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THE EFFECT OF ETHIONINE ON RIBONUCLEIC ACID SYNTHESIS IN RAT LIVER

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1. By 1 h after administration of ethionine to the female rat the appearance of newly synthesized 18 S and 28 S rRNA in the cytoplasm is completely inhibited. This is not caused by inhibition of RNA synthesis, for the synthesis of the large ribosomal precursor RNA (45 S) and of tRNA continues. Cleavage of 45 S RNA to 32 S RNA also occurs, but there was no evidence for the accumulation of mature or immature rRNA in the nucleus. 2. The effect of ethionine on the maturation of rRNA was not mimicked by an inhibitor of protein synthesis (cycloheximide) or an inhibitor of polyamine synthesis [methylglyoxal bis(guanylhydrazone)]. 3. Unlike the ethionine-induced inhibition of protein synthesis, this effect was not prevented by concurrent administration of inosine. A similar effect could be induced in HeLa cells by incubation for 1 h in a medium lacking methionine. The ATP concentration in these cells was normal. From these two observations it was concluded that the effect of ethionine on rRNA maturation is not caused by an ethionine-induced lack of ATP. It is suggested that ethionine, by lowering the hepatic concentration of *S*-adenosylmethionine, prevents methylation of the ribosomal precursor. The methylation is essential for the correct maturation of the molecule; without methylation complete degradation occurs.

Ethionine, the ethyl analogue of the essential amino acid methionine, is a hepatotoxin and carcinogen (Farber, 1963, 1967). Administration of a large dose to the rat inhibits both protein synthesis (Simpson *et al.*, 1950; Villa-Trevino *et al.*, 1963) and RNA synthesis (Villa-Trevino *et al.*, 1966). Ethionine is a good substrate for the ATP–L-methionine *S*-adenosyltransferase (EC 2.5.1.6) of rat liver, but the *S*-adenosylethionine formed is a poor substrate for the enzymes utilizing *S*-adenosylmethionine, either for alkyltransferase reactions such as the alkylation of tRNA (Pegg, 1972) and the synthesis of choline (Simmonds *et al.*, 1950), or (because decarboxylation is inhibited) for the utilization of the main propyl chain of the molecule in the synthesis of polyamines (Raina *et al.*, 1964; Pegg, 1969). The result is an accumulation of *S*-adenosylethionine. This sequesters the adenine in the liver cell at a greater rate than it can be synthesized *de novo* and the ATP concentration is lowered (Stekol *et al.*, 1960; Shull, 1962; Shull *et al.*, 1966). Some of the biological defects in ethionine poisoning in the female rat, e.g. the inhibition of protein synthesis, occur from the same time as the fall in ATP concentration, and the severity of the inhibition is proportional to the amount of the decrease in ATP (Villa-Trevino *et al.*, 1963). If the fall in ATP concentration is prevented or reversed by administration of adenosine or metabolic precursors of adenosine such as inosine, the inhibition of protein synthesis can be prevented (Shull, 1962; Bartels & Hohorst, 1963; Shull & Villa-Trevino, 1964). For this reason it has been suggested that the primary reason for the effect of ethionine on protein synthesis is a decrease in ATP concentration (Farber *et al.*, 1964; Shull *et al.*, 1966; Kisilevsky *et al.*, 1973).

In liver parenchymal cells of the female rat poisoned with ethionine severe morphological changes in the nucleolus occur (Shinozuka *et al.*, 1968). Since the nucleolus is the site of ribosomal RNA synthesis (for reviews see Perry, 1967; Maden, 1971) ethionine was chosen as a positive control for experiments on the effect on RNA metabolism of various carcinogenic *N*-nitroso compounds. It was found that administration of ethionine affected the synthesis of mature 18 S and 28 S rRNA apparently by preventing the final maturation of the ribosomal precursor RNA. A previous report (Vaughan *et al.*, 1967) that in HeLa cells maturation

of the ribosomal precursor RNA does not take place if the cells are deprived of methionine for 6h was confirmed and extended to show that only 1h of methionine deprivation is necessary. The similarity between this effect and the change in RNA metabolism induced by ethionine in rat liver *in vivo* suggest that ethionine produces its effect by preventing methylation of the ribosomal precursor RNA and not through induction of a deficiency of ATP.

Materials and Methods

Animals

Sprague–Dawley rats from the National Institutes of Health stock maintained on Wayne Lab-Blox (Allied Mills, Chicago, Ill., U.S.A.), or Wistar rats from the Courtauld Institute stock maintained on Rowett Research Institute Diet 86, were used.

Treatment of animals

Female rats weighing 100 or 180g were used for the experiments in which DL-ethionine (750mg/kg in 3.5ml of 0.85% NaCl) was given by intraperitoneal injection. The rats were not fed for 18h before each experiment. Intravenous injections were given into the tail while the rat was under light diethyl ether anaesthesia.

