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Molecular Aspects of the In Vivo and In Vitro Effects of Ethionine, an Analog of Methionine

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INTRODUCTION

Ethionine is the *S*-ethyl analog of the essential amino acid methionine. This substitution appreciably increases molecular bulk and length (0.3 nm in residue length) (Fig. 1). It is debatable whether (63, 119) or not (203) ethionine occurs naturally, although the latter is more likely. In any case, it is a dangerous poison. It was used for the first time by Dyer in 1938 (50) to supplement the diet of rats which were lacking methionine. The rats died within 10 days.

Electron microscopic examination of hepatic cells from rats which have received intraperitoneal injections (14, 15, 73, 166, 178) or chronic administration (188) of ethionine showed fat accumulation, necrosis, nucleolar fragmentation, disaggregation of polyribosomes, and numerous other lesions. Since then, the pathogenesis of ethionine has been described in detail. Ethionine induces fatty liver (for review, see reference 55), acute pancreatitis (for review, see reference 120), and hepatocarcinogenesis; prolonged feeding of rats with ethionine results in a high frequency of hepatic carcinoma (for review, see reference 54). Presumably, ethionine blocks one or more of the essential functions of methionine in cells (181), because most of its biochemical and morphological effects are reversed by the administration of methionine or adenine (this fact alone makes it unique when compared with other carcinogens). Any of the following processes could be affected: the biosynthesis of proteins, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and phospholipids or transmethylation reactions in which methionine is the methyl donor (e.g., the synthesis of choline). Each of these possibilities has been investigated.

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INCORPORATION OF ETHIONINE INTO PROTEINS

In the early 1950s, ethionine, an analog of the naturally occurring amino acid methionine, was first used as a tool to study the mechanism of protein biosynthesis. It was shown that ethionine, although inhibiting the incorporation of methionine, was itself incorporated into rat proteins (114, 173). It was unexpected that the biosynthetic mechanism was sufficiently flexible

to allow the incorporation of an unusual amino acid. This observation was confirmed by the incorporation of ethionine into proteins of the protozoon *Tetrahymena pyriformis* (77), ascites cells (146), and yeasts (37, 125, 174) and into proteins secreted by feline pancreas (90). The rate of synthesis of proteins is markedly reduced in the presence of ethionine (56, 173), but subsequent studies with procaryotic cells have revealed, as described below, that the overall mechanism of protein biosynthesis is not appreciably disturbed when ethionine is substituted for methionine and that these ethionine-containing proteins resemble the natural methionine-containing analogs.

Ethionine, which inhibits the growth of *Escherichia coli*, is incorporated into the proteins of this organism at an initial rate of about 50% that of methionine incorporation (30, 145, 150). The inhibition of growth is reversed by methionine (91, 151) and adenosine triphosphate (ATP) (131), which is surprising because ATP is generally assumed not to be readily taken up by bacteria or other cells. Addition of ethionine to a *met rel* strain of *E. coli* produces a slow linear (rather than the normal exponential) increase in optical density of the culture (doubling time, 4 to 5 h), but this apparent growth is due entirely to an increase in size of the bacteria (176); there is no cell division. The [^{14}C-ethyl]ethionine incorporation into proteins and the total protein content of the cells increase proportionately with the increase in cell size (30).

It might be expected that initiation of protein biosynthesis is a step very sensitive to the presence of ethionine, because methionine is always used as the initiator amino acid. But in vitro studies revealed no important discrimination between ethionine and methionine in the following reactions: (i) aminoacylation of $tRNA_m^{Met}$ and $tRNA_f^{Met}$ (although the methionyl-transfer RNA [tRNA] synthetase recognizes ethionine as both a competitive inhibitor and a substrate, with a K_m value two orders of magnitude higher than that for methionine [37, 61, 113, 134–136, 195]); (ii) formylation of ethionyl-$tRNA_f^{Met}$; and (iii) initiation by formyl-ethionyl-$tRNA_f^{Met}$ of the synthesis of polymethionine or polyethionine in the presence of poly(adenylate-usidylate-guanylate) (134). It is even more surprising that the levels of deformylation and removal of the N-terminal (methionine or ethionine) residues in proteins synthesized by *E. coli* were about the same (within approximately 60%), irrespective of whether initiation occurred via formyl-methionyl-$tRNA_f^{Met}$ or formyl-ethionyl-$tRNA_f^{Met}$ (27).

There are several examples of proteins containing ethionine in place of methionine being fully active. The α-amylase of *Bacillus subtilis* containing either ethionine or methionine shows the same physicochemical and catalytic properties as the native protein (218, 219). *E. coli* 30S ribosomal proteins containing ethionine are active (19). *E. coli* 50S ribosomal proteins containing ethionine are also active, as demonstrated by the fact that, when supplemented with 23S and 5S ribosomal RNAs (rRNAs) and ribosomal protein L16 (see below), they yield, on in vitro reconstitution, 50S particles active for polyuridylate-programmed polyphenylalanine synthesis (8). The biological activity of gastrin, a peptide hormone, is not affected by the substitution of ethionine for methionine (135). It should be noted that a few proteins containing ethionine are somewhat more thermolabile than the native proteins, e.g., the glutamate dehydrogenase from *Neurospora crassa* (115), but despite this observation and the markedly reduced rate of protein synthesis observed, it is difficult to imagine that incorporation of ethionine into proteins accounts for the major physiological effects observed in the presence of ethionine.

