 0.5 × 10⁶ cells/dish; diam. 5 cm) containing 5 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn-calf serum and antibiotics. The cells were grown for 24 h in a humidified CO₂ incubator at 37°C. Then GSSeSG was added, and after 1 h, if not stated otherwise, either L-[4,5-³H]leucine (0.5 μCi/ml; 53 Ci/mmol), [G-³H]uridine (0.1 μCi/ml; 6.5 Ci/mmol) or [methyl-³H]thymidine (0.5 μCi/ml; 19 Ci/mmol) was mixed with the culture medium. Incorporation was carried out for 30 min, after which incorporation was stopped by removal of the culture medium. The cells were washed three times with phosphate-buffered saline (Dulbecco A; Oxoid, London E.C.4, U.K.; pH 7.2), trypsin-treated (0.02% trypsin in phosphate-buffered saline) and collected

Abbreviations used: GSSeSG, selenodiglutathione; GSSG, oxidized glutathione; GSH, reduced glutathione.

by centrifugation. Cold perchloric acid (5%, w/v), was added to the cell pellets. The cells were stored overnight at -20°C and subsequently prepared for the different radioactivity measurements.

Assay of [^3H]leucine, [^3H]uridine and [^3H]thymidine incorporation by 3T3-f cells

The cell suspensions in 5% perchloric acid were thawed and centrifuged. For the measurement of [^3H]leucine incorporation, the pellet was suspended in 3 ml of 5% perchloric acid, boiled for 10 min, and centrifuged. The pellet was dissolved in 2 ml of 0.5M-NaOH and precipitated with 1 ml of 2.5M-perchloric acid supplemented with 20 mg of Hyflo. The determination of [^3H]uridine or [^3H]thymidine incorporation was carried out at 0°C . The cell pellets were washed twice with 5% perchloric acid and centrifuged. Next, 3 ml of 5% perchloric acid with 20 mg of Hyflo was added. All precipitates were collected on a paper filter [Schleicher and Schüll, Dassel, Germany: 5892 (white band) for protein; 5893 (blue band) for RNA or DNA]. The filters were washed successively with 5% perchloric acid and a mixture of ethanol/ether (1:1, v/v). The precipitate on the filter was dissolved in 1 ml of Soluene-100, scintillation solution was added and radioactivity was assayed in a liquid-scintillation counter.

Uptake of GS[^{75}Se]SeSG

GS[^{75}Se]SeSG was added to the cell cultures, and after the required time the medium was removed, the cells were washed twice with phosphate-buffered saline, trypsin-treated, collected on Whatman no. 1 filters and washed with phosphate-buffered saline. ^{75}Se radioactivity was measured in a gamma-counter.

Growth of E. coli

E. coli B strain LMD 49.5 (obtained from the Department of General and Applied Microbiology, Delft University of Technology) was grown overnight in M9 medium (Wang *et al.*, 1967) on a rotary shaker at 37°C . The culture was diluted 1:20 into fresh M9 medium and inhibitor was added immediately. The turbidity of the cultures was measured at regular time intervals in a Klett-Summerson photoelectric colorimeter equipped with a no. 54 green filter.

Protein synthesis in E. coli

E. coli B strain LMD 49.5 was inoculated 1:20 from an overnight culture into fresh M9 medium and grown on a rotary shaker at 37°C until a turbidity of about 100 units (Klett-Summerson) was reached. Inhibitors were added to 5 ml samples of this culture and incubation was continued for 30 min. Samples of 1 ml were pipetted into sterile tubes, 0.2 μCi of L-[^{14}C]leucine (180 mCi/mmol) was added, and the tubes were incubated in a water bath at 35°C for 30 min.

Incorporation was stopped by adding 1.2 ml of 1% (w/v) casamino acids. Samples of 1 ml were mixed with 1 ml of 0.06M-Tris/HCl, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate. Cells were lysed by keeping the tubes in a boiling-water bath for 2 min, 2.5 ml of 7% (w/v) trichloroacetic acid containing 0.1% Hyflo was added, and the tubes were placed in ice for at least 1 h. The precipitate was filtered on paper discs [Schleicher and Schüll 5892 (white band)] and washed with 3×5 ml of 3.5% (w/v) trichloroacetic acid and 5 ml of ethanol. The precipitate was dissolved in 1 ml of Hyamine hydroxide, scintillation solution was added and radioactivity was assayed in a liquid-scintillation counter.