Preparation of rat liver nuclear and cytoplasmic RNA for measurement of the incorporation of radioactive orotic acid into RNA species of different electrophoretic mobility

At various times after injection of the toxins or metabolic inhibitors each rat was given [6-^{14}C]orotic acid (100μCi) or [5-^{3}H]orotic acid (200μCi) both in 0.85% (w/v) NaCl by intravenous injection. Later, the rats were decapitated and tissue was removed from the median lobe of the liver of each rat. The liver was homogenized in 10ml of either 0.25M-sucrose–1mM-$MgCl_2$ or 0.32M-sucrose–2mM-$MgCl_2$/g of liver. The homogenate was centrifuged (10000*g*, 30min) and cytoplasmic RNA extracted from the supernatant. The sediment was resuspended in 0.25M-sucrose–1mM-$MgCl_2$ (4ml/g of liver), and nuclei were prepared by the method of Blobel & Potter (1966).

Cytoplasmic RNA was extracted from the 10000*g* supernatant by the addition of sodium dodecyl sulphate (4%, w/v) to give a final concentration of 0.1%. After 3min at room temperature, an equal volume of phenol–*m*-cresol–water (100:14:11, by wt.) was added, and the mixture shaken for 30min, centrifuged (10000*g*, 30min) and the supernatant used for gel electrophoresis. RNA was extracted from the nuclei from 0.6g of liver by resuspending them in 4ml of tri-isopropylnaphthalenesulphonate (2g in 100ml of 1% NaCl), and adding an equal volume of phenol–*m*-cresol–water. The mixture was then treated in the same way as the cytoplasm (Parish & Kirby, 1966; Loening, 1968).

Chemicals

DL-Ethionine (A grade) was purchased from Calbiochem, San Diego, Calif., U.S.A. Tri-isopropylnaphthalenesulphonate and 1-ethyl-2-{3-(1-ethylnaphtho[1,2*d*]thiazolin-2-ylidene)-2-methylpropenyl} naphtho[1,2*d*]thiazolium bromide (Stains all) was from Eastman–Kodak, Rochester, N.Y., U.S.A. SeaKem agarose was from Marine Colloids, P.O. Box 748, Rockland, Maine 04841, U.S.A. Nuclear–Chicago Solubilizer (NCS) was from Amersham/Searle, Chicago, Ill., U.S.A., or Hopkin and Williams, Chadwell Heath, Essex, U.K. Methylglyoxal bis-(guanylhydrazone) {1,1′-[(methylethanediylidene)dinitrilo]diguanidine} was purchased from Aldrich Chemical Co., and cycloheximide and firefly lanterns were from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Radioactive compounds

[5-^{3}H]Uridine (sp. radioactivity 20Ci/mmol), [5-^{3}H]orotic acid (sp. radioactivity 15–27Ci/mmol), [6-^{14}C]orotic acid (sp. radioactivity 60.8mCi/mmol) and L-[4,5-^{3}H]leucine (sp. radioactivity 53Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Measurement of radioactivity

All radioactivity measurements were made in scintillation counters by conventional methods. Corrections from c.p.m. to d.p.m. were made by the addition of [^{3}H]- or [^{14}C]-toluene of known radioactivity (Packard Instrument Co., La Grange, Ill., U.S.A.) or by use of the external standardization equipment of the scintillation counter. The method of determining radioactivity in polyacrylamide gel is given below.

Cell culture

HeLa cells (S3 strain from Dr. M. B. Sporn, Lung Cancer Unit, National Cancer Institute, Bethesda, Md., U.S.A.) were maintained in minimal essential medium (Eagle, 1959) from Gibco–Biocult, Paisley, U.K., containing 5% (v/v) calf serum. In some experiments the medium contained $NaHCO_3$ (2.2g/litre) and the culture was buffered by incubating in air containing 5% CO_2. In other experiments the medium contained 0.35g of $NaHCO_3$/litre, incubation was in air, and the medium was buffered with 20mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)ethanesulphonic acid].

Gel electrophoresis of RNA

RNA was fractionated by electrophoresis for 1–2h, at 200V, at 5°C in gels containing both

acrylamide and agarose (0.5% by wt.) in an EC 470 apparatus (E-C Apparatus Corp., St. Petersburg, Fla. 33709, U.S.A.) (Dingman & Peacock, 1968; Peacock & Dingman, 1968). The position of the major RNA species was found by staining with Stains-all [0.05% (w/v) in formamide–water (1:1, v/v)]. The background stain was removed by rinsing the gel in running tap water. The gel was cut into sections 1 mm in length and these were placed in scintillation counter vials with Nuclear–Chicago Solubilizer (1 ml) and left overnight at room temperature. Radioactivity was determined after the addition of 10 ml of 0.6% 2,5-diphenyloxazole in toluene.

Purification of liver tRNA from rats treated with ethionine and measurement of the incorporation in vivo of radioactivity from orotic acid into purines and pyrimidines

