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The Inhibition of Liver Ribonucleic Acid Synthesis by Ethionine*

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SUMMARY

The administration of DL-ethionine to female rats inhibits liver ribonucleic acid synthesis up to 90 to 95%. The inhibition of RNA synthesis follows in time the decrease in adenosine triphosphate concentration and precedes the inhibition of protein synthesis. The degree of inhibition of RNA synthesis is a function of the dose of ethionine. The curve for this inhibition parallels closely the curves for the decrease in ATP concentration and for the inhibition of protein synthesis with varying dosages of ethionine. The simultaneous administration of methionine reverses to a considerable extent the inhibition of RNA synthesis by ethionine.

Ethionine, the ethyl analogue of methionine (2), when injected into female rats induces a rapid fall in hepatic adenosine triphosphate concentration (3), followed by a marked inhibition of protein synthesis (4) and the accumulation of triglycerides in the liver (5, 6). The main site of ethionine interference with protein synthesis is the polysome (4, 7, 8), which shows a progressive disaggregation into monomers within a few hours after administration of the analogue (9).

Since ATP is one of the substrates for the synthesis of ribonucleic acid, and since ATP is important in the synthesis of the other nucleoside triphosphates, it became of interest to observe whether the decrease in ATP concentration might be accompanied by a decrease in RNA synthesis. Conceivably, such an interference with the synthesis of RNA, including messenger RNA, might play a role in the ethionine-induced inhibition of protein synthesis and in the disaggregation of liver polyribosomes. The striking inhibition of liver RNA synthesis induced by ethionine is the subject of this communication.

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‡ Sarah Mellon Scaife Fellow, 1962 to 1964.

EXPERIMENTAL PROCEDURE

White female rats of the Wistar strain (Carworth Farms) maintained on Wayne Lab Blox and weighing 180 to 220 g, were deprived of food overnight and were given injections of aqueous solutions of 0.153 M DL-ethionine, 0.053 M DL-methionine, or 0.053 M adenine sulfate, as indicated in the tables and figure legends. Control animals received equal volumes of 0.9% NaCl solution. Orotic acid-6-^{14}C (5 mC per mmole, New England Nuclear) was injected into the portal vein as a single dose of 5 μC 15 min before the animals were killed. The injection was performed after laparotomy under Nembutal anesthesia (6 mg/100 g of body weight).

Nuclear, microsomal, and soluble RNAs were isolated from the liver by the following procedure. The rapidly excised liver was homogenized in 2.3 volumes of ice-cold Solution A of Littlefield and Keller (10) with a Teflon-glass homogenizer, and was centrifuged for 15 min at 15,000 rpm in a Spinco model L ultracentrifuge with a No. 40 rotor. The supernatant was saved and the sediment was suspended in 20 volumes of 2.1 M sucrose containing 0.001 M $MgCl_2$, 0.0035 M K_2HPO_4, and 0.0007 M ATP, adjusted to pH 6.7 (11), and was centrifuged for 2 hours at 25,000 rpm to yield a nuclear pellet (12). This supernatant was discarded. To the first supernatant was added an equal volume of Solution A. This was centrifuged for 2 hours at 30,000 rpm to yield a microsomal pellet. This supernatant was adjusted to pH 5.1, and the precipitate was recovered by centrifugation. The nuclear and microsomal pellets and the pH 5.1 precipitate were suspended in 1.35 ml of Solution A per g of original liver. An equal volume of 90% aqueous phenol was added and RNA was extracted by shaking for 1 hour at room temperature. Before shaking, 0.1 ml of 10% sodium dodecyl sulfate for each 10 ml of Solution A was added to the nuclear suspension. After extraction, the samples were centrifuged for 10 min at 10,000 rpm. To the aqueous layers were added 0.1 volume of 20% potassium acetate, pH 5.0, and 3 volumes of ice-cold absolute ethanol. After at least 6 hours in the freezer, the RNA was collected by centrifugation and dissolved in H_2O. Traces of phenol were removed by extraction with ethyl ether. Aliquots of this RNA solution were added to a dioxane mixture (13), and units of radioactivity were counted in a Packard liquid scintillation spectrometer. RNA concentration was measured in a Beckman model DU spectrophotometer at 260 mμ, with the use of an equivalent of 20 optical density units per mg of RNA (14).