Amino acid incorporation in a cell-free system from E. coli

A cell-free protein-synthesizing system was derived from *E. coli* strain LMD 67.11 (identical with KA81, obtained from the Department of General and Applied Microbiology, Delft University of Technology). The procedure of Nirenberg (1963) was followed, with the exception that centrifugation of the cell extract was performed at 11000g and the preincubation step was omitted. The 11000g supernatant was passed through a column of Sephadex G-25 (1.2 cm $\times$ 30 cm), made up with 50 mM-Tris/HCl, pH 7.6, 25 mM-KCl and 10 mM-MgCl₂. Part of the eluate from the first peak corresponding to 20 absorption units at 260 nm served as source for the polyribosomes and enzymes. The incubation mixture further contained 0.25 mM-GTP, 5 mM-phosphoenolpyruvate, 25 μg of pyruvate kinase/ml and 56 μg of L-[U- ^{14}C]leucyl-tRNA (0.161 $\mu\text{Ci}/\text{mg}$)/ml together with the other 19 non-radioactive aminoacyl-tRNA species. The substances tested were added as indicated in the Tables. The ionic conditions were adjusted to 50 mM-Tris/HCl, pH 7.6, 70 mM-KCl, 6 mM-MgCl₂ and 2 mM-CaCl₂. Incubation was carried out for 30 min at 37°C in a final volume of 1 ml.

Results

Inhibition of protein synthesis by GSSeSG in 3T3-f cells

The effect of various concentrations of GSSeSG on amino acid incorporation by 3T3-f cells is presented in Fig. 1. At low concentrations of GSSeSG (1–3 $\mu\text{g}/\text{ml}$) almost no effect was observed. At higher concentrations a rapid decrease of the amino acid incorporation occurred, resulting in practically complete inhibition of protein synthesis at 4 μg of GSSeSG/ml. In these experiments the cells were preincubated for 1 h with GSSeSG followed by incubation with [^3H]leucine for 30 min. If the preincubation time with GSSeSG was extended to 6 h, no change in the percentage inhibition was

Fig. 1. Inhibition of [^3H]leucine incorporation into protein in 3T3-f cells as a function of the GSSeSG concentration. 3T3-f cells were grown for 24 h. Then GSSeSG was added and 1 h thereafter [^3H]leucine. Incorporation was stopped 30 min later and the cells were prepared for the radioactivity measurements.

Table 1. Effect of several compounds on [^3H]leucine incorporation into protein by 3T3-f cells

The indicated amounts were added per ml of culture medium. The incorporation in the control experiment, without any addition, was 4567 d.p.m.

Addition	Amount (μg)	Inhibition (%)
GSSeSG	10	98
GSSG	10	0
GSSG	50	0
Na_2SeO_3	10	0
Na_2SeO_3	50	0
GSSeSG + GSSG	10 + 10	97
GSSeSG + GSSG	10 + 50	98
GSSeSG + GSH	10 + 18	0

observed. This indicates that maximum inhibition is obtained within 1 h of preincubation and that the lack of inhibition by lower amounts of GSSeSG (1–3 $\mu\text{g}/\text{ml}$) is not due to insufficient preincubation. The percentage of inhibition remained the same when after 1 h of preincubation GSSeSG was removed by refreshing the culture medium. When assayed 6 or 20 h later, the cells did not show any restoration of protein synthesis, suggesting that the inhibition by GSSeSG under these conditions is an irreversible process.

Inhibitor role of GSSeSG

The following experiments were performed to investigate whether GSSeSG itself is the inhibitor and whether the inhibition of protein synthesis by GSSeSG is comparable with that in the cell-free situation. First Na_2SeO_3 and GSSG, which were not

inhibitory in the cell-free system (Vernie *et al.*, 1974), served as controls. As shown in Table 1, neither GSSG nor Na_2SeO_3 at molar concentrations exceeding that of GSSeSG by about 6 and 20 times, respectively, inhibited amino acid incorporation. When GSSG and GSSeSG were added simultaneously, GSSG had no effect on the inhibitory action of GSSeSG. In the cell-free system derived from rat liver the inhibitory effect of GSSeSG can be abolished by GSH, provided that GSH is added before GSSeSG can react with elongation factor 2 (Vernie *et al.*, 1975); addition of GSH to GSSeSG results in decomposition of GSSeSG to GSSG and elemental selenium via the intermediate GSSeH (Ganther, 1971). As shown in Table 1, GSH mixed with GSSeSG at a molar ratio of 4:1 failed to inhibit protein synthesis of the 3T3-f cells.