Female rats were starved overnight and then given an intraperitoneal injection of DL-ethionine (750 mg/kg body wt.) or an equal volume of 0.85% NaCl. Then 1 h later the rats were given either [5-^{3}H]- or [6-^{14}C]-orotic acid (200 μCi or 100 μCi/rat respectively), and 3 h after that the rats were killed. An equal weight of liver from a control and an ethionine-treated rat was combined and the cytoplasmic RNA was prepared as described above. To remove the bulk of the ribosomal RNA the aqueous phase (4 ml) after phenol extraction was mixed with water (2 ml) and 6 ml of a solution (pH 4.5) containing 0.8 M-NaCl, 1 mM-EDTA (disodium salt), 0.02 M-sodium acetate and 0.02 M-magnesium acetate, and centrifuged (2.5 h, 120000g). The soluble RNA was precipitated from the supernatant with ethanol (2.5 vol., −20°C, 16 h). The RNA was recovered by filtration (0.45 μm pore size; Millipore Corp., Bedford, Mass., U.S.A.) and redissolved in water (1 ml). After the addition of 5 M-NaCl (0.08 ml) the sample was chromatographed on a column (91 cm × 1.25 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) and eluted with 0.4 M-NaCl (0.2 ml/min). E_{260}^{1cm} in each 2 ml fraction, and radioactivity in a part (0.1 ml) of each fraction, was measured. Fractions containing 4S RNA were pooled and the RNA was recovered by filtration after precipitation with ethanol. The RNA was dissolved in 1 M-HCl and hydrolysed to pyrimidine 2′- and 3′-monophosphates and purine bases, by heating at 100°C for 1 h. The hydrolysate was chromatographed on Whatman 3 MM paper by using propan-2-ol–conc. HCl–water (85:22:16, by vol.) as solvent (Wyatt, 1951). The radioactivity in each part of the paper was measured by scintillation counting.

Measurement of ATP

This was done by the luciferin–luciferase technique, by using a scintillation counter (Strehler, 1965, 1968). HeLa cells (1 ml) were pipetted into 2 ml of boiling water, kept at 100°C for 10 min, then cooled and centrifuged. The supernatant (2 ml) was taken into a scintillation-counter vial and 0.1 M-arsenate buffer (pH 7.4; 0.2 ml) added. Then 0.1 ml of a solution of an extract of 50 mg of dried firefly lanterns in 5 ml of 0.05 M-potassium arsenate–0.02 M-$MgSO_4$ was added. Exactly 10 s later the vial was loaded into the counting chamber of the scintillation counter (Packard 3000). The counts were recorded for 20 s by using the settings 100% gain, window 60–65, coincidence off.

Measurement of the incorporation of radioactive precursors into RNA, DNA and protein in vivo

The rate of RNA, DNA and protein synthesis was estimated by measurement of the incorporation of radioactivity after an intraperitoneal dose of [6-^{14}C]orotic acid (0.025 mCi/kg body wt.) and [*methyl*-^{3}H]thymidine (0.5 mCi/kg body wt.) 15 min before death, or a subcutaneous dose of L-[4,5-^{3}H]-leucine (0.03 mCi/kg body wt.) 30 min before death. RNA, DNA and protein were separated by a modification of the method of Schneider (1945) as described by Munro & Fleck (1966). Protein was measured by the modified Lowry method (Layne, 1957), and DNA by the Burton (1956) method.

Extraction of RNA from HeLa cells and measurement of the incorporation of [5-^{3}H]uridine

HeLa cells growing in exponential phase at 2×10^5–4×10^5 cells/ml were concentrated to approx. 6.7×10^5 cells/ml by centrifugation and resuspension in a portion of the same medium. After the addition of [5-^{3}H]uridine (5 μCi/ml) they were incubated further in a shaker incubator. After the labelling period, the cells from 3 ml portions were collected by centrifugation in a micro homogenizer tube (Dounce pattern; Blaessig Glass Co., Rochester, N.Y., U.S.A.) and treated with 200 μl of chilled sodium dodecyl sulphate (0.5%, w/v) dissolved in the electrophoresis buffer. The mixture was homogenized with 15 passes of the pestle and mixed with 200 μl of water-saturated phenol on a vibration mixer. The mixture was centrifuged in a Beckman Spinco micro centrifuge for 2 min and the aqueous layer applied to the gel for electrophoretic analysis.

Results

Administration of DL-ethionine (750 mg/kg body wt.) to female rats (both 100 and 180 g) had a marked effect on the transport in the liver of rRNA from nucleus to cytoplasm. [5-^{3}H]Orotic acid was given by intravenous injection 1 h after the ethionine and the rats were killed at 30, 90 or 180 min later. At 1 h after each rat had received 5 μCi of [5-^{3}H]orotic acid the radioactivity incorporated into RNA in the nuclei of the control animals was significantly

