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SYNTHESIS OF 4-THIAMETHIONINE AND ITS EFFECT ON ENERGY METABOLISM AND AMINO ACID INCORPORATION INTO PROTEIN OF EHRLICH ASCITES TUMOUR CELLS

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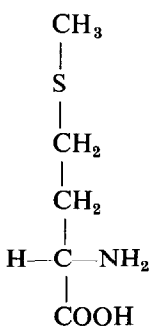
Abstract—The methionine analog, 4-thiamethionine, was synthesized and found to be an inhibitor of incorporation of several amino acids into Ehrlich ascites protein *in vitro*. The inhibition was not prevented by methionine. Glutathione prevented the inhibition but could not bring about a reversal once the block was initiated. Glucose both prevented and reversed the inhibition, thus indicating that 4-thiamethionine interfered with the oxidative energy supply supporting protein synthesis.

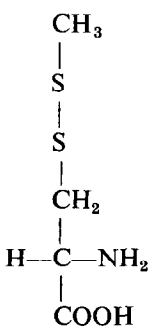
INTRODUCTION

THE possibility of introducing a permanent lesion within the protein synthetic mechanism of cells through the use of amino acids bearing highly reactive functional groups has received attention in various laboratories.[1–3] Since there is inherent in the process of protein synthesis a marked specificity with regard to the structure and configuration of amino acids utilized,[4] such inhibitors should bear a close structural resemblance to those found in protein.

One alteration in the structure of a natural amino acid which has produced an effective antagonist is the substitution of sulfur for the fourth carbon of lysine, to form S(β:aminoethyl) cysteine or 4-thialysine. This analog is a potent antimetabolite of lysine in bone marrow cells[5] and also in the Ehrlich ascites tumor.* Accordingly, we have synthesized the corresponding analog of methionine, 4-thiamethionine, which is the mixed disulfide of methyl mercaptan and cysteine.



Methionine



4-Thiamethionine

* M. Rabinovitz and J. M. Johnson, unpublished observations.

This analog, which bears the reactive disulfide function, might be expected to participate in disulfide interchange[6] with sulfhydryl groups at potential sites specific to methionine at intermediate stages in protein synthesis. A study of the characteristics of its inhibition of protein synthesis, however, indicated that the analog did not function as a methionine antagonist, but reduced the oxidative energy supply available for protein synthesis.

MATERIALS AND METHODS

Synthesis of 4-*thiamethionine*

The compound was prepared by the dismutation between dimethyl disulfide and cysteine, a method previously reported to be applicable for the general synthesis of unsymmetrical disulfides.[7]

Lithium hydroxide monohydrate, 1·85 g (0·045 moles), in 21 ml of oxygen-free water, was slowly added over a period of 20 min to a stirred refluxing solution of L-cysteine hydrochloride monohydrate, 7·0 g (0·04 moles), and dimethyl disulfide, 12 g (0·128 moles), in methanol (50 ml). The solution became turbid upon addition of a few milliliters of alkali and a heavy precipitate had formed by the time all the lithium hydroxide was added. The reaction mixture was cooled and the precipitate was filtered under suction, washed with ethanol and ether and dried. The yield of crude product was 4·8 g. A small sample was dissolved in water and analysed by descending paper chromatography with butanol–water–acetic acid (10 : 5 : 2).* The principal ninhydrin-reactive spot had an R_f of 0·49; L-methionine gave an R_f of 0·48. The compound gave the typical violet coloration with ninhydrin. A cystine contaminant was also recognized, which amounted to 20 per cent of the reaction product.

The crude product was purified by removing the cystine at the pH of its minimum solubility.[8] It was suspended in 200 ml of water and brought into solution by dropwise addition of hydrobromic acid. Cystine was precipitated by adjusting the solution to pH 3 with lithium hydroxide. The pH of the filtrate was raised to pH 6·7, acetone was added and the product was obtained as white microcrystals. It was filtered under suction and washed with ethanol and acetone.