Some samples of RNA were run in a linear sucrose gradient

from 0.5 to 1 M, containing 0.005 M Tris buffer, pH 7.6, in the Spinco SW 25.1 rotor for 40 hours at 25,000 rpm. At the end of this period the tubes were punctured and were automatically analyzed at 260 mμ. Fractions (1 ml) were collected and their radioactivity was counted.

The acid-soluble fraction was obtained by homogenizing a suitable aliquot of liver (approximately 0.5 g) in 10 volumes of 5% perchloric acid and centrifuging the suspension at 2000 rpm for 30 min. For determination of total acid-soluble radioactivity an aliquot (1 ml or less) of the supernatant was mixed with Bray's solution (13) and was counted in the liquid scintillation spectrometer. For determination of radioactivity in UMP and CMP, the acid extract was heated at 100° for 30 min. This procedure hydrolyzes the various uracil and cytosine nucleotides to UMP and CMP. On cooling, the extract was neutralized with 38% KOH and was centrifuged. The supernatant, after removal of the potassium perchlorate, was added to a column (1 × 4 to 5 cm) of Dowex 1-X2, Cl^- form. The column was washed with H_2O until the 260 mμ absorption of the eluate reached a constant minimal level. The column was then eluted with 0.005 M HCl to obtain CMP and UMP. The amount of each nucleotide and its radioactivity were measured.

RESULTS

The administration of ethionine to female rats consistently inhibits the labeling by orotic acid-6-^{14}C of nuclear as well as of microsomal and supernatant RNA in the liver (Table I). The degree of inhibition in the nuclear fraction varied between 88 and 94%. The magnitude of the inhibition is somewhat less in the cytoplasmic RNA fractions. The decrease in radioactivity in RNA is not due to differences in the uptake of orotic acid by the liver, since the total acid-soluble fractions in both control and experimental groups were of the same order of magnitude.

Since the decreased labeling of RNA by orotic acid in the ethionine-treated rats could be due to a decrease in the specific activity of the precursor pyrimidine nucleotides, UMP and CMP were isolated from the total acid-soluble fraction of the liver 15 min after the intraportal administration of orotic acid to animals treated with ethionine or NaCl 4 hours previously. As recorded in Table II, there is no inhibition of the conversion of orotic acid to UMP in the ethionine-treated animal. The increased specific activity of the isolated UMP in the experimental animals is probably a reflection of the decrease in the utilization of UMP. All

TABLE I

Effect of ethionine upon labeling in vivo of liver RNA fractions by orotate-6-^{14}C

The animals in the ethionine group received intraperitoneally 1.23 mmoles of DL-ethionine, and animals in the control group received 0.9% NaCl solution, both at zero time. Both groups received 5 μC of orotic acid-6-^{14}C at 3 hours and 45 min. The livers were rapidly removed from the anesthetized animals at 4 hours. Each value was obtained from two pooled livers in Experiments 1 and 2, and from three pooled livers in Experiment 3.

Groups	Radioactivity in liver			
	Nuclear RNA	Microsomal RNA	Soluble RNA	Total acid-soluble fraction
	cpm/mg (%)	*cpm/mg* (%)	*cpm/mg* (%)	*cpm/50 mg liver*
Experiment 1				
Control....	42,151 (100)	313 (100)	1,116 (100)	1,355 (100)
Ethionine..	2,591 (6)	62 (20)	189 (17)	1,496 (112)
Experiment 2				
Control....	39,970 (100)	212 (100)	1,043 (100)	
Ethionine..	3,505 (9)	67 (31)	246 (24)	
Experiment 3				
Control....	43,668 (100)	449 (100)	671 (100)	1,126 (100)
Ethionine..	5,124 (12)	96 (21)	225 (34)	981 (87)

TABLE II

Radioactivity in liver acid-soluble uracil nucleotides after administration of orotic acid-6-^{14}C to ethionine-treated and control rats

The animals received 1 mg of DL-ethionine per g of body weight or the same volume of NaCl and were killed 4 hours later. Orotic acid-6-^{14}C (5 μC) was injected intraportally 15 min before the animals were killed. In this experiment, nuclear RNA in the ethionine-treated rats had 90% less radioactivity than in the control animals.