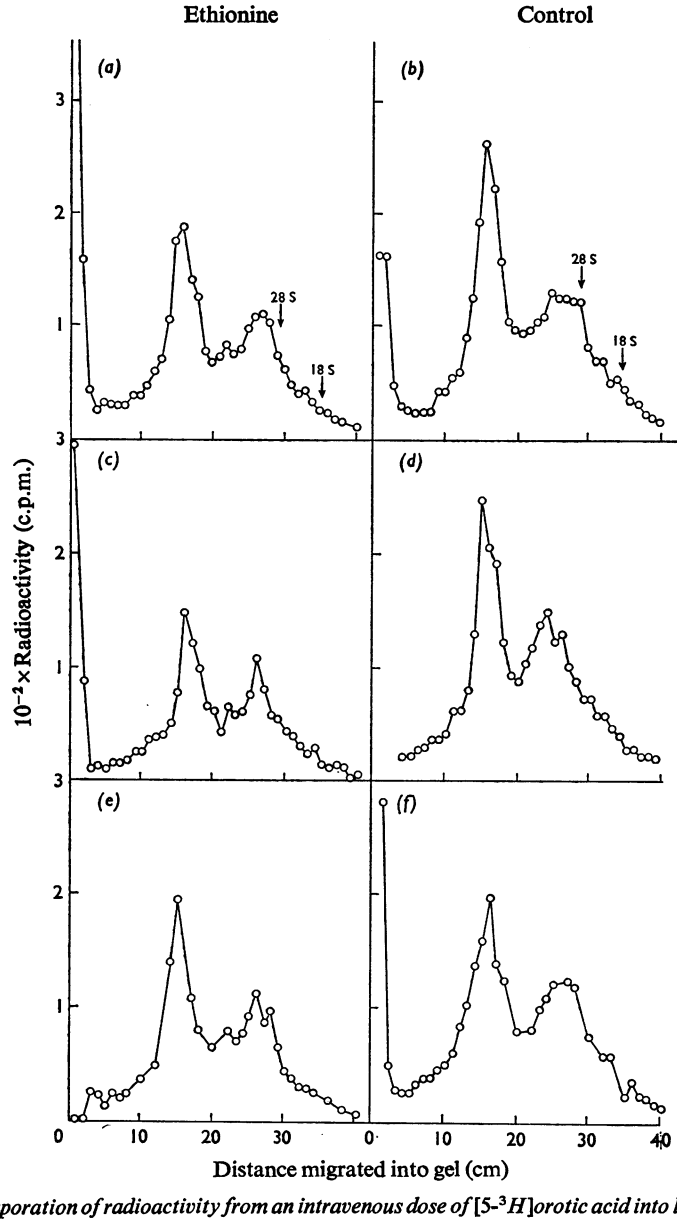

Fig. 1. *Incorporation of radioactivity from an intravenous dose of* [5-^{3}*H*]*orotic acid into liver nuclear RNA*

The controls (*b*, *d*, *f*) were given 0.9% NaCl and the ethionine-treated rats 0.75mg of ethionine/g body wt. (*a*, *c*, *e*) in the same volume of NaCl, by intraperitoneal injection 1h before the dose of labelled orotic acid. The rats were killed 30 (*a*, *b*), 90 (*c*, *d*) and 180 (*e*, *f*) min later and nuclear RNA was prepared as described in the Materials and Methods section. The RNA species were separated by electrophoresis on a 1.5% (w/v) acrylamide–0.5% agarose gel (8.8V/cm, 5°C, 1.5h). The gels were cut into slices and the radioactivity of eachw as determined: 28S and 18S show the positions of the marker rRNA species.

($P<0.01$) but only slightly greater (2.19±0.16c.p.m./μg of DNA) than in the ethionine-treated rat (1.66±0.07), and at these times no marked difference was seen in the relative incorporation of radioactivity into RNA of different molecular weights when the nuclear RNA was analysed by gel electrophoresis (Fig. 1). However, this high-molecular-weight RNA did not migrate from the nucleus to form cytoplasmic rRNA. In the control rat, incorporation into the 18S and 28S rRNA was seen 90min after giving [^{3}H]-

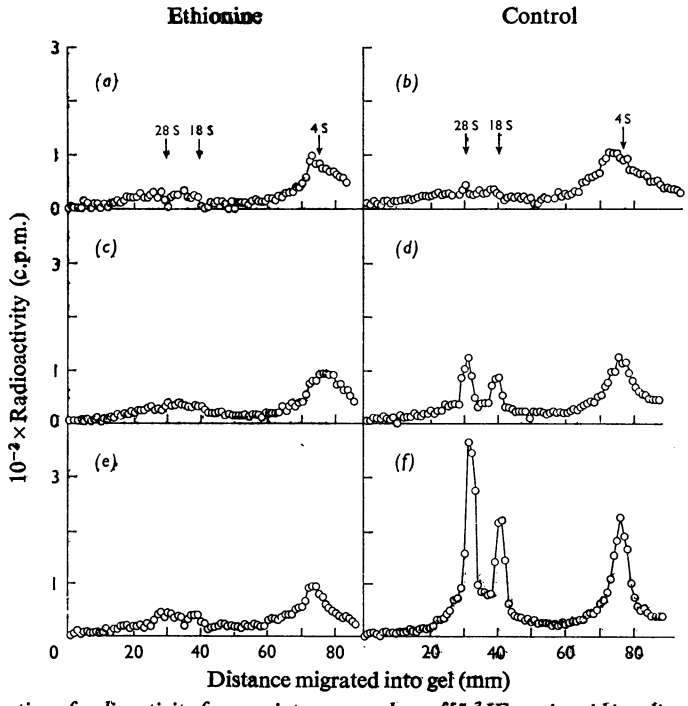

Fig. 2. *Incorporation of radioactivity from an intravenous dose of [5-^{3}H]orotic acid into liver cytoplasmic RNA*

The same conditions were used as in Fig. 1. Control (*b*, *d*, *f*) and ethionine-treated (*a*, *c*, *d*) rats were killed 30 (*a*, *b*), 90 (*c*, *d*) and 180 (*e*, *f*) min after injection.