The effect of the culture medium on the inhibitory action of GSSeSG was studied next. Because GSSeSG is a relatively unstable component (Ganther, 1968) the possibility exists that during incubation it decomposes to harmless products such as GSSG and Se. Furthermore it may react with some factor in the medium to yield the ultimate inhibitor. To test this possibility GSSeSG was added to cell-free medium and after various periods of incubation, the medium was transferred to the cells. The cells were then incubated for 1 h, followed by further incubation with [^3H]leucine for 30 min. It is obvious from Table 2 that as the period of preincubation of GSSeSG with culture medium was increased, there was a correlative decrease in the percentage of inhibition; after 60 min, no effect was seen. The breakdown of GSSeSG in the culture medium was dependent on the serum concentration of the medium (Table 3). At the critical concentration of 4 μg of GSSeSG/ml and increasing serum concentrations from 5 to 20%, a decrease of inhibition of protein synthesis was recorded, resulting in complete loss of inhibition at 20%. The effect of cell number on the inhibitory action of GSSeSG is also illustrated in Table 3; the higher the cell number the lower the percentage of

Table 2. Inactivation of GSSeSG in the culture medium as measured by the inhibition of [^3H]leucine incorporation

Indicated is the incubation time of GSSeSG in the culture medium before this medium had been transferred to the cells (10 $\mu\text{g}/\text{ml}$ of GSSeSG was used). The incorporation in the control experiment was 4373 d.p.m.

Time (min)	Inhibition (%)
0	99
15	90
30	44
45	13
60	0

Table 3. *Effect of serum concentration or cell number on the inhibition by GSSeSG of [³H]leucine incorporation*

[GSSeSG] ($\mu\text{g/ml}$)	Inhibition (%)									
	Serum (%)	...	5	10	20	Cell no.	...	1×10^6	2×10^6	4×10^6
4			45	23	0			56	22	0
6			97	99	99			92	49	23

Fig. 2. *Uptake of ⁷⁵Se from GS[⁷⁵Se]SeSG by 3T3-f cells (●), and inhibition of [³H]leucine incorporation by GSSeSG (○) as a function of time*

[³H]Leucine incorporation of the cells, for 30 min, was measured after the cells were incubated for the indicated time with GSSeSG (10 $\mu\text{g/ml}$) and GSSeSG had been removed by refreshing the culture medium. The uptake of ⁷⁵Se was determined after the incubation of the cells for the indicated time with GS[⁷⁵Se]SeSG (10 $\mu\text{g/ml}$). The radioactivity of GS[⁷⁵Se]SeSG amounted to 1612 c.p.m. per μg .

inhibition. This corresponds with the observed decrease in inhibition when GSSeSG was added to cells 48 h (instead of 24 h) after their inoculation. With excess of GSSeSG, these effects could not be detected.

Time course of the uptake of, and inhibition of protein synthesis by, GSSeSG

The uptake of GSSeSG by the cells was measured, and its correlation with the extent of inhibition of protein synthesis, was studied. Addition of GS[⁷⁵Se]SeSG resulted in a gradual increase of ⁷⁵Se activity in the cells as shown in Fig. 2.

In a control experiment in which GS[⁷⁵Se]SeSG was preincubated for various times in culture medium followed by addition of this medium to the cells, washing and fixation, no radioactivity was bound to the cells. This excludes non-specific absorption of radioactive material to the cells. Concomitant with the uptake of ⁷⁵Se, the incorporation of [³H]leucine was measured as a function of the incubation time with GSSeSG. As shown in Fig. 2, amino acid incorporation was not affected during the first 10 min of incubation, but after

Table 4. *Effect of GSSeSG on [³H]uridine and [³H]-thymidine incorporation*
GSSeSG (5 $\mu\text{g/ml}$) was added.

