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A Possible Mechanism of Inhibition of Protein Synthesis by Dimethylnitrosamine

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1. The incorporation of [^{14}C]leucine into liver proteins of rats was measured *in vivo* at various times after treatment of the animals with dimethylnitrosamine and was correlated with the state of the liver ribosomal aggregates. Inhibition of incorporation ran parallel with breakdown of the aggregates. 2. Inhibition of leucine incorporation into protein and breakdown of ribosomal aggregates were not preceded by inhibition of incorporation of [^{14}C]orotate into nuclear RNA of the liver. 3. Evidence was obtained of methylation of nuclear RNA in the livers of rats treated with [^{14}C]dimethylnitrosamine. 4. Zonal centrifugation analysis of radioactive, nuclear, ribosomal and transfer RNA from livers of rats treated with [^{14}C]dimethylnitrosamine revealed labelling of all centrifugal fractions to about the same extent. 5. It is suggested that methylation of messenger RNA might occur in the livers of dimethylnitrosamine-treated rats and the possible relation of this to inhibition of hepatic protein synthesis is discussed.

Dimethylnitrosamine, a chemical carcinogen, causes acute liver injury and early inhibition of protein synthesis in the liver of the rat. Inhibition of protein synthesis, glycogen depletion and accumulation of liver fat can be detected a few hours after the administration of the compound and precede the development of necrosis (Emmelot & Benedetti, 1960; Magee, 1962). Similar changes have been reported to follow treatment with other hepatotoxic agents. The pattern of hepatic change with carbon tetrachloride is closely similar to that with dimethylnitrosamine (Smuckler, Iseri & Benditt, 1962; Bassi, 1960) and, with the exception of necrosis, the response by the female rat liver to ethionine administration is also similar (Farber, Shull, Villa-Treviño, Lombardi & Thomas, 1964; Farber, 1963).

In the present paper some biochemical changes induced in rat liver by administration of dimethylnitrosamine are described and compared with those reported in poisoning by carbon tetrachloride and ethionine.

MATERIALS AND METHODS

Dimethylnitrosamine. This was obtained from British Drug Houses Ltd., Poole, Dorset, and was purified by distillation (b.p. 151°). It was estimated in rat liver by the polarographic method of Heath & Jarvis (1955).

Isotopically labelled compounds. [^{14}C]Dimethylnitrosamine (specific activity 4·6mc/m-mole) was prepared by Dr D. F. Heath by the method of Dutton & Heath (1956). L-[1-^{14}C]Leucine (specific activity 8·5mc/m-mole) and [6-^{14}C]orotic acid (specific activity 30mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

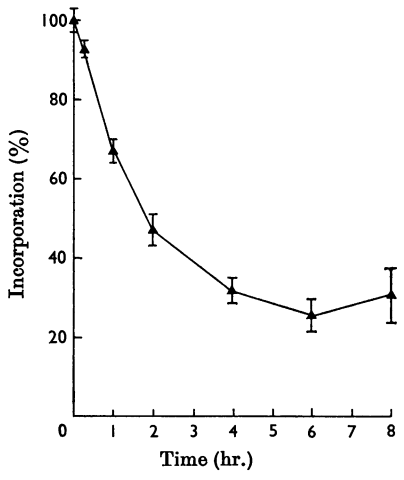

Fig. 1. Incorporation of [^{14}C]leucine into liver proteins of rats at different times after treatment with dimethylnitrosamine (30mg./kg. body wt.). Each point represents the mean ± S.E.M. of five determinations.

