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Mitochondrial Protein Synthesis: Inhibition by Emetine Hydrochloride

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SUMMARY

Emetine hydrochloride has previously been described as an inhibitor of ribosomal protein synthesis in eukaryotic cells but not in bacteria [A. P. Grollman, *Proc. Nat. Acad. Sci. U. S. A.* 56, 1867 (1966)]. I wish to report that this ipecac alkaloid inhibits protein synthesis in mitochondrial systems derived from mouse liver. Structural relatives of emetine, such as isoemetine hydrobromide and *O*-methylpsychotrine oxylate, which are relatively ineffective as inhibitors of ribosomal protein synthesis, also inhibit mitochondrial protein synthesis but at concentrations that are about an order of magnitude greater than those required for inhibition by emetine hydrochloride. Anisomycin, which also inhibits ribosomal protein synthesis in eukaryotic cells but not in bacteria, fails to inhibit mitochondrial protein synthesis. These findings reveal new structural and conformational subtleties with respect to the susceptibility of various protein-synthesizing systems to ipecac alkaloids and their conformational analogues, the glutarimide antibiotics.

INTRODUCTION

Eukaryotic cells contain at least two distinct systems for protein synthesis, one located within mitochondria and the other in the cytosol. A description of the similarities and differences in properties of these two systems is emerging, in part as a result of inhibitor studies (1–4). It is especially interesting that the mitochondrial process appears to be more closely analogous to that of bacteria than to that of the cytosol. Thus *N*-formylmethionyl transfer ribonucleic acid, which is believed to be responsible for the initiation of bacterial protein synthesis, has been found in mitochondria but not in the cytosol (5, 6); the ribosomes of mitochondria are about the same size as bacterial ribosomes whereas those external to the mitochondria are larger (7–9); and the ribosomal ribonucleic acids of mitochondria are similar in molecular weight to bacterial ribosomal ribonucleic acids (9–12). Inhibitors that preferentially affect bacterial protein synthesis (chloramphenicol and the tetracyclines) are also inhibitors of mitochondrial protein synthesis, whereas cycloheximide, which inhibits eukaryotic cytosol protein synthesis, does not affect the mitochondrial systems (13–18).

Grollman has found that emetine hydrochloride is an inhibitor of protein synthesis in subcellular systems derived from several eukaryotic cells (*Saccharomyces cerevisiae* and *pastorianus*, *Anemia phylitidis*, rabbit reticulocytes, and HeLa cells) but not in those derived from *Escherichia coli*. This similarity to the inhibition spectrum of

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cycloheximide was ascribed to structural and conformational similarities of emetine to the glutarimide antibiotics (19). The present study extends the inhibition spectrum of emetine hydrochloride to include mitochondrial protein synthesis. Since cycloheximide does not inhibit the mitochondrial system, a differential effect has been demonstrated. Furthermore, emetine has been shown to be capable of selectively inhibiting mitochondrial but not bacterial protein synthesis.

EXPERIMENTAL PROCEDURE

Animals. Adult (25–30-g) male mice of the Swiss albino strain (HRA/ICR obtained from Hazleton-Carbia, Rockville, Md.) were fed a diet of Purina laboratory chow and water ad libitum, bedded in Sani-Chips (Chesapeake Feed Company, Beltsville, Md.), and maintained in rooms at 22–24° with a 12-hr light cycle.

Isolation of mitochondria. The mice were killed by stunning followed by cervical dislocation. After washing the abdominal wall with 95% ethanol, the livers were quickly excised, the gallbladders were removed, and the livers were rinsed three times with ice-cold homogenization medium M (0.3 M mannitol–0.1 mM EDTA, adjusted to pH 7.4 with NaOH). After weighing, each liver was added to 9 volumes of the same cold medium and homogenized with five strokes of a Teflon pestle rotating at 1725 rpm in a glass vessel. The homogenate was then centrifuged at 2000 × *g* for 7 min to remove unbroken cells and nuclei. The supernatant fluid was collected, and the mitochondria were recovered by centrifugation at 6000 × *g* for 10 min. The fluffy, lighter-colored layer overlying the tan pellet was gently agitated after addition of about 1 ml of fresh cold homogenization medium, and removed by aspiration. The pellet of mitochondria was then suspended by gentle homogenization in cold homogenization medium, the protein concentration was measured by the method of Lowry *et al.* (20), and the concentration was adjusted to 25 mg of mitochondrial protein per milliliter with homogenization medium. This method of mitochondrial isolation is similar to that described by Honjo *et al.* (21) for the isolation of mitochondria with good respiratory control.