Similarly, it seems unlikely that the carcinogenic properties of ethionine are attributable to its general effect on protein biosynthesis, since one would expect neoplasma to be present in many different tissues and not only in liver. Liver has a high rate of protein synthesis, but so do several other tissues that do not become neoplastic. However, since the liver makes a variety of tissue-specific proteins, it cannot be totally excluded that their modification by introduction of ethionyl residues leads to specific hepatocarcinogenesis.

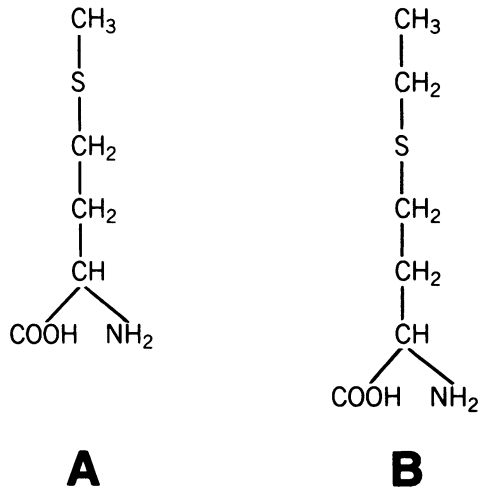

FIG. 1. Configuration of methionine (α-amino-γ-thiomethyl butyric acid) (A) and ethionine (α-amino-γ-thioethyl butyric acid) (B).

SYNTHESIS OF S-ADENOSYL-L-ETHIONINE OCCURS IN EUCARYOTES BUT NOT IN BACTERIA: EFFECTS OF S-ADENOSYL-L-ETHIONINE

Methionine is a special amino acid because of its unique role as an initiator of protein biosynthesis (see above) and as methyl group donor and propylamine group donor via S-adenosyl-L-methionine (SAM). The SAM synthetases (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6) are of particular interest because of the unusual chemistry of the reaction which they catalyze (complete dephosphorylation of ATP and transfer of an adenosyl group to the sulfur atom of methionine [124]) and also because of the existence of two isozymes in yeasts (32) and of three in the mammalian liver (116; see below). It is thus not surprising that all possible analogs of L-methionine, including ethionine, have been systematically used (39, 121, 158, 186) to study the mechanism and specificity of the SAM synthetases isolated from various sources (bacteria, yeasts, rat liver).

Bacterial S-Adenosyl-L-Methionine Synthetases Do Not Recognize Ethionine

Ethionine is not a substrate for the enzymes from either *E. coli* (95, 121, 144) or *Salmonella typhimurium* (12) and does not inhibit the SAM formation by the enzyme from *S. typhimurium* (39). This is in contrast to the SAM synthetases from yeasts or rat liver, as described below. The reason for the increased specificity of the bacterial enzymes is unknown. It could be due simply to steric exclusion because of the bulky ethyl group of ethionine. Furthermore, the *E. coli* enzyme requires a *cis*-like configuration of methionine around the sulfur atom, whereas the eucaryotic enzymes function with a *trans*-like configuration (*cis* and *trans* refer to the relative positions of the terminal methyl group of methionine and of the methylene group adjacent to it) (175). The necessity of a *cis*-like configuration could be the mechanism by which bacterial SAM synthetases discriminate against ethionine; this would suggest an important evolutionary divergence between the procaryotic and eucaryotic enzymes. A third possibility is that isozymes of SAM synthetases allow the use of a greater range of substrates than the single bacterial enzyme.

Similarly, other methionine-specific bacterial enzymes do not recognize ethionine. Ethionine does not inhibit the transport of L-methionine in *E. coli* (98), nor does it repress the synthesis of SAM synthetase (which methionine does) (95). However, non-methionine-specific *E. coli* enzymes can recognize ethionine: the D-amino acid dehydrogenase uses D-ethionine as well as D-methionine as a substrate (214), and the uracil-tRNA methyltransferase is inhibited by ethionine in a noncompetitive manner with respect to SAM (196).

In *E. coli*, the combination of two properties of ethionine metabolism, i.e., incorporation into proteins and nonsynthesis of S-adenosyl-L-ethionine (SAE), has a practical application. The growth of a methionine auxotrophic strain in minimal medium in the absence of methionine and in the presence of ethionine eliminates methylation of all cellular components. The synthesis of nucleic acids, proteins (containing ethionyl instead of methionyl residues; see above), and all other macromolecular components, as well as their assembly into organelles (such as ribosomes), occurs under these conditions. However, there is neither synthesis of SAM (absence of methionine) nor of SAE (lack of reaction of the procaryotic SAM synthetase with ethionine), and hence all of the cellular postsynthetic methylation reactions are blocked. Another advantage of this situation is that, in a subsequent step, ethionine can be removed from the culture medium by washing, and bacteria can be returned to complete medium (plus methionine) to allow methylation in vivo of the accumulated, unmethylated components (cf. reference 8, Fig. 1, and below).