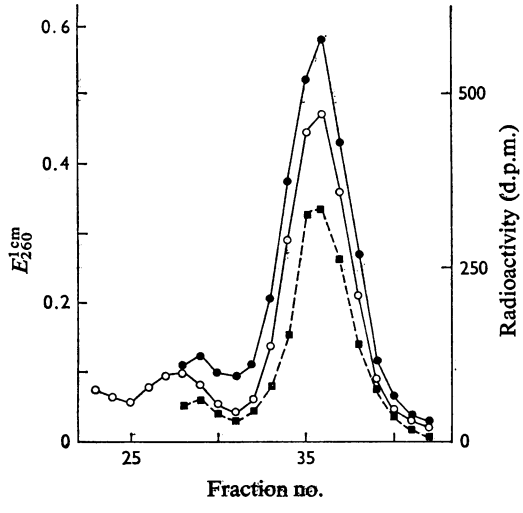

Fig. 3. *Elution of radioactivity in 4S RNA from a column of Sephadex G-100*

[6-^{14}C]Orotic acid was given to a rat that had been given DL-ethionine 1 h earlier, and [5-^{3}H]orotic acid was given to a control. The rats were killed 3 h after injection of the orotic acid, and the 4S RNA was prepared and chromatographed as described in the Materials and Methods section. ■, ^{14}C (ethionine-treated); ●, ^{3}H (control); ○, E^{1cm}_{260}.

orotic acid and the amount of radioactivity in these species of RNA increased threefold in the next 90 min (Fig. 2). In the ethionine-treated rat there was no detectable incorporation into these rRNA species up to 3 h after injection of the [^{3}H]orotic acid. In contrast, incorporation into 4S RNA, although on average 70% of that in the control, was still easily measured (Figs. 2 and 3).

Orotate is converted *in vivo* into uridine 5'-triphosphate, part of which is further metabolized to cytidine 5'-triphosphate. To decide whether the incorporation of radioactivity into 4S RNA was through the addition of cytosine to the CCA end of pre-existing tRNA molecules, rather than by synthesis of complete new molecules, the 4S RNA was isolated and analysed for the content of radioactive uracil and cytosine. Rats were taken in pairs, one treated with DL-ethionine, one with 0.85% NaCl, and 1 h later one rat of each pair was given [5-^{3}H]orotic acid, the other [6-^{14}C]orotic acid. Then, 3 h after injection of the orotic acid, both rats were killed, and equal weights of liver from the control and the ethionine-treated rat were combined. The 4S RNA was prepared as described in the Materials and Methods section. The elution of radioactivity from Sephadex G-100 column closely paralleled the elution of the u.v.-absorbing material in both the ethionine-treated and the control rat (Fig. 3). The amount of radioactivity

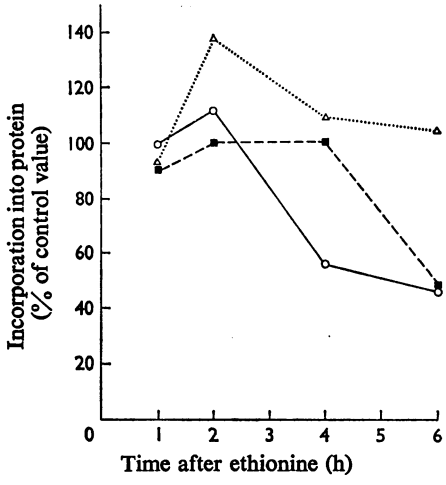

Fig. 4. *Incorporation of* L-[4,5-^3H]*leucine into rat liver protein*

Female rats (155–175g) were given an intraperitoneal injection of ethionine (○; 750mg/kg body wt.), inosine (△; 570mg/kg body wt.) or both ethionine and inosine (■), in each case dissolved in 3.0ml of 0.85% NaCl. At 30min before the rats were to be killed, each rat was given L-[4,5-^{3}H]leucine (5μCi, 0.2ml) by subcutaneous injection. Protein was prepared as described in the Materials and Methods section. Results are expressed as a proportion of the value in saline-treated controls; three or four rats were killed at each time-point for each treatment. The times shown are the time of death. The standard deviation of the controls was ±12%.

in the 4S RNA of the ethionine-treated rat was 70% of that in the control, but the proportion of the radioactivity found in uracil was the same in the ethionine-treated and the control rats. The relative proportion found in uracil depended on the specific radioactivity of the precursor. In rats given [5-^{3}H]orotic acid, which had a high specific radioactivity (15Ci/mmol) the amount of radioactivity found in uracil was 3.2 times greater than that found in cytosine, but in rats given [6-^{14}C]orotic acid, with a specific radioactivity (0.061 Ci/mmol) much lower than that of the tritiated material, the amount in uracil was 1.6 times that in cytosine.

Administration of inosine (570mg/kg body wt.) has been shown to provide sufficient synthesis of adenine to prevent the ATP deficiency induced by ethionine (Shull & Villa-Trevino, 1964; Farber *et al.*, 1965). Inosine administration did delay for 4h the inhibition induced by ethionine of the incorporation of leucine into protein of the liver (Fig. 4), but this dose of inosine did not prevent the specific effect of ethionine on the maturation of rRNA (Fig. 5). The extent of inhibition of protein synthesis was less than that found by Oler *et al.* (1969), but the difference may have been caused by differences in technique. They injected the radioactive amino acid into the hepatic portal vein of anaesthetized rats and killed the rats 15min after this injection.