Paper chromatography indicated that the pH 3-precipitate contained almost all of the cystine, together with much of the product. No attempts were made to recover additional material from the pH 3 precipitate. The product, 4-thiamethionine, (1·2 g), contained only a trace level of cystine, as indicated by paper chromatography, and was used in all subsequent studies. It melted with decomposition at 209–210 °C (corr.), in a sealed tube. *Anal.*, Calc. for $C_4H_9O_2NS_2$: C, 28·72; H, 5·42; N, 8·38; S, 38·34. Found: C, 28·78; H, 5·52; N, 8·37; S, 38·09. Although L-cysteine was used as a starting material, some racemization may have occurred under the alkaline conditions of the reaction.

Metabolic studies

Ehrlich ascites cells were obtained from C_3H mice which had been inoculated 1 week previously. The animals were sacrificed by cervical dislocation, and the ascitic fluid was drained through an abdominal incision into beakers placed in ice. The cells were then washed by suspension and sedimentation in ice-cold Krebs–Ringer-phosphate (KRP) buffer containing 0·12 M sodium chloride, $1{\cdot}3 \times 10^{-2}$ M potassium chloride,

* All paper chromatography reported in this article was performed under these conditions.

$1{\cdot}8 \times 10^{-3}$ M calcium chloride, $6{\cdot}5 \times 10^{-4}$ M magnesium sulfate, and 0·01 M sodium phosphate, pH 7·2. The cells were then suspended in two volumes of buffer and filtered through glass wool to remove small clots. The radioactive amino acids used were DL-methionine-2-C^{14} and the uniformly labeled, natural isomers of isoleucine, leucine, lysine, phenylalanine and valine. These were mixed with corresponding non-radioactive material to make solutions of appropriate concentration and specific radioactivity.

The main compartment of Warburg flasks contained radioactive amino acid, a freshly prepared solution of 4-thiamethionine or other additives in buffer in a volume of 1·5 ml; the sidearms contained 0·5 ml of the Ehrlich tumor cell suspension. After temperature equilibration at 37·5 °C for 7 min, the cells were tipped into the main compartment and incubated for various periods. At times alkali and filter paper strips were added to the center well and oxygen consumption was determined with air as the gas phase.

Anaerobic glycolysis was studied with 95% nitrogen, 5% carbon dioxide as the gas phase, in buffer containing sodium chloride, 0·10 M; potassium chloride, $1{\cdot}3 \times 10^{-2}$ M; calcium chloride, $1{\cdot}8 \times 10^{-3}$ M; magnesium sulfate $6{\cdot}5 \times 10^{-4}$ M; glucose $1{\cdot}5 \times 10^{-2}$ M; sodium bicarbonate, 2×10^{-2} M and sodium phosphate 5×10^{-3} M, pH 7·4. When glucose was added as substrate under aerobic conditions, the above buffer with a gas phase of 95% oxygen and 5% carbon dioxide was used.

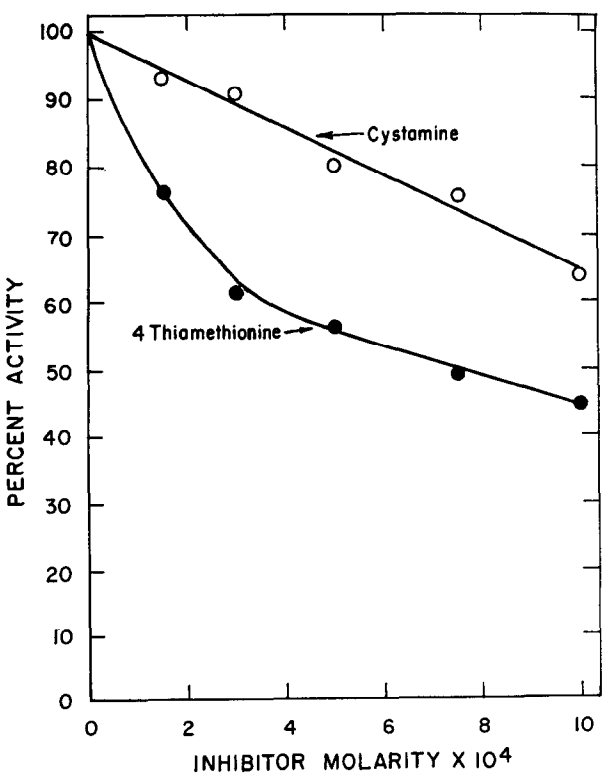


FIG. 1. The inhibition by several concentrations of 4-thiamethionine and cystamine of lysine incorporation into Ehrlich ascites cell protein. The uninhibited control incorporated $1{\cdot}38 \pm 0{\cdot}01$ μmoles lysine per g protein during the 15-min incubation period. The L-lysine concentration was 5×10^{-4}M.