Synthesis of S-Adenosyl-L-Ethionine in Yeasts and Rat Liver

In contrast with the bacterial enzymes, the SAM synthetases from the yeasts *Saccharomyces cerevisiae* (37, 157) and *Candida utilis* (139, 157) and from mouse (93) and rat (72, 177) liver synthesize SAE from ATP and ethionine, although in mouse and rat liver the rate of SAM formation is much higher than that of SAE (2, 83). The rate of synthesis of SAE has been used as a measure of the activity of SAM synthetase in mouse liver (94), since the ethyl analog is a poor substrate for methyltransferases and accumulates in the livers of rats fed ethionine. If ethionine is administered to rats in the form of a complex with cupric acetate, SAE is found in the liver at a significantly higher concentration than when animals are fed only ethionine in the diet (24). However, only in the liver can one demonstrate sizable amounts of SAE synthesis (83).

In passing, it may be noted that ethionine as well as methionine is recognized by some other eucaryotic systems; for example, ethionine acts in yeasts as a repressor of the methionine biosynthetic pathway (37) and is an active substrate for the glutamine transaminase from rat brains (200).

Ethylation of Macromolecules in Rat Liver Cells

Administration of ethionine to rats causes the ethylation of liver DNA (by the formation of 7-

ethylguanosine residues) (190); tRNAs (138, 143, 152), especially $tRNA_2^{Lys}$ (107, 165); and nuclear proteins (by the formation of ethylated arginine residues [65]). Some indirect arguments suggest that not all of the ethylation of tRNA occurs via SAE, but no alternative ethyl donor has been suggested (138, 143). Nonenzymatic ethylation reactions, analogous to the nonenzymatic methylation of DNA by SAM recently described, could also occur (154).

In vitro, SAE can be an active substrate for: (i) the cytoplasmic histone arginine methyltransferase from rat livers leading to the ethylation of arginine residues in histones (16) (the nuclear histone lysine methyltransferase is also able to transfer ethyl groups from SAE to histone but with very low efficiency compared with that of the cytoplasmic histone arginine methyltransferase [17]); (ii) the tRNA methylases from rat liver, although the rate of ethylation is 20 times less than the rate of methylation with SAM (143); and (iii) the reovirus-associated messenger RNA (mRNA) methyltransferase leading to ethylated cap structures e^7GpppG^e and e^7GpppG at the 5′ termini of newly synthesized viral mRNAs (69). However, it is less effective as a substrate for the viral methyltransferase than SAM. These viral mRNAs with e^7G caps bind to wheat germ ribosomes and are translated essentially as well as viral mRNAs with m^7G caps, suggesting that ethylation of mRNA, if it occurs in vivo, is not responsible for the inhibition of protein synthesis (69).

The mechanism of ethionine hepatocarcinogenesis in rats is obscure, but because of the synthesis of SAE only in liver, it has been proposed that an initiating event in the induction of liver cancer could be the enzymatic or nonenzymatic (154) ethylation of some cellular component(s) at sites which are not normally methylated (42). A major advance in the formidable problem of determining the target molecules (lipids, rRNA, tRNA, proteins, etc.) has been achieved recently (107, 165) with the demonstration that, in the case of tRNA, ethylation is not random but specific for $tRNA_2^{Lys}$. Two alternative explanations for this observation are possible: either (i) ethionine in the diet, which, as is well documented (see below), increases the tRNA methylase activities of liver cells, also changes their specificity of action, or (ii) a tRNA transethylase exists in the liver which is specific for one of the two isoacceptors $tRNA^{Lys}$.

Metabolism of *S*-Adenosyl-L-Ethionine

SAE has the same effect as SAM in a few cases; for example, it inhibits the transport of leucine in *S. cerevisiae*, as does SAM (111). Also, in the in vitro synthesis of cytoplasmic polyhedrosis virus mRNA, where SAM is required as a methyl donor as well as an allosteric-like stimulator of mRNA synthesis, SAE functions as a stimulator, although it is less effective than SAM (68).