Isolation of microsomes and pH 5 fraction. The mice were killed and the livers removed as described for the isolation of mitochondria. After two rinses in homogenization medium A (0.35 M sucrose; 70 mM KCl; 50 mM Tris-HCl, pH 7.8; 40 mM $MgCl_2$; and 6 mM β-mercaptoethanol), the livers were homogenized in 2.5 volumes of the same medium and centrifuged at 1600 × *g* for 15 min. The pellet was discarded, and the supernatant fluid was centrifuged at 27,000 × *g* for 15 min. The pellet was again discarded, and the supernatant fluid was centrifuged at 105,000 × *g* for 2 hr after the addition of 2 volumes of medium B (homogenization medium A without Tris-HCl). This microsomal pellet was rinsed with homogenization medium A and suspended in the same medium to a protein concentration of 15 mg/ml.

The supernatant fluid from the last centrifugation was adjusted to pH 5 with 1 M acetic acid and stirred while on ice for 15 min. After centrifugation, the pellet was suspended in homogenization medium A to a final protein concentration of 20 mg/ml to provide the pH 5 fraction.

Isolation of E. coli S-30 fraction. An S-30 fraction of *E. coli* A19 (deficient in ribonuclease) was prepared as described by Nathans (22).

Reaction system. Initial reaction rates were utilized in each case by demonstrating linearity as a function of time for at least twice the time interval used. In addition, the reaction mixtures chosen exhibited linearity as a function of the concentration of mitochondria, microsomes, or S-30 fraction over the ranges used and saturation with respect to the substrate, L-phenylalanine.

Materials. The scintillation fluid contained 1125 ml of ethanol, 1000 ml of xylene, 1000 ml of dioxane, 240 g of naphthalene, 15 g of 2,5-diphenyloxazole, and 0.15 g of *p*-bis[2-(5-phenyloxazolyl)]benzene. Emetine hydrochloride, isoemetine hydrobromide, anisomycin, and *O*-methylpsychotrine oxylate were gifts from Dr. Arthur P. Grollman

of The Albert Einstein College of Medicine. MS2 RNA was a gift from Dr. Daniel Nathans of The Johns Hopkins University School of Medicine. Chloramphenicol was purchased from Calbiochem, cycloheximide from Nutritional Biochemicals Corporation, polyuridylic acid from Miles Laboratories, and ^{14}C-L-phenylalanine (uniformly labeled) from New England Nuclear Corporation.

RESULTS

The effects of various levels of emetine hydrochloride on mitochondrial protein synthesis are shown in Table 1. Emetine hydrochloride is clearly capable of inhibiting mitochondrial protein synthesis, as shown by a representative experiment described in this table. Fifty per cent inhibition is achieved at a concentration of emetine of about 0.1 mM. The degree of inhibition obtained with 1 mM emetine is comparable to that achieved with the same level of chloramphenicol, and considerably greater than that produced by 1 mM cycloheximide. Thus extension of the inhibition spectrum to include mitochondrial protein synthesis as well as prokaryotic and extramitochondrial eukaryotic protein synthesis permits a clear differentiation between emetine hydrochloride and cycloheximide.