An effect on the passage of rRNA from nucleus to cytoplasm similar to that induced by ethionine was not produced by administration of methylglyoxal bis(guanylhydrazone) in a dose (80mg/kg body wt.) reported to be sufficient to give immediate and virtually complete inhibition of spermidine synthesis in the liver (Pegg, 1973) (Fig. 6), nor could the phenomenon be induced by administration of cycloheximide (1.5mg/kg body wt.), which gave about 90% inhibition of the incorporation of L-[4,5-^{3}H]leucine into liver protein at the time the radioactive orotic acid was given. Both these compounds did have an effect on the distribution of radioactivity among

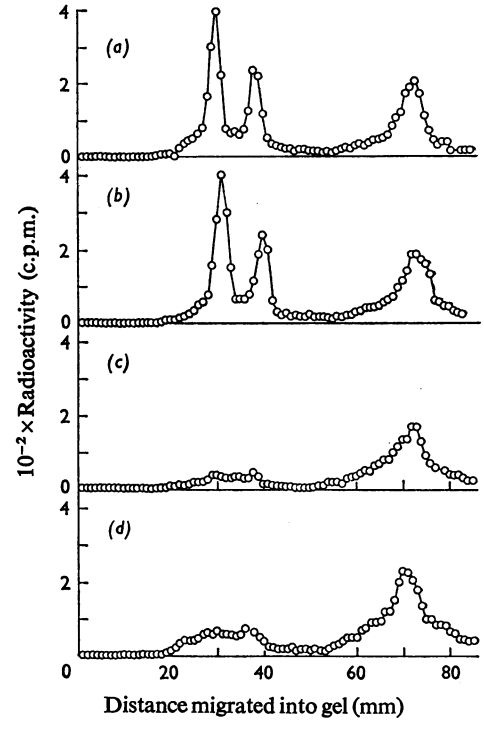

Fig. 5. *Incorporation of* [5-^3H]*orotic acid into the liver cytoplasmic RNA of rats treated with ethionine* (*c*; 750*mg/kg body wt.*), *inosine* (*b*; 570*mg/kg body wt.*) *or both ethionine and inosine* (*d*) 1*h before injection of the* [5-^3H]*orotic acid*

The treated and control (*a*; saline-treated) rats were killed 3h after injection of the labelled orotic acid and cytoplasmic RNA was prepared and analysed by gel electrophoresis. Conditions were as in Figs. 1 and 2.

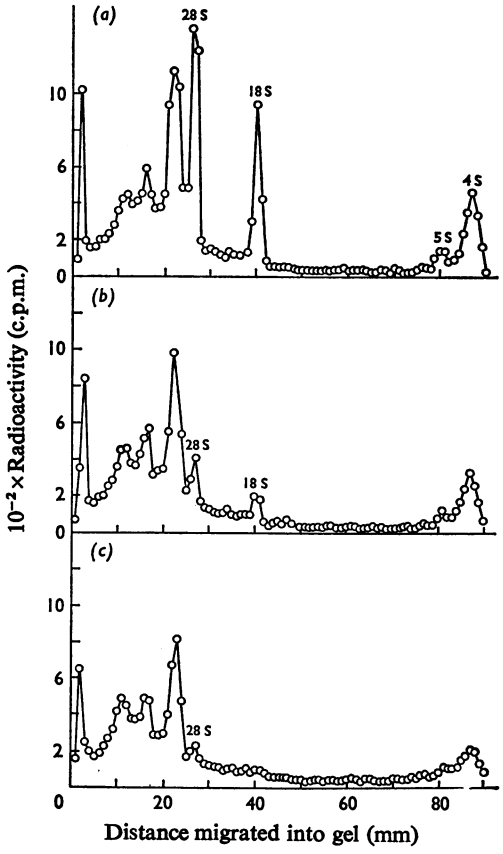

Fig. 6. *Incorporation of radioactivity from [5-^{3}H]uridine into total cellular RNA extracted from HeLa cells*

The cells were suspended in a minimal essential medium containing methionine (15mg/litre) (*a*, control), or in the same medium with added DL-ethionine (1mg/ml; *b*), or in medium which did not contain methionine (*c*). Then 1h later [5-^{3}H]uridine (5μCi/ml) was added and 2h after this the cells were harvested and the RNA was prepared and analysed by electrophoresis as described in the Materials and Methods section.

the cytoplasmic RNA species. In the cycloheximide-treated rats the amount of radioactivity incorporated into 4S RNA was approx. 40% greater than that incorporated into the untreated rats, and in the methylglyoxal bis(guanylhydrazone)-treated rats some radioactive RNA with an electrophoretic mobility similar to that of 32S RNA was found in the cytoplasm of all the treated rats. This was not found in any untreated rat. The amount of this RNA varied greatly in different rats, from the large amount shown in Fig. 6 to a very small amount. It is not known whether this high-molecular-weight RNA is identical with nuclear 32S RNA.