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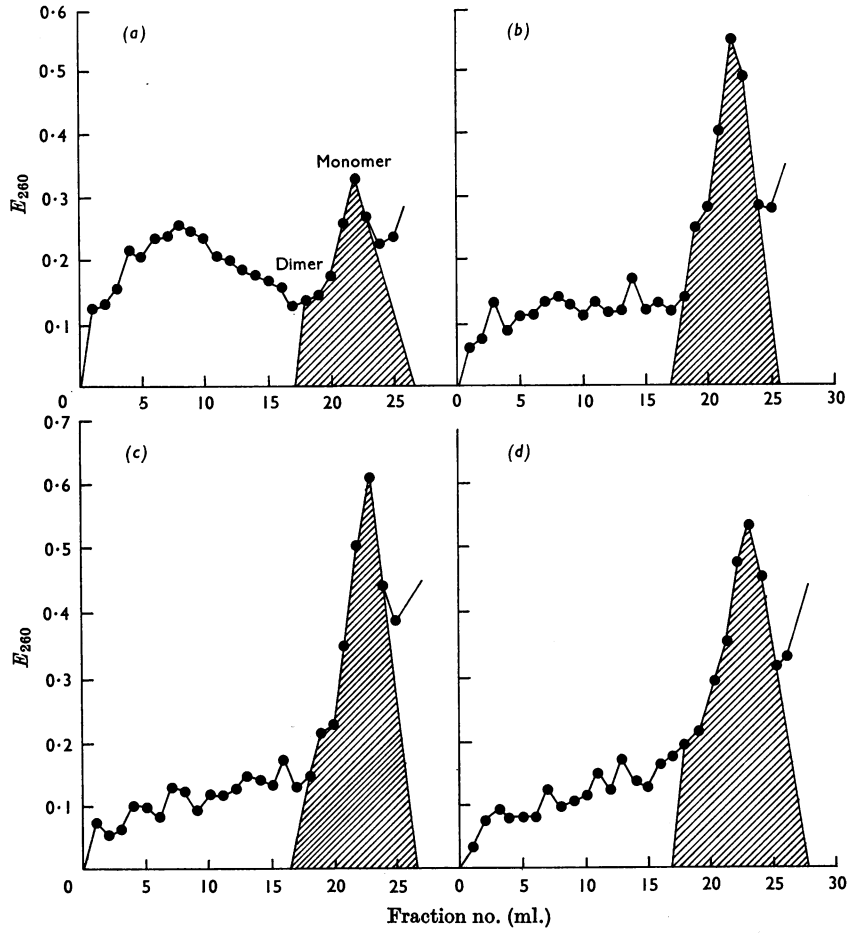

Fig. 2. Ribosomal patterns from post-mitochondrial supernatant fractions of livers from rats killed before (*a*, control) and after treatment with dimethylnitrosamine (30mg./kg. body wt.) for 1hr. (*b*), 4hr. (*c*) and 8hr. (*d*). The hatched areas represent monomers plus dimers.

Animals. Female Wistar rats of the Porton strain, weighing about 200g., were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956). They were starved overnight and given an aqueous solution of dimethylnitrosamine containing a dose of 30mg./kg. body wt. by intraperitoneal injection.

Leucine incorporation into proteins. Rats under Nembutal anaesthesia (60mg./kg. body wt.) were injected via the portal vein with 0·5ml. of an aqueous solution containing 1μc of [1-^{14}C]leucine 15min. before they were killed. For incorporation at 15min. (Fig. 1), dimethylnitrosamine was also administered into the portal vein, control rats receiving water alone. The livers were excised, homogenized in 25ml. of 10% (w/v) trichloroacetic acid and proteins were prepared and assayed for radioactivity as described by Villa-Treviño, Shull & Farber (1962).

Orotate incorporation into RNA. At different times after administration of dimethylnitrosamine a procedure similar to that with leucine was followed, 0·5ml. of an aqueous solution containing 2·5μc of [6-^{14}C]orotate being injected. A purified fraction of liver nuclei was isolated and RNA extracted by a sodium dodecyl sulphate–phenol method as reported by Villa-Treviño, Shull & Farber (1966). After three reprecipitations, fractions of the samples in aqueous solution were taken for concentration and radioactivity determinations, and for these purposes a solution of 1mg. of RNA/ml. was assumed to have $E_{260}^{1\,cm.}$ 20.

Ribosomal aggregates. These were analysed by zone centrifugation. A sample (0·3ml.) of the deoxycholate-treated post-mitochondrial supernatant was layered on the surface of a convex sucrose gradient (0·3–1·0M) in 25mM-KCl–4mM-magnesium acetate–50mM-tris–HCl buffer, pH7·6, and was centrifuged at 25000rev./min. (53000*g*) for 195min. at 0° in the SW25.1 rotor of a Spinco model L ultracentrifuge. Fractions (1ml.) were collected manually after perforation of the bottom of the tube and their E_{320} and E_{260} values measured with a spectrophotometer. The sedimentation patterns were plotted (Fig. 2) and the

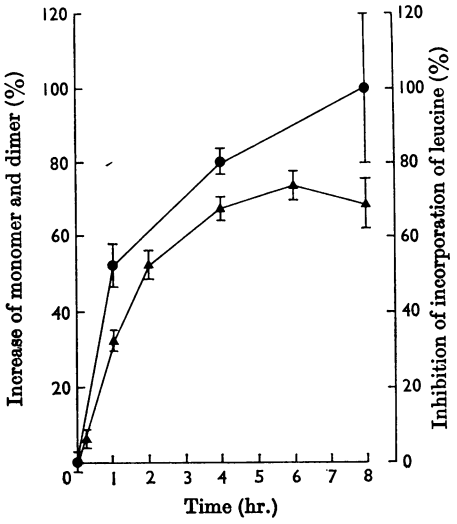

Fig. 3. Parallel changes in ribosomal pattern and inhibition of incorporation of [^{14}C]leucine induced by dimethylnitrosamine. ●, Relative increase in area of monomers plus dimers (hatched areas, Fig. 2); points at 1hr. and 4hr. represent the means of three observations and that at 8hr. the mean of two observations, the vertical lines representing the range. ▲, Inhibition of incorporation of [^{14}C]leucine as in Fig. 1.