The effectiveness of emetine hydrochloride as an inhibitor of a eukaryotic microsomal system has been confirmed, as shown in Table 2, using mouse liver. In addition, it is

TABLE 1

Inhibition of protein synthesis in mouse liver mitochondria: effects of emetine, chloramphenicol, and cycloheximide

Reactions were carried out for 30 min at 30° in a final volume of 1.0 ml. Final concentrations in each reaction mixture were as follows: potassium phosphate buffer, pH 7.2, 200 mM; potassium chloride, 50 mM; magnesium chloride, 10 mM; bovine plasma albumin, 1 mg/ml; phosphoenolpyruvate, 5 mM; pyruvate kinase, 25 μg/ml; adenosine triphosphate, 2 mM; 19 L-amino acids, excluding phenylalanine, 200 μM each; ^{14}C-phenylalanine, 0.3 μM (41.6 μCi/μmole); mitochondrial protein, 2.5 mg/ml. Reactions were terminated by adding 5 ml of 10% trichloracetic acid containing 0.1% DL-phenylalanine. The precipitates were heated for 15 min at 90°, washed twice with 5% trichloracetic acid containing 0.1% DL-phenylalanine, once with ethanol–ether (1:1 by volume), and once with ether, then dissolved in 0.5 ml of 88% formic acid, added to 15 ml of scintillation fluid, and counted at approximately 53% efficiency in a liquid scintillation counter. Zero time controls, of about 100 cpm, have been subtracted.

Inhibitor	Inhibitor concentration	Phenylalanine incorporation	Inhibition
	mM	*μμmoles/mg mitochondrial protein*	%
None		13.5	0
Emetine hydrochloride	0.01	10.9	19
	0.05	8.0	41
	0.1	6.6	51
	0.5	3.4	75
	1.0	2.7	80
Chloramphenicol	1.0	2.6	81
Cycloheximide	1.0	12.1	10

TABLE 2

Inhibition of protein synthesis in mouse liver microsomes: effects of emetine, chloramphenicol, and cycloheximide

Reactions were carried out for 20 min at 37° in a total volume of 0.5 ml. Final concentrations in each reaction mixture were as follows: Tris-HCl, pH 7.8, 5.9 mM; potassium chloride, 8.2 mM; magnesium chloride, 3.3 mM; phosphoenolpyruvate, 12.3 mM; pyruvate kinase, 40 μg/ml; adenosine triphosphate, 1 mM; 19 L-amino acids, excluding phenylalanine, 100 μM each; ^{14}C-phenylalanine, 40 μM (41.6 μCi/μmole); sucrose, 41 mM; mercaptoethanol, 700 μM; guanosine triphosphate, 200 μM; polyuridylic acid, 0.2 mg/ml; microsomal protein, 1.5 mg; pH 5 fraction, 5 mg of protein. Reactions were terminated and precipitates prepared and counted as described in Table 1. Zero time controls, of about 120 cpm, have been subtracted.

Inhibitor	Inhibitor concentration	Phenylalanine incorporation	Inhibition
	mM	*μμmoles/mg protein*	%
None		8.1	0
Emetine hydrochloride	0.01	6.2	23
	0.1	2.1	74
	1.0	0.8	89
Chloramphenicol	1.0	10.1	0
Cycloheximide	1.0	3.2	60

TABLE 3

Inhibition of bacterial (E. coli) protein synthesis: effects of emetine, chloramphenicol, and cycloheximide

Reactions were carried out for 15 min at 37° in a total volume of 0.040 ml. Final concentrations in each reaction mixture were as follows: Tris-HCl, pH 7.8, 54 mM; potassium chloride, 45 mM; magnesium acetate, 14 mM; phosphoenolpyruvate, 8 mM; pyruvate kinase, 1.08 μg/0.04 ml of reaction mixture; adenosine triphosphate, 2.8 mM; 19 L-amino acids, excluding phenylalanine, 9 μM each; ^{14}C-phenylalanine, 6 μM (475 μCi/μmole); glutathione, 14 mM; guanosine triphosphate, 15 μM; *E. coli* soluble ribonucleic acid, 10.7 μg/0.040 ml of reaction mixture; poly U or MS2 RNA, 13 μg (included as indicated above); S-30 fraction, 0.28 mg of protein. Reactions were stopped by spotting 0.020 ml of the reaction mixture onto a Whatman No. 3MM filter paper disc 2.4 cm in diameter. The discs were then processed as described by Bollum (23) and counted in a liquid scintillation counter at about 61% efficiency. Zero time controls, of about 100 cpm, have been subtracted.