In other cases, SAE is not able to fulfill the metabolic functions of SAM, e.g., it cannot stimulate germination and outgrowth of yeast ascospores (26). In rat livers, it inhibits the synthesis of polyamines, an important metabolic pathway in which SAM plays the role of propylamine group donor; administration of ethionine to rats stops the synthesis of spermine and spermidine (147). This is because SAE is both a very bad substrate for SAM decarboxylase, the first enzyme of the polyamine biosynthetic pathway, and an inhibitor of the formation of spermidine from decarboxylated SAM (140, 141). Also, SAE is a competitive inhibitor of most of the methyltransferases, such as DNA- (38) and histone- (16, 17) methyltransferases of rat liver, tRNA-methyltransferases (129, 130, 142) of bacteria and rat liver, and protein carboxymethylase (EC 2.1.1.24) of mouse pancreas (70). The inhibition by SAE of this last enzyme could be the explanation for the inhibition observed in mice fed ethionine of the export of the amylase, a pancreas-specific digestive enzyme (70). In plant tissues, the first step in the biosynthesis of ethylene, a plant hormone, is the conversion of SAM to 1-aminocyclopropane carboxylate and methylthioadenosine. SAE serves as a substrate (although it is only 30% as active as SAM) for the 1-aminocyclopropanecarboxylate synthase and, when both SAM and SAE are present, competitively inhibits the enzyme (220). This could be the mechanism of inhibition of ethylene production in plant tissues after ethionine application (118).

In addition to the small number of ethylation reactions described above, SAE is also used in the synthesis of ethylated metabolites, such as triethylcholine (182–184) and ethyl analogs of creatinine (182), creatine (184, 198), and anserine (216). However, the rate of use of SAE is much less than that of SAM, and this imbalance between the rates of formation and of breakdown of SAE leads to its accumulation in the rat liver.

Adenosine-Trapping Activity of Ethionine in Rat Liver Cells

Normally, the rapid turnover of SAM produces a continuous supply of an adenyl derivative (methylthioadenosine) which is reused for the synthesis of ATP, but the accumulation in rat liver of SAE (see above) traps the ATP in a non-metabolizable form. Yeasts are capable of counteracting this intracellular trapping of adenine groups by ethionine by the intense de novo synthesis of adenine groups (160), but in liver

cells, the rate of de novo ATP synthesis from available precursors is much slower than that of the reaction of ATP with ethionine to form SAE. This results in a marked reduction in the hepatic ATP concentration (59, 133, 168). Adenine, adenosine, and inosine, which is a precursor of adenine nucleotides, counteract the effect of ethionine upon the ATP levels and protein synthesis (169). It is possible that ATP deficiency accounts for the many diverse effects observed in rat livers after administration of ethionine, such as the inhibition of the biosynthesis of proteins (205), RNA (58), and also choline (172) and accumulation of lipids (14, 55, 60). These effects are much less marked in male than in female rats (56, 57, 184), possibly because the reduction in ATP concentration is more pronounced in female rats than in male rats (181).

ALTERATIONS IN THE TRANSLATIONAL APPARATUS

Administration of ethionine elicits many alterations in the translational apparatus of procaryotic and eucaryotic cells, such as disaggregation of polyribosomes, inhibition of and defects in the biosynthesis of ribosomal subunits, and inhibition of postsynthetic modifications (especially methylations) of both nucleic acids and proteins constituting the translational machinery.

Inhibition of Ribosomal Ribonucleic Acid Synthesis

In bacteria (30, 76), yeast (174), and rat liver cells (206), an inhibition of RNA synthesis amounting to 90% is observed upon ethionine administration. This is mostly accounted for by rRNA. A possible explanation for the inhibition of RNA synthesis in ethionine-fed rats is the decrease in the concentration of ATP (see above) and consequently of pyrimidine triphosphates (206) (ATP is required in the following reactions: uridine monophosphate → uridine diphosphate → uridine triphosphate → cytidine triphosphate). This in vivo effect of ethionine on RNA synthesis is reversed by administration of methionine or adenine or both (58). However, in yeast cells, the sizes of the ATP (160) and pyrimidine triphosphate (174) pools are not greatly affected, and the inhibition of RNA synthesis by ethionine is not reversed by supplying exogenous adenine or methionine (174), showing that in yeast cells there is another, perhaps specific, effect on RNA formation. Moreover, in an in vitro RNA synthetic system to which all of the nucleoside triphosphates have been added, the isolated nuclei from ethionine-treated rats are less active than those from control rats (178). Thus, even in rat liver, the inhibition of RNA synthesis is not due only to the decreased nucleoside triphosphate pools.

The synthesis of tRNA is little affected (189), although the disappearance of a minor species of $tRNA^{Leu}$ (13, 211) and a change in the relative abundance of other, different species of tRNA (41) have been reported to occur in the livers of rats fed diets containing ethionine.

In wild-type bacteria, RNA synthesis is inhibited when the availability of any aminoacyl-tRNA species becomes limiting (the so-called "stringent response"). Supplying ethionine instead of methionine to a *met* strain induces this response because ethionine is a far worse substrate for methionyl-tRNA synthetase than methionine (see above). This effect could constitute a severe limitation of the production of unmethylated rRNA by ethionine-grown bacteria (described above). However, this problem can be avoided by using a mutant strain (*rel*) in which the obligatory coupling between RNA and protein synthesis is eliminated (relaxed phenotype), i.e., by incubating a *met rel* strain starved for methionine in the presence of ethionine.