Table 1. *Incorporation of* [6-^{14}C]*orotate into nuclear RNA of livers of rats treated with dimethylnitrosamine*

Dimethylnitrosamine (30mg./kg. body wt.) was injected intraperitoneally, followed after increasing intervals by [6-^{14}C]orotate (2·5μc) via the portal vein. Rats (numbers/group in parentheses) were killed 15min. after injection of orotate. Specific radioactivities are given as means ± S.E.M. *P* values were determined by Student's *t* test.

Time after injection of dimethylnitrosamine (hr.)	10^{-3} × Sp. activity of nuclear RNA (disintegrations/min./20*E* units)	% of control
Control (saline-injected)	59·5 ± 2·2 (5)	(100)
1	71·4 ± 5·5 (4) ($P < 0·1$)	120
2	50·1 ± 2·8 (4) ($P < 0·05$)	85
5	28·6 ± 4·4 (4) ($P < 0·001$)	48

relative areas of the monomers plus dimers (hatched) and the larger aggregates were determined by cutting out and weighing the sections, the results being expressed as percentages of the total areas.

Incorporation of radioactivity from [^{14}C]*dimethylnitrosamine.* Labelled dimethylnitrosamine was administered intraperitoneally in a dose of 30mg./kg. body wt. and 180μc or 10μc/rat. Nuclei, microsomes and pH5 fractions of liver were prepared and nucleic acids and proteins were isolated from these fractions as described by Villa-Treviño *et al.* (1966) and portions were taken for radioactivity measurement.

Table 2. *Incorporation of radioactivity from* [^{14}C]*dimethylnitrosamine into protein, RNA and DNA of cellular fractions of rat liver*

Rats (five/group) received [^{14}C]dimethylnitrosamine (30mg./kg. body wt., 10μc) by intraperitoneal injection, except in the 15min. group, when the injection was intraportal, and were killed after the intervals indicated. The specific radioactivities are given as means ± S.E.M.

Fraction	Sp. activity (disintegrations/min./mg.) Time after injection of [^{14}C]dimethylnitrosamine 15 min.	60 min.	180 min.
Microsomal protein	23 ± 3	144 ± 4	271 ± 11
Soluble protein	11 ± 1	105 ± 1	263 ± 14
Nuclear protein	14 ± 4	58 ± 2	196 ± 13
Microsomal RNA	44 ± 6	359 ± 34	749 ± 38
Transfer RNA	46 ± 4	513 ± 60	268 ± 38
Nuclear RNA	81 ± 16	277 ± 12	592 ± 34
DNA	24 ± 3	172 ± 12	407 ± 28

RNA gradients. A 0·5mg. sample of the RNA labelled by [^{14}C]dimethylnitrosamine *in vivo* was layered on a linear sucrose gradient (0·5–1·0M) in 5mM-tris–HCl buffer, pH7·6, and centrifuged for 30hr. at 25000rev./min. (53500*g*) at 0° in the SW25.1 rotor of the Spinco model L centrifuge. Samples (1ml.) were collected manually, read at 260mμ and assayed for radioactivity after the addition of liquid scintillator.

Ion-exchange chromatography. Acid hydrolysates of nuclear RNA, heavily labelled *in vivo* by [^{14}C]dimethylnitrosamine, with added non-labelled carrier (5mg. of total liver RNA), were passed through a Dowex 50 (H^+ form) column (1cm. × 10cm.), elution being with a continuous 1–4N-HCl gradient (Magee & Farber, 1962). Samples (10ml.) were collected, read at 260mμ, evaporated and assayed for radioactivity.

Radioactivity assays. Solid samples, after solution in Hyamine hydroxide [*p*-(di-isobutylcresoxyethoxyethyl)-dimethylbenzylammonium chloride, hydroxide form], and liquid samples were counted in conventional toluene scintillation mixtures, with a Packard Tri-Carb scintillation spectrometer. [^{14}C]Toluene was used as internal standard for determination of counting efficiency.