At the end of the incubation period the reaction was terminated by the addition of an equal volume of 20% trichloroacetic acid (TCA). Precipitated material was washed three times with 5% TCA, once with 5% TCA at 90 °C for 15 min, once with 5% TCA at room temperature, once with absolute ethanol, three times with 3 : 1 ethanol : ether mixture at 62 °C for 5 min, and twice with absolute ether. The protein was plated on aluminum discs and counted in a flow-gas counter.

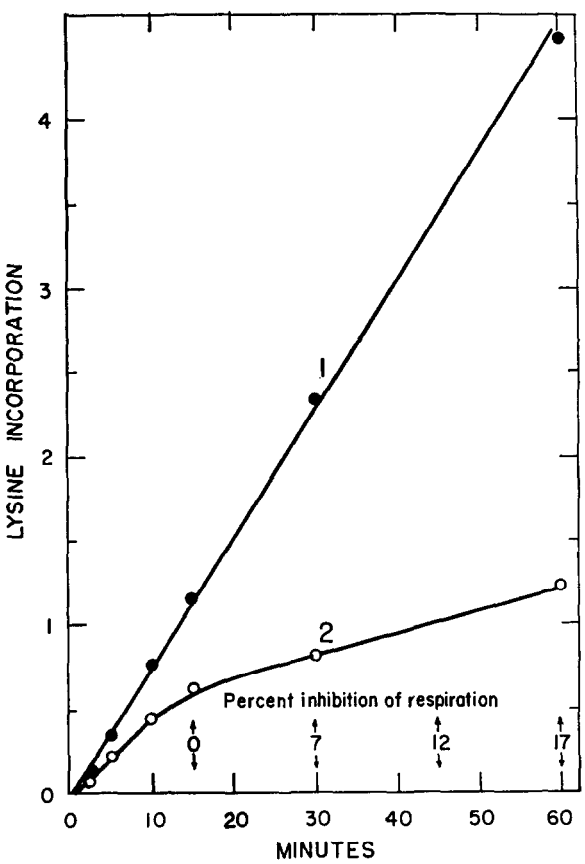


FIG. 2. Progressive increase in inhibition of lysine incorporation by 4-thiamethionine: (1) uninhibited control; (2) inhibition by 5×10^{-4} M 4-thiamethionine. The L-lysine concentration was 5×10^{-4} M. The per cent inhibition of respiration is indicated for four time intervals. Incorporation is expressed as μmoles lysine incorporated per g of protein.

RESULTS

General characteristics of the inhibition

4-Thiamethionine inhibited the incorporation of all the amino acids tested into proteins of the Ehrlich ascites cell. The effect of several concentrations of the inhibitor on lysine incorporation during a 15-min incubation period is shown in Fig. 1. Higher concentrations could produce complete inhibition during this period. The lower concentrations, however, are more effective during longer incubation periods (Fig. 2). This phenomenon is similar to that previously observed with dinitrophenol.[9] The inhibition is not due to an interference in the passage of radioactive amino acid into

the cell, since the incorporation of intracellular isoleucine was inhibited to an equal extent (Table 1).

A strong odor of methyl mercaptan was recognized after incubation of cells with the inhibitor. As shown in Fig. 1, another disulfide, cystamine, was a less effective inhibitor at equivalent concentrations. Dimethyl disulfide was not an inhibitor.