Addition	Incorporation (d.p.m.)	
	Control	GSSeSG
Uridine	5214	4114
Thymidine	20539	28378
Leucine	6682	552

15 min, amino acid incorporation had dropped to 17%. Protein synthesis is thus already inhibited before a maximum in the amount of selenium taken up by the cells is reached.

Effect of GSSeSG on RNA and DNA synthesis

In most experiments RNA and DNA synthesis when measured by [³H]uridine and [³H]thymidine incorporation respectively was not inhibited after 1 h incubation with GSSeSG. A typical experiment is shown in Table 4. In some experiments an increase and an incidental decrease in the incorporation of [³H]uridine and [³H]thymidine was observed. This is probably due to pool effects of the used precursors. However, from the experiments the conclusion can be drawn that GSSeSG, independent of RNA and DNA synthesis, primarily acts on the protein-synthesizing system.

Effect of GSSeSG on the growth and protein synthesis of E. coli

The effect of various concentrations of GSSeSG on the growth of *E. coli* in a synthetic medium is illustrated in Fig. 3. Up to a concentration of 50 μg of GSSeSG/ml, exceeding the inhibitory concentration on 3T3-f cells by more than 10 times, no inhibition of the growth of *E. coli* was observed.

The effect of GSSeSG on protein synthesis by *E. coli* was studied by preincubating these cells for 30 min with GSSeSG followed by incubation with [¹⁴C]leucine.

As shown in Table 5, addition of GSSeSG resulted even in a more-than-2-fold increase of amino acid incorporation. This increase, being dependent on the concentration of GSSeSG, was also brought about by another selenium compound, Na₂SeO₃. Chloram-

Fig. 3. Influence of GSSeSG on the growth of *E. coli*. To the *E. coli* B culture, GSSeSG or chloramphenicol was added and the turbidity of the culture was measured at the indicated time. ●, No addition; ○, 10 µg of GSSeSG/ml; □, 50 µg of GSSeSG/ml; ▲, 10 µg of chloramphenicol/ml.

Table 5. Effect of GSSeSG on the incorporation of [14 C]-leucine in *E. coli*

Addition	Concn. (µg/ml)	Expt.	Incorporation (d.p.m.)	
			1	2
None (control)			1113	2248
GSSeSG	10		2093	3231
	50		2742	5226
Chloramphenicol	10		215	488
	50		—	171
Na ₂ SeO ₃	3		—	3955
	10		—	4533

phenicol, an inhibitor of protein synthesis in the bacterial system (Vazquez, 1974), used as a control, caused a strong decrease in both growth and amino acid incorporation.

Effect of GSSeSG on amino acid incorporation in a cell-free system derived from *E. coli*

GSSeSG was also tested in a cell-free amino acid-incorporating system derived from *E. coli*. In Table 6 it is shown that even at the highest concentration of GSSeSG used, which exceeded the inhibitory concentration in the rat liver system by about 10

Table 6. Effect of GSSeSG on leucine incorporation from [14 C]leucyl-tRNA into protein by a cell-free system derived from *E. coli*

Addition	Concn. (µg/ml)	Expt.	Incorporation (d.p.m.)	
			1	2
None (control)			924	1008
GSSeSG	37		933	1006
	74		916	1046
	148		867	1060
	60		—	431
Chloramphenicol	250		—	565
Fucidin	1000		—	327

times, no decrease of the incorporation of amino acids (from aminoacyl-tRNA) into protein was observed. Because GSSeSG was suspected to be an inhibitor of elongation factor G (as it is of elongation factor 2) and hence of translocation, the translocation inhibitor fucidin [the sodium salt of fusidic acid (Vazquez, 1974)], was used as a control. This compound turned out to be inhibitory (Table 6). Since GSSeSG does not inhibit the protein-synthesizing system, it can be concluded that it does not inactivate elongation factor G of *E. coli*, the bacterial counterpart of elongation factor 2.