Inhibition of Some Postsynthetic Modifications to the Macromolecular Components of the Translational Apparatus

Methylation. An increasing variety of postsynthetic methylations of DNA, tRNA, mRNA, rRNA, and protein molecules in procaryotic and eucaryotic cells has been discovered in the past few years. Among rRNAs, only 5S rRNA has no methyl groups added post-transcriptionally. All of these methylations are mediated via highly specific methyltransferases and SAM, with only one exception described until now, i.e., the tetrahydrofolate-dependent biosynthesis of ribothymidine in tRNAs of gram-positive bacteria (161). The postsynthetic methylations have been found to be impaired after ethionine treatment in all cases studied so far.

Postsynthetic methylations of DNA (108, 110), tRNA (207), rRNA (18, 19, 29, 30), and ribosomal proteins L3 and L11 (5–7, 112) are inhibited in ethionine-grown *E. coli*. As described previously, the maximal effect is obtained by cultivating a *met rel* strain of *E. coli* in a medium in which ethionine is substituted for methionine. Under these conditions, macromolecular components are unmethylated, and subsequent methylation in vivo or in vitro with radiolabeled methionine provides a method of detecting methylated cellular components and of studying the role of these postsynthetic modifications. In addition, ethionine inhibits (in a noncompetitive manner with respect to SAM) the uracil tRNA methylating enzymes from *E. coli* (196).

In ethionine-treated eucaryotic cells, the synthesis of SAE (see above) and the reduction in concentrations of ATP and thus of SAM observed in rat liver (see above), together with the

direct competitive inhibition by SAE of DNA methyltransferases (38), tRNA methyltransferases (129, 130, 142), and histone methyltransferases (16, 17), lead to a great reduction in the postsynthetic methylations of DNA in rat liver (38) and other cell types (21, 22, 34, 35, 180), of tRNA in rat liver (66, 67, 71, 78, 96, 100, 122, 197, 208, 209) and in Friend erythroleukemia cells (34), and of rRNA (4 [see also 215], 189, 217), mRNA (74), and histones (16, 17, 40) in rat liver. The tRNAs from livers of rats injected with ethionine are deficient in methylated nucleosides, even though tRNA methyltransferase activities are highly elevated (see below). These changes in tRNA and in enzyme levels are not contradictory, because they are time dependent. A possible sequence of events after administration of ethionine could be: (i) production of inhibitor(s) of methylation, such as SAE; (ii) reduction in methylation of tRNA; (iii) derepression of tRNA methyltransferases; and (iv) nearly normal methylation due to excess enzyme (208). In *S. cerevisiae*, however, ethionine has no effect on the methylation of tRNA and only a limited effect on that of rRNA (174), perhaps because there is no important decrease in the ATP concentration (160). When assayed by injection into oocytes, tRNAs from ethionine-treated animals are severely impaired in their aminoacylation capacity as well as in their ability to participate in protein synthesis, but the effect of ethionine treatment varies from one specific tRNA species to another (71). The cause(s) (undermethylation, altered maturation, or ethylation or all three) of this impaired biological activity of tRNA has not yet been determined.

Methylation of sterol side-chains in ergosterol biosynthesis is also inhibited in yeast cells grown in the presence of ethionine (10).

Phosphorylation. Phosphorylation of eucaryotic ribosomal protein S6, where ATP is probably the phosphate donor, is abolished after administration of ethionine to rats (193, 194).

Ribosomal ribonucleic acid processing. Ethionine inhibits in vivo the processing of eucaryotic rRNA (174, 189, 217), as do other treatments which inhibit methylation (11, 28, 81, 117, 202, 212). From results like these, it is generally agreed that methylation of the 45S precursor of rRNA is necessary for its processing into 18S and 28S rRNAs. But, contrary to this conclusion, the processing of certain steps, in particular the initial 45S → 32S transition, has been observed in the presence of ethionine (217) or cycloleucine (28), although there was extensive degradation and only a low level of specific cleavage.

In *E. coli*, 16S, 23S, and 5S rRNAs labeled with ^{32}P in the presence of ethionine are at the same time unmethylated (as shown by the absence of characteristic methylated oligonucleotides in their fingerprint analyses) but of the correct size (as shown by the presence of 5′ and 3′ ends characteristic of mature 16S, 23S, and 5S rRNAs and not of those characteristic of their precursors) (29). Similarly, the 16S rRNA of the *E. coli* mutant *ksgA* (resistant to kasugamycin) is not methylated in the sequence $m_2^6Am_2^6AC$-CUG but is the correct size (92). Thus, it is clear that, in *E. coli*, methylation and processing of rRNAs are independent.

The different effects of methylation on the maturation of rRNA in procaryotic and eucaryotic cells may be due to the very different degrees of ribose methylation in rRNA precursors in these two cell types. In eucaryotes, ribosomal RNA methylation takes place mainly (>90%) on the 2′-hydroxyl groups of ribose residues, whereas in *E. coli*, rRNA methylation is confined almost entirely to purine and pyrimidine bases. Thus, these different effects of ethionine observed for procaryotic and eucaryotic systems are not necessarily contradictory, and it may be suggested that, as in the case of procaryotic rRNA precursors, base methylation in eucaryotic rRNA precursors is not essential for their maturation, but that ribose methylation (which contributes only about 10% of the total RNA methylation in *E. coli*) is necessary for eucaryotic RNA processing.