TABLE 1. INHIBITION BY 4-THIAMETHIONINE AND CYSTAMINE OF INCORPORATION OF INTRACELLULAR *iso*LEUCINE INTO PROTEIN OF THE EHRLICH ASCITES CELL

	Increase in protein specific activity after second incubation (Counts/min per mg protein)	Inhibition (per cent)
Uninhibited control	394±1*	—
4-Thiamethionine, 5×10^{-4} M	193±6	51
Cystamine, $1{\cdot}5 \times 10^{-3}$ M	210±1	47

The cells were isolated from ascitic fluid and washed once with KRP buffer. Two milliliters of cells were then supplied with an intracellular pool of radioactive isoleucine by incubating them for 2 min with 4·8 μmoles L-*iso*leucine-U-C^{14}, assaying $1{\cdot}15 \times 10^8$ counts/min in a total volume of 8·4 ml. The cells and medium were cooled to 0 °C in ice, the medium was removed by centrifugation and the cells washed twice with cold KRP buffer to remove all remaining extracellular *iso*leucine-U-C^{14}. They were then resuspended in buffer for a second incubation under standard conditions.

* Deviation from the mean of duplicate incubations.

Prevention of the inhibition

The inhibition of methionine incorporation by 4-thiamethionine was non-competitive at several inhibitor concentrations. This is illustrated for two inhibitor concentrations in Fig. 3. Also, as shown in Table 2, methionine did not prevent the inhibition of incorporation of other amino acids.

Reduced glutatione prevented the action of 4-thiamethionine (Table 3) by reacting with the inhibitor,* but it could not effect a reversal after the inhibition had been initiated.

Inhibition in relation to energy source

Higher concentrations of 4-thiamethionine caused a progressively increasing inhibition of respiration of the Ehrlich ascites cells. However, at a concentration of 5×10^{-4} M, its effect on oxygen uptake became apparent only after inhibition of protein synthesis was complete (Fig. 2).

Under conditions of anaerobic glycolysis, 4-thiamethionine had comparatively little effect on lysine incorporation (Fig. 4) in a concentration at which it significantly inhibited incorporation supported by respiration. The analog stimulated glycolysis by 20 per cent when present in the medium at this concentration (5×10^{-4} M). A similar stimulation of anaerobic glycolysis by dinitrocresol[10] and dinitrophenol[11] has been reported. Furthermore, the inhibitor was ineffective aerobically in the presence of glucose (Table 4), which can also reactivate the completely inhibited system (Fig. 5). Under similar conditions no reactivation could be achieved by the addition of reduced glutathione, 0·01 M, or cysteamine, 0·005 M.

* This reaction can be followed by paper chromatography of the 4-thiamethionine–glutathione mixture.

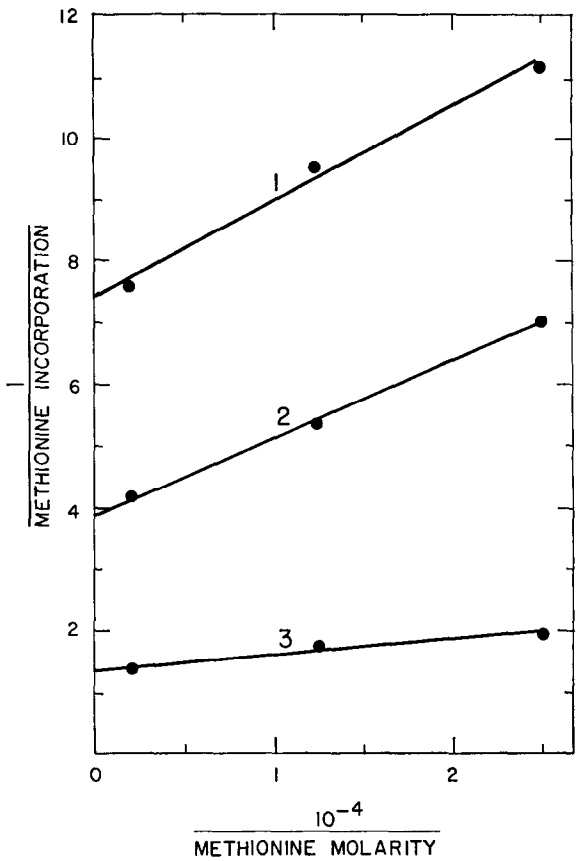


FIG. 3. Double reciprocal plot showing non-competitive inhibition of methionine incorporation into Ehrlich ascites cell protein by two concentrations of 4-thiamethionine: (1) inhibition by 5×10^{-3} M 4-thiamethionine; (2) inhibition by 5×10^{-4} M 4-thiamethionine; (3) uninhibited control. Incorporation is expressed as μmoles methionine incorporated per g of protein during the 15-min incubation period.