Inhibitor	Inhibitor concentration	Phenylalanine incorporation	Inhibition
	mM	*mμmoles/0.28 mg S-30 protein*	%
With poly U			
No inhibitor		144	
Emetine hydrochloride	1.0	150	
Chloramphenicol	1.0	109	27
Cycloheximide	1.0	151	
With MS2 RNA			
No inhibitor		9.4	
Emetine hydrochloride	1.0	9.1	3
Chloramphenicol	1.0	1.2	88
Cycloheximide	1.0	8.1	13

apparent that the concentrations of emetine hydrochloride needed for inhibition of microsomal protein synthesis are similar to those found to effect a similar degree of inhibition in the mitochondrial system. The effect on this microsomal protein-synthesizing system permits a clear differentiation in the spectrum of activity of emetine hydrochloride and chloramphenicol, since the latter does not affect the microsomal system.

TABLE 4

Inhibition of mouse liver mitochondrial protein synthesis: effects of emetine hydrochloride, isoemetine hydrobromide, and O-methylpsychotrine oxylate

Reactions were carried out as described in Table 1.

Inhibitor	Inhibitor concentration	Phenylalanine incorporation	Inhibition
	mM	*μmoles/mg protein*	%
None		13.1	0
Emetine hydrochloride	0.01	11.1	15
	0.1	5.9	55
	1.0	2.7	79
Isoemetine hydrobromide	0.01	12.3	7
	0.1	11.2	15
	1.0	6.5	50
O-Methylpsychotrine oxylate	0.01	12.0	8
	0.1	11.8	10
	1.0	6.5	50

The lack of effect of emetine on protein synthesis in a bacterial system is illustrated in Table 3. The relative ineffectiveness of chloramphenicol when poly U is used as a messenger has been previously documented by Kucan and Lipmann (24). Emetine hydrochloride is the first inhibitor of protein synthesis that has been shown to inhibit both microsomal and mitochondrial protein synthesis in eukaryotic cells but to be without inhibitory effect in prokaryotic cells.

Since it has been proposed that emetine hydrochloride inhibits protein synthesis in extramitochondrial eukaryotic systems because of its structural similarity to cycloheximide, other structural and conformational analogues of ipecac alkaloids were tested in the mouse liver mitochondrial system. The results for two of these analogues are shown in Table 4.

Although both isoemetine hydrobromide and *O*-methylpsychotrine oxylate inhibit mitochondrial protein synthesis, the concentrations required for 50% inhibition are about 10 times those necessary with emetine

TABLE 5

Inhibition of mouse liver mitochondrial and microsomal protein synthesis: effects of anisomycin, chloramphenicol, and cycloheximide

Mitochondrial protein synthesis was studied as described in Table 1, and microsomal protein synthesis as described in Table 2.

Inhibitor	Inhibitor concentration	Phenylalanine incorporation	Inhibition
	mM	*μμmoles/mg protein*	%
Mitochondrial system			
No inhibitor		7.68	
Anisomycin	0.01	7.26	5
	0.1	7.08	8
	1.0	6.29	18
Chloramphenicol	1.0	3.55	54
Cycloheximide	1.0	6.65	13
Microsomal system			
Without poly U			
No inhibitor		7.40	
Anisomycin	0.01	4.09	45
	0.1	2.11	71
	1.0	1.35	82
Chloramphenicol	1.0	8.19	
Cycloheximide	1.0	1.46	80
With poly U (0.2 mg/ml)			
No inhibitor		25.96	
Anisomycin	0.01	13.05	50
	0.1	8.41	68
	1.0	2.30	91
Chloramphenicol	1.0	27.01	
Cycloheximide	1.0	4.24	84

hydrochloride. In rabbit reticulocytes more than 100 times as much of either of these agents is needed to achieve 50% inhibition of protein synthesis *in vitro*, and more than 1000 times as much *in vivo* (19). Jondorf *et al.* (25) have demonstrated marked differences between emetine and either isoemetine or *O*-methylpsychotrine in experiments on hepatic protein synthesis in the rat both *in vivo* and *in vitro*, confirming the stereochemical requirements for biological activity proposed by Grollman (19). These requirements appear to be less stringent in the present mitochondrial system.