Animal	Treatment	Radioactivity in total acid-soluble fraction	Specific radioactivity in acid-soluble UMP	Total radioactivity in UMP in total acid-soluble fraction	Radioactivity in acid-soluble fraction as UMP
		cpm/ml	*cpm/μmole* × 10^{-3}	*cpm/ml*	%
1	NaCl	12,774	49	15,080	118
2	NaCl	14,348	58	14,382	100
3	Ethionine	22,182	95	23,324	105
4	Ethionine	13,032	130	14,769	113

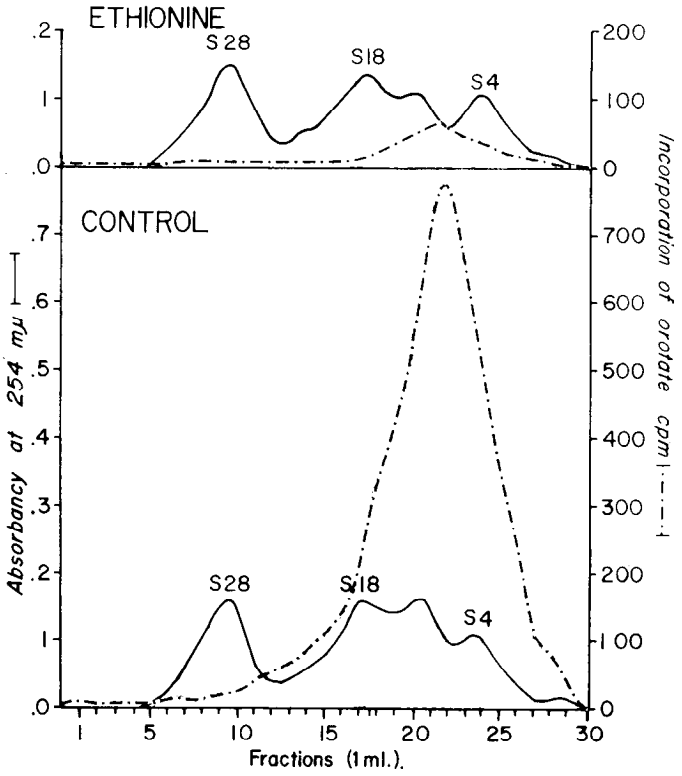

FIG. 1. Patterns of nuclear RNA from control (*bottom*) and ethionine-treated (*top*) female rat liver. The animals received 1.23 mmoles of DL-ethionine or NaCl intraperitoneally at zero time and 5 μC of orotate-6-^{14}C in the portal vein at 4¾ hours, and were killed at 5 hours. The peak in optical density between S18 and S4 is mostly DNA.

the radioactivity appears to be in UMP, since it accounted for 100% of the radioactivity of the total acid-soluble fraction. This result was expected, since at the time interval and under the experimental conditions used, all the radioactivity in the acid-soluble fraction of liver has been reported to be in the form of uracil nucleotides, with essentially none in cytosine derivatives (15, 16).

As seen in Fig. 1, most of the radioactivity in the total nuclear RNA from a control animal is present in a peak between *S18* and *S4*. This peak is very small in the RNA from the ethionine-treated animal.