Addition of polyadenylate tails. The size distribution of polyadenylate [poly(A)] sequences at the 3′ end of eucaryotic mRNAs is not affected by ethionine, but their metabolism (addition and removal of AMP units) is increased, perhaps because mRNA in a nonpolysomal state (see above, this section) is a better primer for cytoplasmic poly(A) polymerase than mRNA in the form of polysomes (45, 46).

Processing of viral polyproteins. The cleavage of precursor viral polypeptides synthesized in the presence of ethionine in echovirus type 12-infected cells is not impaired, whereas it is if viral proteins are synthesized in the presence of other amino acid analogs (153). Thus, incorporation of ethionine residues into the precursor viral polypeptides does not change their three-dimensional configuration sufficiently to affect the recognition by the specific protease(s).

Major changes in the ribosomal subunits

Owing presumably to the lack of postsynthetic modifications of some of their components (see above), eucaryotic ribosomal subunits synthesized and assembled in the presence of ethionine are partially inactive in protein synthesis (1) and show morphological defects which can be attributed to a measurable change in conformation

(103, 104, 191a) or to the lack of one or a small number of ribosomal proteins (102) or both.

Somewhat more work has been done on the effect of ethionine on bacterial ribosomes. Ribosomal protein L16 is completely absent, whereas L6, L27, L28, and L30 are present in reduced amounts in 50S ribosomal subunits isolated from ethionine-treated *E. coli* cells. These subunits possess a more labile and flexible structure than the normal 50S subunits and are totally inactive in protein biosynthesis (8), suggesting that methylation of some ribosomal component(s), RNA (18, 19, 29, 30) or proteins (5–7, 112) or both, is essential for assembly or activity or both of ribosomes. These changes are reversible; when *E. coli* cells which have been grown in the presence of ethionine are transferred to a medium containing methionine, the abnormal ribosomes which had been synthesized in the presence of ethionine regain activity (8, 18, 30), even in the absence of protein biosynthesis. Of course, ethionyl residues which have been incorporated into the ribosomal protein chains are not removed (18), but presumably one or several methylations occur which reestablish the activity of these subunits.

Disaggregation of Rat Hepatic Polyribosomes

Inhibition of protein biosynthesis after the administration of ethionine to rats is accompanied by breakdown of hepatic polysomes (15, 52, 53, 171, 204) into "runoff" monosomes and released mRNA. However, this effect is not observed in all cases (75) and can be modified by the long- and short-term administration of other hepatocarcinogens (e.g., acute administration of ethionine to rats already dosed with another hepatocarcinogen has less effect on polyribosome structure and protein synthesis than ethionine alone) (170). When the dissociation of polysomes is observed, the mRNA molecules (as ribonucleoprotein [mRNP] particles) remain attached to the endoplasmic reticulum independently of the ribosomes and of the nascent polypeptide chains (52). This methyl-deficient (74), poly(A)-containing (45, 46) mRNA is quite stable (see below) but differs in its translational properties from mRNA isolated from nontreated animals in an in vitro protein synthesizing system from wheat germ (74). The inhibition of RNA synthesis (see above, this section) is not the cause of the dissociation of polysomes, since addition of adenine (14) or methionine allows polysomes to reform even when new RNA synthesis is blocked by actinomycin D (171). For this recovery from ethionine intoxication, membrane-bound mRNA appears to be reutilized for the reformation of the membrane-bound polysomes (171) without ever being detached from the membranes, an observation which does not agree with the "signal" hypothesis, according to which membrane-bound polysomes are attached via the nascent polypeptide (52). It should be noted that the possibility of reversing the effect of ethionine becomes progressively impaired with a longer period of ethionine treatment because of a decrease in mRNA concentration (45).

INHIBITION OF DEOXYRIBONUCLEIC ACID REPLICATION

In a methionine auxotroph of *E. coli*, addition of ethionine instead of methionine causes the replication of DNA to cease after one doubling of the cellular DNA content. The rate of DNA synthesis is reduced considerably but remains measurable (76, 108–110, 176). However, it is not completely clear whether the residual DNA synthesis observed in the presence of ethionine is of the same type (i.e., de novo synthesis rather than repair) as that occurring in the presence of methionine. One hypothesis that has been proposed but not yet proven is that methylation of the DNA template is necessary for replication (108, 110). It is clear that the origin of the replication site of *E. coli* DNA is heavily methylated (148). The problem which is inherent in the use of ethionine is that it is difficult to distinguish between an effect of ethionine on the methylation of DNA itself or of other macromolecules involved in replication and the synthesis of proteins essential for replication. More recent in vitro results show that DNA synthesis is reduced in lysates of *E. coli* cells grown in the presence of ethionine. Incubating these lysates with SAM and ATP restored DNA synthesis which was dependent on DNA polymerase I, whereas DNA polymerase III was not required. This suggests that methylation of DNA is indeed involved in DNA synthesis at a step mediated by DNA polymerase I (109).