A similar though less marked inhibition of the synthesis of mature 18S and 28S rRNA was produced in HeLa cells when incubated in a culture medium containing the normal L-methionine concentration (15mg/litre) and DL-ethionine (1mg/ml) (Fig. 7). A number of experiments showed that, when [5-^{3}H]uridine was added to the culture 1h after the cells had been suspended in the medium containing ethionine, the amount of radioactivity that had been

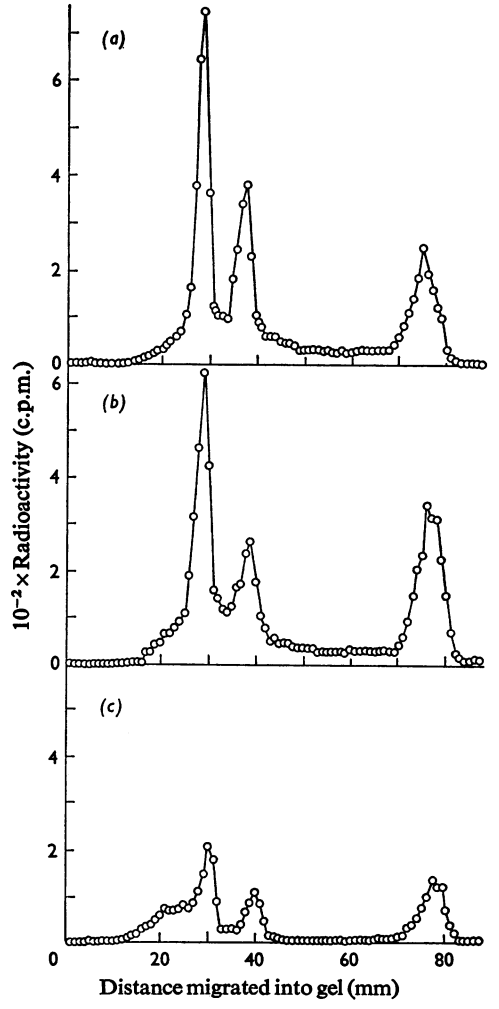

Fig. 7. *Incorporation of [5-^{3}H]orotic acid into rat liver cytoplasmic RNA after a dose of 0.9% NaCl (a), cycloheximide (1.5mg/kg body wt.; b), or methylglyoxal bis(guanylhydrazone) (80mg/kg body wt.; c)*

The rats were given the labelled orotic acid by intravenous injection 1h after each compound. They were killed 3h later and cytoplasmic RNA was prepared and analysed by gel electrophoresis. Conditions were as for Fig. 1.

incorporated into 18S rRNA 2h later was 61% of that incorporated into the control, and that into 28S RNA was 66% of that in the control. By contrast, the amount of radioactivity incorporated into 4S RNA was the same as that of the control. A similar though much greater preferential inhibition of synthesis of 18S and 28S rRNA was produced when the cells were incubated in a medium that did not contain methionine (Fig. 7). The synthesis of 5S RNA was not studied systematically, but the distribution of radioactivity in the gel suggested that neither incubation in ethionine nor methionine deprivation inhibited synthesis of 5S RNA.

The concentration of ATP in cells incubated in a medium without methionine, or in the medium containing ethionine, was not appreciably different from the concentration found in the control (0.005–0.01 pmol/cell in various experiments).

Discussion

Ribosomes in mammalian cells contain three different RNA species. The large ribosomal subunit contains two RNA species, a small molecule with a sedimentation coefficient of 5S, and a large RNA (mol.wt. 1.75×10^6) (28S) which consists of a long sequence with a small (7S) sequence of complementary RNA hydrogen-bonded to part of it. The small ribosomal subunit contains a single RNA (18S) of mol.wt. approx. 0.7×10^6 (see review by Maden, 1971). Two of these molecules, the 18S and 28S (with the attached 7S), are formed by a series of maturation stages, involving methylation and cleavage, from a single much larger molecule of mol.wt. 4.1×10^6 (45S RNA) (Maden, 1971). This large ribosomal RNA precursor is synthesized in the nucleolus, but it is probable that the 5S rRNA as well as tRNA are synthesized on extranucleolar genes (Ritossa *et al.*, 1966; Brown & Weber, 1968).

Ethionine administration had a considerable effect on RNA metabolism, preferentially affecting the formation of cytoplasmic 28S and 18S RNA. Many of the toxic changes induced by ethionine administration are thought to be caused by the rapid fall in concentration of liver ATP that it is known to induce (Farber, 1967), but there is the possibility that some of the biochemical changes are caused by an interference with the transmethylation reactions, which require *S*-adenosylmethionine. ATP deficiency probably plays a dominant role in the inhibition of protein synthesis in rat liver after ethionine poisoning (Farber *et al.*, 1964, 1965; Shull & Villa-Trevino, 1964; but the inhibition of choline synthesis (Simmonds *et al.*, 1950) is probably caused by an inhibition of transmethylation (Gordon & Farber, 1965).

In studies of the effects of ethionine on RNA metabolism previous reviews and reports have emphasized the inhibition of overall RNA synthesis and have suggested that this is caused by a lack of ATP (Villa-Trevino *et al.*, 1966; Stewart & Farber, 1968; Oler *et al.*, 1969; Farber, 1971), but the published evidence does not entirely support that view. The ethionine-induced nucleolar lesions can be reversed by administration of adenine (Shinozuka *et al.*, 1968), but the same nucleolar lesions are not produced by fructose-induced ATP deficiency (Goldblatt *et al.*, 1970). In the guinea pig, where an ATP deficiency can be induced by administration of methionine as well as of ethionine, the effects of the two compounds on RNA synthesis are not the same (Cox *et al.*, 1973).