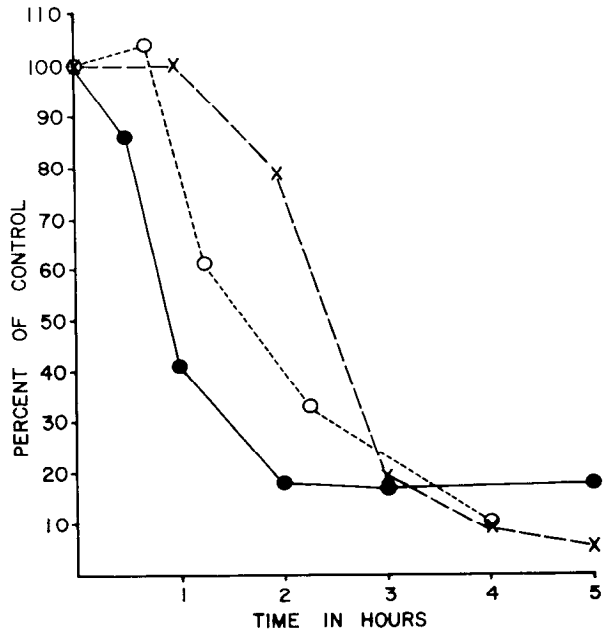

FIG. 2. Time course of changes in hepatic ATP concentration (●——●), labeling of nuclear RNA with orotate-6-^{14}C (○-----○), and ribosomal protein synthesis (^{14}C-leucine incorporation) *in vitro* (×- - -×). The methods and data for ATP and protein synthesis were described previously (4). The animals received 1.23 mmoles of DL-ethionine at zero time.

Since characteristic relationships between the inhibition of protein synthesis and the levels of ATP have been found as functions of both dosage and duration of action of ethionine (4), it became of importance to determine the effects of duration and dosage of ethionine upon the inhibition of labeling of RNA by orotic acid. It is apparent from Fig. 2 that the inhibition of incorporation of orotic acid into nuclear RNA follows the decrease in ATP concentration and precedes the inhibition of incorporation *in vitro* of leucine into protein by ribosome preparations. The minimal level of labeling of RNA is found when orotic acid is injected between 2 and 4 hours after the administration of ethionine.

The parallelism in the responses of orotate labeling of nuclear RNA, ATP concentration, and incorporation *in vitro* of leucine into ribosomal protein to various doses of ethionine is evident in Fig. 3. Each parameter reaches a minimum value between 0.8 and 1 mg of ethionine per 100 g of body weight.

Methionine, adenine, and other ATP precursors are effective in preventing the decrease in ATP concentration (3, 4, 17), the inhibition of protein synthesis (4, 8, 17), and the accumulation of liver triglyceride (5, 18, 19) following ethionine administration. Therefore, it was of interest to observe whether adenine or methionine would have the same effect on the inhibition of orotate incorporation into liver RNA. As is evident in Table III, adenine *per se* decreases the labeling of liver RNA by orotate as much as does ethionine, and therefore its effect on ethionine inhibition cannot be tested in this way. This effect of adenine upon labeling of RNA is reproducible, and remains as yet unexplained. Methionine reverses the ethionine inhibition to a major degree. The failure to prevent inhibition completely may be due to an effect of this amino acid on nucleotide pool size, since methionine does interact with ATP to form *S*-adenosylmethionine.

DISCUSSION

It is evident from the results of this study that the labeling of nuclear, microsomal, and soluble RNAs by radioactive orotate is markedly decreased in female rats given ethionine. The labeling of RNA by orotate is dependent upon the following reactions.