In mitogen-stimulated lymphocytes, ethionine inhibits DNA synthesis without blocking nucleotide transport or synthesis or depleting the ATP pool. This effect is completely reversible. Under the conditions used, ethylation of macromolecules or substitution of ethionine for methionine in proteins was presumed to be minimal and unlikely to be responsible for the observed inhibition. It is thus tempting to speculate that ethionine inhibits a methylation reaction critical to initiation of DNA synthesis. However, morphological changes characteristic of lymphoblast formation and complete commitment to DNA synthesis can occur in the presence of ethionine, even though the synthesis of DNA is blocked. Thus, ethionine permits a quiescent lymphocyte to enter the G1 stage of the cell cycle and to progress toward the S phase (221).

In rats, administration of ethionine provokes,

after a delay, an enhancement in DNA repair replication, presumably owing to DNA damage produced by SAE or ethionine (43, 201). DNA damage by ethionine has also been observed in Chinese hamster cells (191).

INDUCTION OF VARIOUS CELLULAR FUNCTIONS

Despite its general inhibitory effect on protein synthesis, ethionine induces in rat liver cells an elevation in the activities of several enzymes, such as tRNA methylases (2, 84, 85, 87, 185, 197), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34) (89), cysteine desulfhydrase (105), adenylate cyclase (and the cyclic adenosine monophosphate content) (44), guanylate cyclase (and the cyclic guanosine monophosphate content), cyclic guanosine monophosphate phosphodiesterase (47), histone methylases, protein kinases (86), 5'-nucleotidase (in female rats only) (3), and even RNA polymerase (199). However, the behavior of the two SAM synthetase isozymes (α and β forms) is very different after ethionine administration. The activity of the α form increases temporarily (2 days) and then decreases, whereas the activity of the β form decreases gradually (2, 197). A similar gradual decrease is observed in the activity of glycine methyltransferase (EC 2.1.1.20) (2), an enzyme which competes with the methylases for the available common substrate SAM (101). In ethionine-fed rats, the feedback repression by exogenous heme on δ-aminolevulinic acid synthetase (the first and rate-limiting enzyme of the heme biosynthetic pathway) is lost. However, it is still observed in vitro, i.e., in liver cells isolated from ethionine-treated rats (51).

Ethionine also induces the synthesis of some other proteins, e.g., embryonic α-fetoprotein in rat liver (this newly synthesized α-fetoprotein is rapidly secreted into the blood circulation [64, 88, 167, 179, 210]), and ovalbumin and conalbumin in immature chicken oviducts (162, 164, 165), and of the steroids progesterone and cholesterol in the sera of immature chickens (163, 165). In addition, ethionine can be an agent which provokes differentiation of cells in culture. When it is used at concentrations sufficient to inhibit in vivo methylation of DNA and tRNA, ethionine is an inducer of erythroid maturation (shown by the induction of globin synthesis) in Friend erythroleukemia cells (34, 35) and of myeloid maturation in human promyelocytic leukemia cells (126).

The mechanism of the induction of the synthesis of these different compounds might or might not have a common origin and could be due to ethylation or undermethylation of target molecules (DNA) (34, 35) or to the reduction in the intracellular ATP concentration. It could be an effect of either ethionine itself or one of its metabolic derivatives (24, 25), e.g., via their binding to a specific cellular polypeptide (such an interaction has been observed for ethionine in rat livers [20]). Such compounds could inhibit the synthesis or the methylation of a repressor (64) or the synthesis of a corepressor (possibly SAM), or they might directly derepress certain genes. All of these possibilities are still to be investigated, but the recent demonstration (21) that there occurs in the presence of ethionine a preferential hypomethylation of the inverted repetitive DNA sequences (compared with unique sequences) in the genome of mouse mastocytoma cells suggests that normally these sequences have a high degree of methylation. This is perhaps indicative of a regulatory role for the repetitive sequences and explains the fact that ethionine can induce reexpression of certain genes from these differentiated cells. Furthermore, in these ethionine-treated cells, the decrease in enzymatic DNA methylation (which varies among different methylation sites) correlates with the presence of new transcriptional products (22). Finally, activation (80) or reactivation (36, 128) of certain genes by treatment with 5-azacytidine (97), a cytosine analog which is integrated into DNA and leads to hypomethylation of DNA, or 3-deazaadenosine (33) supports the hypothesis that inhibition of gene expression is linked to DNA methylation at highly specific sites.

ETHIONINE AS A TOOL FOR THE ELUCIDATION OF METABOLIC PATHWAYS

One practical use of ethionine, namely, that of producing unmethylated bacterial macromolecules in vivo which can be subsequently used to study methylation, has already been described (see above). It is worthwhile to mention some other practical applications of ethionine.