TABLE 2. 4-THIAMETHIONINE INHIBITION OF PHENYLALANINE AND *iso*LEUCINE INCORPORATION INTO EHRLICH ASCITES CELL PROTEIN, AND ITS INDEPENDENCE OF METHIONINE CONCENTRATION

Amino acid incorporated	4-Thiamethionine concentration	Inhibition			
		No methionine added	L-methionine / 4-thiamethionine (molar ratio)		
			5	10	25
	(M)	(% of uninhibited control)			
L-Phenylalanine	5×10^{-4}	66	—	66	51
L-*iso*Leucine	3×10^{-4}	43	46	57	—

The L-phenylalanine and L-*iso*leucine were added to give a final concentration of 5×10^{-4} M.

TABLE 3. PREVENTION OF 4-THIAMETHIONINE INHIBITION OF LYSINE INCORPORATION BY REDUCED GLUTATHIONE

Lysine incorporation with			
Buffer only	4-Thiamethionine 5×10^{-4} M	Glutathione 5×10^{-3} M	4-Thiamethionine 5×10^{-4} M and glutathione 5×10^{-3} M
(μmoles/g protein per 15 min incubation period)			
1·80 ± 0·01*	0·96 ± 0·00	1·83 ± 0·01	1·70 ± 0·03

* Deviation from the mean of duplicate incubations. The L-lysine concentration was 5×10^{-4} M.

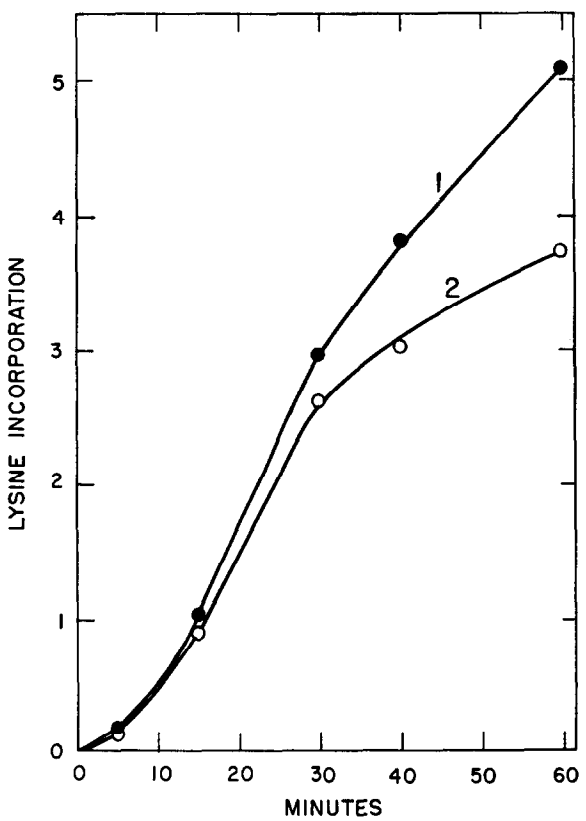


FIG. 4. Inhibition by 4-thiamethionine of lysine incorporation under conditions of anaerobic glycolysis: (1) uninhibited control; (2) inhibition by 5×10^{-4} M 4-thiamethionine. The L-lysine concentration was 5×10^{-4} M. Anaerobic glycolysis was stimulated by 20 per cent in the presence of 4-thiamethionine. Incorporation is expressed as in Fig. 2.

The results are in accord with the view that the analog or its metabolic products interfere with amino acid incorporation of respiring tumor cells by reducing the energy supply made available through oxidative phosphorylation, as do dinitrophenol[9] and other compounds.[12]

TABLE 4. PREVENTION BY GLUCOSE OF 4-THIAMETHIONINE INHIBITION OF LYSINE AND VALINE INCORPORATION UNDER AEROBIC CONDITIONS