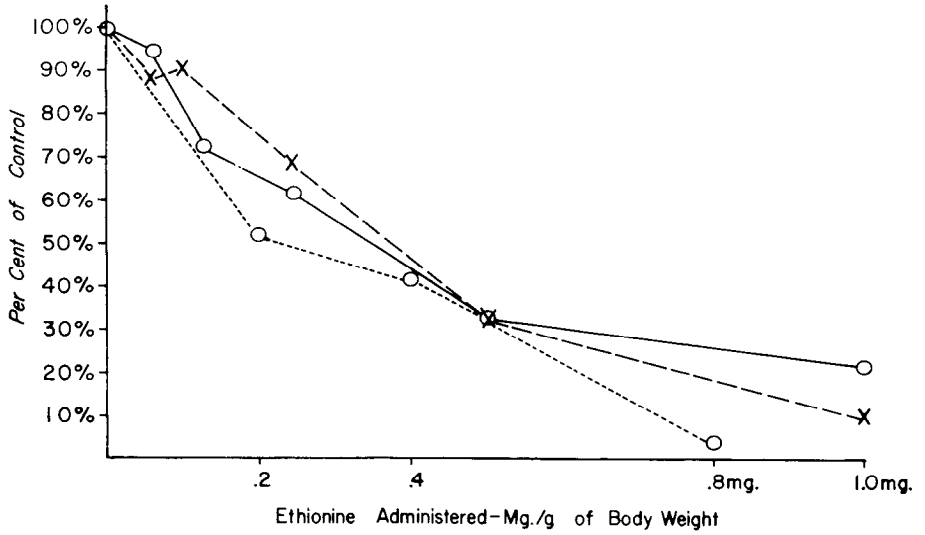

FIG. 3. Hepatic ATP concentration (○——○), nuclear RNA synthesis (orotate-6-^{14}C incorporation) (○-----○), and ribosomal protein synthesis *in vitro* (^{14}C-leucine incorporation) (×- - -×) as functions of the dosage of ethionine. The animals received the ethionine at zero time and were killed 5 hours later. The methods and data for ATP and protein synthesis were described previously (4).

TABLE III

Effects of methionine or adenine upon incorporation in vivo of orotate-6-^{14}C into liver RNA fractions in rats given ethionine

The NaCl, ethionine, adenine, and methionine were injected intraperitoneally at time zero. The orotate-6-^{14}C was injected intraportally at 4¾ hours, and the animals were killed at 5 hours.

Treatment	Radioactivity in liver RNA		
	Nuclear	Microsomal	Supernatant
	cpm/mg (%)	*cpm/mg* (%)	*cpm/mg* (%)
Experiment 1[a]			
NaCl	39,970 (100)	212 (100)	1,043 (100)
Adenine	3,756 (9)	28 (13)	213 (20)
Ethionine	3,505 (9)	67 (31)	246 (24)
Ethionine plus adenine	6,696 (16)	72 (33)	245 (24)
Experiment 2[b]			
NaCl	27,027 ± 1,902 (100)	86 ± 14 (100)	857 ± 174 (100)
Methionine	31,084 ± 3,866 (115)	86 ± 9 (100)	911 ± 248 (106)
Ethionine	1,572 ± 682 (5)	27 ± 8 (31)	189 ± 45 (22)
Ethionine plus methionine	16,644 ± 1,640 (62)	74 ± 14 (86)	514 ± 57 (60)
Experiment 3[b]			
NaCl	34,147 ± 3,658 (100)	125 ± 19 (100)	1,036 ± 64 (100)
Methionine	21,180 ± 4,091 (62)	140 ± 14 (112)	928 ± 204 (90)
Ethionine	2,480 ± 432 (7)	26 ± 2 (20)	261 ± 24 (25)
Ethionine plus methionine	18,286 ± 1,665 (54)	48 ± 2 (38)	469 ± 66 (45)

[a] Each value is the mean from two separate animals.

[b] Each value is the mean from three animals ± the standard error of the mean.

$$\text{Orotate} \rightarrow \text{orotidylate} \rightarrow \text{UMP} \rightarrow \text{UDP} \rightarrow \left.\begin{matrix}\text{UTP} \\ \downarrow \\ \text{CTP}\end{matrix}\right\} \rightarrow \text{RNA}$$

In the time intervals used in this study, no labeling of cytosine nucleotides was found, in agreement with previous reports (15, 16). Also, no inhibition of labeling of uracil nucleotides was found in the ethionine-treated rats. Therefore, it must be concluded that ethionine is affecting labeling of RNA by interference with one or more steps between UMP and RNA. Since ATP is required for phosphorylation of UMP to UTP and is an essential substrate for RNA polymerase activity, it is reasonable to suggest that the inhibition of labeling of RNA by ethionine is truly an inhibition of RNA synthesis mediated via the large and rapid decrease in the concentration of ATP resulting from excessive trapping of the adenosine moiety of this nucleotide as *S*-adenosylethionine (20–22).

Further evidence for the conclusion that RNA synthesis is inhibited in the ethionine-treated rat is the finding of a net decrease in the RNA content of rat liver nuclei several hours after ethionine administration (23), similar to that observed with actinomycin (24, 25). The administration of adenine, methionine, or both completely prevented this loss or reversed it even when these compounds were administered many hours after the time of injection of ethionine.