Discussion

We have demonstrated that in the cell-free system derived from rat liver, GSSeSG blocks amino acid incorporation through an inactivation of elongation factor 2 (Vernie *et al.*, 1975). In the present studies we show that GSSeSG inhibits protein synthesis in 3T3-f cells. This inhibition is not due to a product formed during incubation of GSSeSG in the culture medium; on the contrary, GSSeSG is inactivated after a longer incubation period. When GSSeSG was used at the concentration of critical inhibition, i.e. 4 µg/ml, it was observed that both cell number and serum concentration affect this process. Because thiols like GSH are capable of degrading GSSeSG, these factors are probably involved. It is likely that the serum contained thiols, and it is possible that GSH is excreted by the cells, as has been shown in perfused rat liver (Bartoli & Sies, 1978).

The similarity between the effect of GSSeSG on amino acid incorporation in the cell-free system derived from rat liver and in 3T3-f cells suggests that the same mechanism is involved and that elongation factor 2 is blocked. From the activity of the added GS[75 Se]SeSG, it can be calculated that maximally 31 ng of Se is taken up by approx. 1×10^6 cells. It is probable that not all Se is bound to elongation factor 2 but is also present in elemental form after

degradation of GSSeSG to GSSG and Se, or is to some extent bound to other proteins. The possibility of incorporation of selenium from GSSeSG into protein is considered by Ganther & Corcoran (1969).

The titration-like effect of GSSeSG on protein synthesis in 3T3-f cells, both with regard to time and GSSeSG concentration, suggests that some intracellular factor acts to detoxify GSSeSG. Such a process is found in the reduction of GSSeSG by glutathione reductase (Ganther, 1971; Hsieh & Ganther, 1975) according to the equation:

The sudden decline of protein synthesis caused by GSSeSG as presented in Figs. 1 and 2 can have a more general significance for the toxic effects of selenium compounds in animals. As shown by Ganther (1971), GSSeSG is the first intermediate in the metabolism of selenious acid in animal tissues. Because all forms of selenium are considered to follow a common metabolic pathway (Palmer *et al.*, 1970), it is probable that GSSeSG arises as an intermediate. As long as GSSeSG can be properly reduced to selenides, selenium can be excreted or incorporated into organo-selenium compounds having essential biological functions (Hsieh & Ganther, 1975). If, however, an excess of GSSeSG is formed, inhibition of protein synthesis is likely to follow, and GSSeSG may thus contribute to the toxicity of selenium compounds.

In contrast with the severe inhibition of protein synthesis in 3T3-f cells are the results with GSSeSG in the *E. coli* system. Most important, GSSeSG did not inactivate elongation factor G. The different effects of GSSeSG on the mammalian and bacterial ribosomal translocation factors elongation factor 2 and elongation factor G point to a specific reaction of GSSeSG with elongation factor 2. Previously (Vernie *et al.*, 1975) we made the assumption that a selenium atom is inserted by GSSeSG between two SH groups on elongation factor 2, according to the reaction of the selenotrisulphide of mercaptoethanol with reduced ribonuclease shown by Ganther & Corcoran (1969). A special configuration of two SH groups on the enzyme is obligatory for this reaction. Because elongation factor 2 and elongation factor G considerably differ in the half-cysteine content, 22 residues for elongation factor 2 (Robinson & Maxwell, 1972) and six for elongation factor G (Kaziro *et al.*, 1969), it is possible that GSSeSG cannot react with elongation factor G in an inhibitory manner. Further studies are needed to establish the mechanism of inactivation of elongation factor 2

by GSSeSG. The inhibition of protein synthesis in cells in tissue culture, however, opens a new perspective on counteracting tumour cells with seleno compounds of the general formula RSSeSR. These compounds are easy to synthesize from Na₂SeO₃ and RSH, and R could confer biological specificity, i.e., differential toxicity. The reaction products of cysteine or 2-mercaptoethanol and Na₂SeO₃ have already been shown to be inhibitory on amino acid incorporation *in vitro* (Vernie *et al.*, 1974).

In comparing other inhibitors of translocation with GSSeSG, a striking resemblance between the actions of GSSeSG and diphtheria toxin is noticed. Diphtheria toxin is a specific inhibitor of elongation factor 2 by ADP-ribosylation (Honjo *et al.*, 1971), and just like GSSeSG does not inhibit elongation factor G (Pappenheimer, 1977).

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