The results reported now suggest that the inhibition of RNA synthesis is not the first, or the most important, effect of ethionine administration on RNA metabolism. At 1h after administration of ethionine to the rat the rate of synthesis of the ribosomal precursor RNA and 4S RNA are not greatly changed, but the maturation of the ribosomal RNA and the appearance of this RNA in the cytoplasm is completely blocked. This effect of ethionine administration on the maturation of the rRNA precursor appears to be caused by the inhibition of a methylation reaction depending on *S*-adenosylmethionine rather than a lack of ATP. The evidence for this view is that the effect was not prevented by administration of a dose of inosine large enough to gives ufficient adenine synthesis to prevent the inhibition of protein synthesis induced by ethionine (Farber *et al.*, 1965) (Figs. 4 and 5). An apparently identical effect could be induced in cells in tissue culture by methionine starvation for 1h (Fig. 6), and a similar, though less marked, effect was produced when the cells were incubated in a medium containing both methionine and DL-ethionine (1 mg/ml) (Fig. 6). The ATP content measured by the luciferin–luciferase method was the same in cells incubated without methionine or in the ethionine-containing medium as in normal growth medium. This method of ATP measurement was not very reproducible, and the results should be accepted with caution, but the ATP concentrations found were in the same range as those found in Krebs ascites cells by Gumaa & McLean (1969).

The alternative possibility, that this effect on the maturation of the rRNA precursor was secondary to an inhibition of protein synthesis or polyamine synthesis by ethionine, was not supported by a comparison of the effect on RNA maturation of ethionine and an inhibitor of protein synthesis (cycloheximide) or of spermidine synthesis [methylglyoxal bis(guanylhydrazone)]. Both inhibitors did affect the synthesis of cytoplasmic RNA (Fig. 7 and the Results section), with the administration of cycloheximide increasing the amount of radioactivity appearing as 4S RNA, and methylglyoxal bis(guanylhydrazone) administration causing some

inhibition of RNA synthesis and causing some RNA with an electrophoretic mobility similar to that of 32S RNA to appear in the cytoplasm. These peripheral effects have not been studied systematically. Neither inhibitor had the same effect as ethionine. A previous report that this dose of cycloheximide (1.5mg/kg body wt.) inhibits protein synthesis more than 90% (Verbin *et al.*, 1969) was confirmed in the rats used in the experiment shown in Fig. 7. Previous authors (Muramatsu *et al.*, 1970; Yu & Feigelson, 1972) have shown that very high doses of cycloheximide (20 or 30mg/kg) have a great effect on nucleolar RNA metabolism, but more recent studies (Farber & Farmar, 1973; Timberlake & Griffin, 1974) suggest that these are caused by cycloheximide itself and not primarily as a result of inhibition of protein synthesis. The experiment with methylglyoxal bis(guanylhydrazone) was begun because it is known that polyamine synthesis coincides with increased RNA synthesis in growth (see Cohen, 1972), but it was found that virtually complete inhibition of spermidine synthesis with methylglyoxal bis(guanylhydrazone) (Pegg, 1973; Corti *et al.*, 1974) produces only a partial inhibition of the synthesis of rRNA (Fig. 7). The observation of an inhibition of RNA synthesis does not entirely agree with previous results showing that neither inhibition of putrescine synthesis with α-hydrazino-ornithine nor the inhibition of spermine and spermidine synthesis inhibit RNA synthesis in rat liver or lymphocytes (Fillingame & Morris, 1973; Kay & Pegg, 1973; Harik *et al.*, 1974).

The conclusion that the major effect of ethionine on RNA metabolism in the liver is to block maturation of the rRNA precursor would suggest an explanation for the similarity in the morphological changes in the nucleolus produced by ethionine and by toyocamycin (Monneron *et al.*, 1970), for toyocamycin also blocks the maturation of the ribosomal RNA precursor (Tavitian *et al.*, 1968; Weiss & Pitot, 1974).

There was no evidence that 32S RNA, 21S RNA, 28S RNA or 18S RNA accumulates in the liver nuclei of the ethionine-poisoned rat, so presumably, after cleavage of the ribosomal precursor RNA to give 32S RNA, all the RNA is completely degraded. It is known that the parts of the large ribosomal precursor RNA that are conserved and appear in the cytoplasm are methylated, and that the large part, which is degraded during the normal maturation process, is not methylated. These results with ethionine agree with the conclusion of Vaughan *et al.* (1967) and of Perry & Kelley (1972) that the methylation is essential if complete degradation of the molecule is to be prevented.

If nucleotides from the ribosomal precursor RNA were not catabolized but were reutilized for the synthesis of new RNA, these results could be reconciled with those showing inhibition of the incorporation of labelled orotic acid into nuclear RNA 4h after ethionine had been given to the rat (Farber *et al.*, 1974), and earlier experiments showing that RNA leaves the nucleus at a slower rate after administration of ethionine than after administration of actinomycin D (Stewart & Farber, 1968). If the nucleotides were reutilized, a cycle of nucleotides would exist: in the untreated rat only the nucleotides from the non-conserved regions of the 45S would be reutilized; in the ethionine-poisoned rat all the nucleotides from the 45S RNA would be reutilized. Thus, even if synthesis of RNA were proceeding at a normal rate, the requirement for the input of nucleotides from the cytoplasm would be decreased. Thus the apparent rate of 'synthesis' would be decreased (Farber *et al.*, 1974) and the labelled nucleotides in RNA would remain longer in the nucleus in the ethionine-poisoned than in the actinomycin D-poisoned rat (Stewart & Farber, 1968).

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