(i) Measurements of the incorporation of ethionine into proteins have been used to estimate the aging of cells. Aging, according to the theory of Orgel (137), is due to the accumulation of errors by the translational system. If this is so, an evaluation of aging can be made by testing the ability of this system to discriminate between two amino acid analogs, e.g., methionine and ethionine. Any increase in ethionine incorporation will indicate a change in the translational specificity and be a measure of the error frequency (23, 115, 132). The key enzyme likely to be involved in determining the relative incorporation ratio of methionine to ethionine is methionyl-tRNA synthetase. In the case of bacteria (*E. coli* and *Bacillus stearothermophilus*), this enzyme has an editing mechanism for the rejec-

tion of amino acids smaller than methionine but which does not function with ethionine, which is larger (see above). However, it has been reported (61) that an aged sample of methionyl-tRNA synthetase from *E. coli* can mischarge $tRNA^{Met}$ with α-aminobutyrate, whereas the normal enzyme cannot, implying that aging reduces the editing capacity of the enzyme.

(ii) Since SAM represses the synthesis of methionine, a mutant with reduced SAM synthetase activity will overproduce methionine, causing the strain to be ethionine resistant. Thus, ethionine-resistant mutants are a likely source of mutants in SAM synthetase or methionine biosynthesis (overproduction) (31, 32, 82, 99, 127, 149) and of mutants with undermethylated components, such as tRNAs (62) or polymethylpolysaccharides (123).

(iii) Ethionine specifically blocks the growth of *S. cerevisiae* at the point in the G1 phase of the cell cycle called "start" (174), without being as highly cytotoxic as it is in HeLa cells (213). After treatment with ethionine, the yeast cells can still complete any division cycle already initiated. Since the blocked cells are viable, this appears to be a potential method of producing synchronous growth.

(iv) Ethionine can be used as an inducer of differentiation and of various enzymatic activities in eucaryotic cells, as described above.

(v) Besides ethionine, other analogs of methionine, such as α-methylmethionine (79, 159), L-2-amino-4-methoxy-*trans*-3-butenoic acid (155, 156, 192), L-2-amino-4-methoxy-*cis*-but-3-enoic acid (187), glutamyl-γ-methylester (106), and cycloleucine (1-aminocyclopentane-1-carboxylic acid) (9, 12, 28, 48, 121, 143), are currently used to study the regulation of methionine metabolism and the roles of the postsynthetic methylations.

CONCLUSION

Ethionine is the nonnaturally occurring *S*-ethyl analog of methionine. Their similarity allows ethionine to be incorporated into proteins instead of methionine. However, it is extremely toxic for the cells, since it interferes with a large number of other biosynthetic and regulatory processes, causing numerous malfunctions. Below is a list of the actual known effects of ethionine in which the distinction is made between those effects which are general and those which are specifically exhibited in eucaryotic cells.

In Vivo Effects of Ethionine Occurring in Procaryotes and Eucaryotes

(i) Incorporation of ethionine into proteins
(ii) Inhibition of synthesis of SAM and of postsynthetic methylations to macromolecules
(iii) Inhibition of rRNA synthesis
(iv) Major changes in the ribosomal subunits and dissociation of polysomes
(v) Inhibition of DNA replication

In Vivo Effects of Ethionine Occurring Only in Eucaryotes

(i) Synthesis of SAE
(ii) Inhibition of synthesis of ATP and polyamines
(iii) Postsynthetic ethylation of macromolecules
(iv) Inhibition of phosphorylation of ribosomal protein S6
(v) Inhibition of the maturation of rRNA
(vi) Induction of various enzymatic activities

The effects of ethionine can be considered in three classes. First, certain effects of ethionine are general for both procaryotic and eucaryotic systems, e.g., incorporation of ethionyl residues into proteins. Second, there are a series of effects of ethionine seen only in eucaryotic cells, e.g., the synthesis of SAE and the occurrence of certain ethylated components. Third, there are what appear to be highly specific effects occurring within a differentiated cell type, e.g., induction of the synthesis of embryonic α-fetoprotein in rat liver or of globin synthesis in Friend erythroleukemia cells, and ultimately of carcinogenesis. Classification in this manner does not preclude the possibility that the specialized effects of ethionine are in fact dependent on a general effect, e.g., hypomethylation or ethylation of a sensitive component.

The multitude of effects observed with ethionine, the majority of which are likely to be mediated via its action at the level of methylation, show the importance of this type of postsynthetic modification in all cells. The most dramatic effects, however, have been observed with differentiated eucaryotic cells. Many observations, only some of which have been described here, point to the importance of methylation as a manner of controlling gene expression (49). Ethionine has been used often in the past to study in vivo carcinogenesis and recently, in a few isolated cases, to study differentiation in vitro. Its large sphere of action makes the interpretation of the experiments sometimes difficult. Nevertheless, because of its known interference with the methylation process, its use will probably increase in studies on cellular regulation and differentiation. Ethionine has perhaps not yet been maximally exploited.

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