C^{14}-Amino acid 5×10^{-4} M	Incorporation with			
	Buffer only	4-Thiamethionine 5×10^{-4} M	Glucose $1{\cdot}5 \times 10^{-2}$ M	4-Thiamethionine 5×10^{-4} M and glucose $1{\cdot}5 \times 10^{-2}$ M
	(μmoles/g protein per 15 min incubation period)			
L-Lysine	1·66	0·83	1·68	1·50
L-Valine	1·72	0·75	1·69	1·47

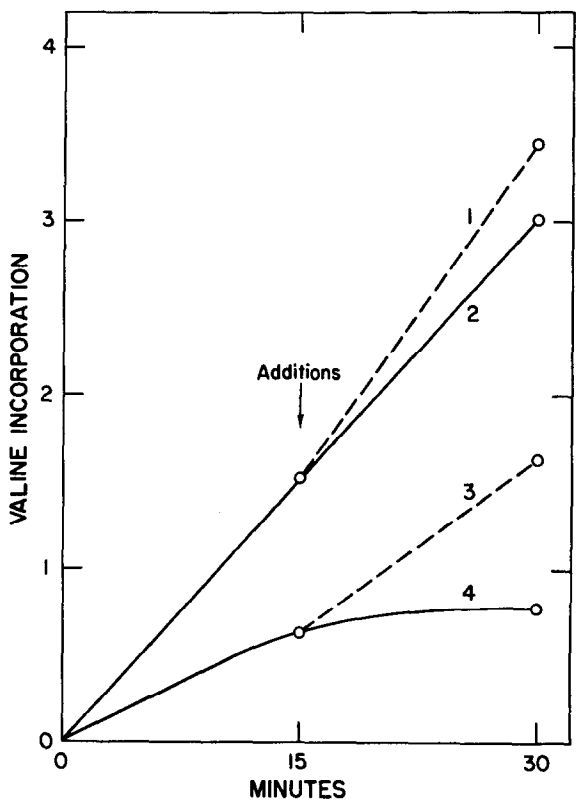


FIG. 5. Relief by glucose of 4-thiamethionine inhibition of valine incorporation: (1) uninhibited control; glucose, 0·015 M final concentration, added at 15-min interval; (2) uninhibited control; buffer only added at 15-min interval; (3) inhibition by 5×10^{-4} M 4-thiamethionine; glucose, 0·015 M final concentration, added at 15-min interval; (4) inhibition by 5×10^{-4} M 4-thiamethionine; buffer only added at 15-min interval. Incorporation is expressed as μmoles valine incorporated per g protein. The L-valine concentration was 5×10^{-4} M.

DISCUSSION

The results indicate that 4-thiamethionine interferes primarily with the energy supply of the cells made available by respiration, and not with the protein synthetic mechanism of the cell. The ability of glucose to relieve, as well as to prevent, the

inhibition indicates that this substrate acts by furnishing the cells with an alternate energy source through glycolysis. Quastel and Bickis[12] have suggested that tumor cells may be resistant to a variety of inhibitors through their ability to utilize alternately either oxidative or glycolytic energy sources. It is of interest, also, that glutathione and cysteamine could not reverse the inhibition, although such reversal can be demonstrated in disulfide-inhibition of individual enzymes.[13]

The mechanism for protein synthesis is capable of introducing each amino acid, many close analogs of one another, into the proper sequence of individual proteins. Recent studies have indicated that specificity is shown in reactions that may represent early stages of protein biosynthesis, such as amino acid activation and transfer to soluble ribonucleic acid.[14] These considerations would appear to run counter to the view[15] that effective analogs of amino acids may deviate considerably in structure from the natural metabolite. Although this view is valid for other systems[16] the high specificity required for competitive amino acid antagonism to protein synthesis would indicate that this concept is not applicable here. The alternative, that protein synthesis may be blocked* by an amino acid bearing a highly reactive functional group which is also a close structural analog of a natural amino acid, imposes severe restrictions on the number of potential compounds which could be designed.

* Studies with labeled amino acids and their antagonists indicate that most amino acid antagonists do not inhibit the protein synthetic mechanism, but to some extent replace the corresponding metabolite during the synthesis of protein.[4] The resultant formation of unnatural proteins and enzymes within the cell possibly leads to inhibition of growth and decrease in the associated net increase in protein. A direct reversible block of globin synthesis at the microsomal site has been reported.[17]

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