The inhibition by ethionine of hepatic RNA synthesis, no doubt including messenger RNA synthesis, previously was considered to be a likely mechanism for the breakup of polyribosomes to ribosome monomers and for the accompanying decrease of protein synthesis (9). However, recent work in our laboratory[1] has clearly shown that this is not the probable mechanism. The adenine- or methionine-induced reassembly of ribosomes into polyribosomes in the ethionine-treated animals occurs to an equal degree in control animals and in animals in which the return of RNA synthesis to normal is prevented by the administration of actinomycin D.

The inhibition of RNA synthesis in rat liver by ethionine is another tool with which one may study the metabolism of various moieties of RNA under controlled conditions in an intact animal. Since the degree of inhibition is a function of the dosage of ethionine administered and since the inhibition can be readily prevented or counteracted by adenine, methionine, or both, it becomes feasible to investigate several phases of the metabolism of RNA in a system which can be readily reversed. Hopefully, new insight into some aspects of the regulation of RNA by living cells may be facilitated by the use of this tool (26).

[1] G. A. Stewart and E. Farber, unpublished work.

REFERENCES

1. VILLA-TREVINO, S., SHULL, K. H., AND FARBER, E., *Federation Proc.*, **22**, 237 (1963).
2. DYER, H. M., *J. Biol. Chem.*, **124**, 519 (1938).
3. SHULL, K. H., *J. Biol. Chem.*, **237**, PC1734 (1962).
4. VILLA-TREVINO, S., SHULL, K. H., AND FARBER, E., *J. Biol. Chem.*, **238**, 1757 (1963).
5. FARBER, E., SIMPSON, M. V., AND TARVER, H., *J. Biol. Chem.*, **182**, 91 (1950).
6. FARBER, E., SHULL, K. H., VILLA-TREVINO, S., LOMBARDI, B., AND THOMAS, M., *Nature*, **203**, 34 (1964).
7. FARBER, E., AND CORBAN, M. S., *J. Biol. Chem.*, **233**, 625 (1958).
8. VILLA-TREVINO, S., AND FARBER, E., *Biochim. Biophys. Acta*, **61**, 649 (1962).
9. VILLA-TREVINO, S., FARBER, E., STAEHELIN, T., WETTSTEIN, F. O., AND NOLL, H., *J. Biol. Chem.*, **239**, 3826 (1964).
10. LITTLEFIELD, J. W., AND KELLER, E. B., *J. Biol. Chem.*, **224**, 13 (1957).
11. BARONDES, S. H., DINGMAN, C. W., AND SPORN, M. B., *Nature*, **196**, 145 (1962).
12. CHAUVEAU, J., MOULE, Y., AND ROULLIER, C., *Exptl. Cell Res.*, **11**, 317 (1956).

13. Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
14. Wettstein, F. O., Staehelin, T., and Noll, H., *Nature*, **197**, 430 (1963).
15. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195**, 257 (1952).
16. Fujioka, M., Koga, M., and Lieberman, I., *J. Biol. Chem.*, **238**, 3401 (1963).
17. Shull, K. H., and Villa-Trevino, S., *Biochem. Biophys. Res. Commun.*, **16**, 101 (1964).
18. Farber, E., Lombardi, B., and Castillo, A. E., *Lab. Invest.*, **12**, 873 (1963).
19. Farber, E., Shull, K. H., McConomy, J. M., and Castillo, A. E., *Biochem. Pharmacol.*, **14**, 761 (1965).
20. Farber, E., and Castillo, A. E., *Federation Proc.*, **22**, 370 (1963).
21. Smith, R. C., and Salmon, W. D., *Arch. Biochem. Biophys.*, **111**, 191 (1965).
22. Raina, A., Janne, J., and Siimes, M., *Acta Chem. Scand.*, **18**, 1804 (1964).
23. Stewart, G. A., Farber, J. L., Villa-Trevino, S., and Farber, E., *Federation Proc.*, **24**, 655 (1965).
24. Revel, M., and Hiatt, H. H., *Proc. Natl. Acad. Sci. U. S.*, **51**, 810 (1964).
25. Dingman, C. W., and Sporn, M. B., *Science*, **149**, 1251 (1965).
26. Stewart, G. A., and Farber, E., *Federation Proc.*, **25**, 646 (1966).