Another inhibitor of protein synthesis, anisomycin, has also been noted to share a similar inhibition spectrum with cycloheximide and emetine (26). The effects of anisomycin on both the mouse liver mitochondrial system and the mouse liver microsomal system are shown in Table 5.

Anisomycin is ineffective in inhibiting mitochondrial protein synthesis as compared to its effect on a microsomal system, with or without the addition of poly U as an exogenous messenger. Anisomycin has recently been shown to inhibit the peptidyltransferase associated with 80 S ribosomes but not that of 70 S ribosomes, and thus appears to act at a different site from that affected by cycloheximide (27, 28). Its inactivity in the mitochondrial system may be related to the difference between mitochondrial ribosomes and those in the extramitochondrial cytoplasm.

DISCUSSION

Emetine hydrochloride inhibits protein synthesis in mitochondria isolated from mouse liver. This finding has several implications.

It has been suggested that emetine hydrochloride acts as an inhibitor of protein synthesis because of its structural and conformational similarity to cycloheximide (19). Thus the mechanism of action should be the same for these two agents, and one might expect the spectrum of systems inhibited by or resistant to each to be identical.

Cycloheximide (28, 29) and emetine (28) have been shown to be capable of inhibiting the translocation of peptidyl-tRNA from the acceptor site to the donor site on eukaryotic ribosomes. Recently, however, Baliga *et al.* (30) have shown that cycloheximide also inhibits the aggregation of ribosomes and does this at a much lower concentration. They have suggested that these two sites of action of cycloheximide are distinct.

The present experiments have confirmed that bacterial systems are resistant to both agents while eukaryotic extramitochondrial systems are susceptible to both. The protein-

synthesizing system of isolated mouse liver mitochondria, however, clearly distinguishes between emetine and cycloheximide, being inhibited by the former but not by the latter. This selectivity must reside in structural differences between the two agents. Several explanations for this selectivity are possible.

It is possible that the structural differences between cycloheximide and emetine allow an interaction between emetine and analogous bacterial, mitochondrial, and extramitochondrial sites but restrict the interaction with cycloheximide to the extramitochondrial site. This possibility would remain compatible with an identity in the mechanism of action of these two agents based on structural similarities. It is also possible, however, that in spite of certain similarities of molecular structure, the mechanism of action of emetine differs from that of cycloheximide.

The selectivity need not, however, be at the final site of action of the drugs. Thus the mitochondrion might permit the entry of emetine but not of cycloheximide. In this case the selectivity would be shown by some transport process and not by the protein-synthetic system itself. Somewhat contrary to this possibility is the report that a submitochondrial system from yeast remains resistant to cycloheximide (31). Nevertheless, it remains possible that cycloheximide fails either to enter the mouse liver mitochondrion or to localize at a specific site within the mitochondrion.

The present findings could also be explained by a mitochondrial inactivation of cycloheximide but not of emetine. Again the selectivity would be based on a molecular difference, but would be located at some site other than one directly concerned with protein synthesis.

Anisomycin, on the other hand, fails to inhibit mitochondrial protein synthesis. Thus, at least with respect to its spectrum of activity, anisomycin continues to parallel cycloheximide, and the mitochondrial system apparently can distinguish between anisomycin and emetine whereas the microsomal system cannot.

The effectiveness of emetine as an inhibitor of mitochondrial protein synthesis and its ineffectiveness in bacterial systems may be of value in distinguishing mitochondrial protein-synthesizing systems from contaminating bacteria.

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