RESULTS

The administration of dimethylnitrosamine decreases the incorporation of L-leucine into total liver proteins progressively (Fig. 1). The maximum effect is at 5–7hr., but some inhibition is detected after 15min. Since the injection at 15min. was by a

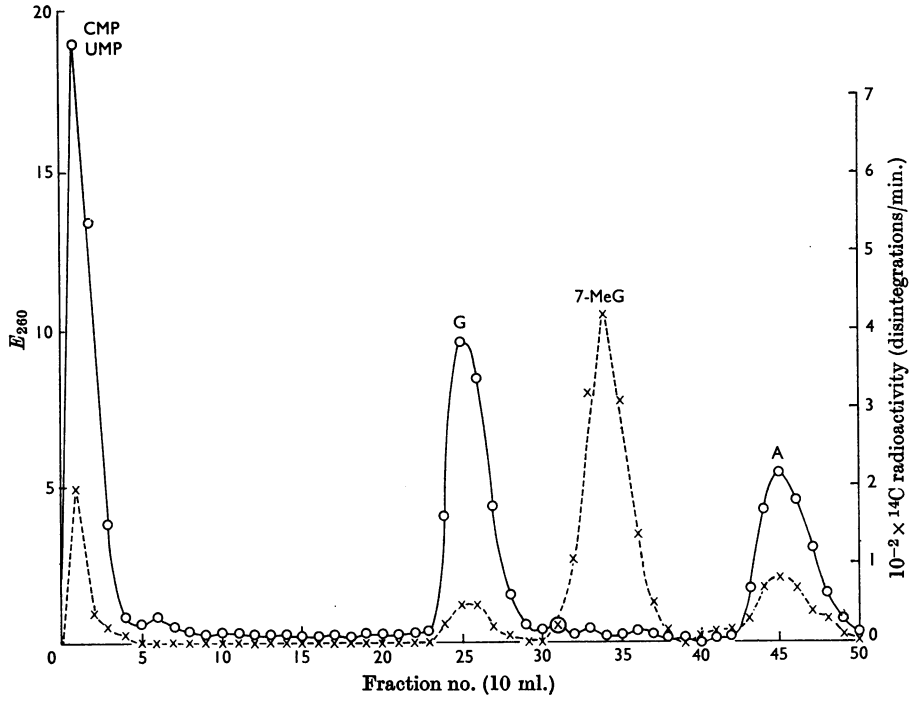

Fig. 4. Ion-exchange chromatography of acid hydrolysate of nuclear RNA from livers of rats killed 5hr. after treatment with [^{14}C]dimethylnitrosamine (30mg./kg. body wt.; 180μc). Carrier RNA was added (see the text). Dowex 50 (H^+ form) resin was used, with gradient elution with 1–4N-HCl. ○, E_{260}; ×, ^{14}C radioactivity. G, Guanine; A, adenine; 7-MeG, 7-methylguanine.

different route, the concentration of dimethylnitrosamine in the liver under similar conditions was determined in four rats. Concentrations of 26·6, 26·6, 31·3 and 29·8μg. of dimethylnitrosamine/g. wet wt. of liver were found, these being of the same order as those observed after oral administration by Hultin, Arrhenius, Löw & Magee (1960). Starting at 1hr., the ribosomal aggregate patterns show an increase of the monomer-plus-dimer area with a decrease of the polysome area (Fig. 2). By 6hr., there is a maximum breakdown of the ribosomal aggregate. The rate of this breakdown parallels the degree of inhibition of amino acid incorporation (Fig. 3) into total liver protein. Some hepatotoxic drugs, like ethionine or actinomycin, which inhibit protein synthesis, show a preceding inhibition of RNA synthesis (Villa-Treviño, Farber, Staehelin, Wettstein & Noll, 1964; Staehelin, Wettstein & Noll, 1963). Dimethylnitrosamine administration has no early effect on the incorporation of orotate into nuclear RNA and at 1hr., where leucine incorporation is decreased by 32% (Fig. 1), orotate incorporation is not different from the control. At 2hr. orotate is 15% inhibited whereas leucine incorporation is 52% inhibited, but at 5hr. the incorporation of orotate is significantly decreased, being only 45% of the control values (Table 1).

One of the biochemical changes produced by dimethylnitrosamine in the liver is methylation of cellular components, including nucleic acids and proteins (Magee & Farber, 1962). At 5hr. after injection of [^{14}C]dimethylnitrosamine (30mg./kg. body wt.) the greater part of the radioactivity incorporated into rat liver RNA is found in 7-methylguanine. The fraction of the total radioactivity so incorporated may be as high as 85% (P. Brookes & P. D. Lawley, personal communication). Table 2 shows that DNA and all the cellular fractions of RNA and protein are labelled at 15min. after the intraportal injection and that the degree of labelling increases progressively with time except in the transfer RNA. Microsomal RNA has the fastest labelling rate and, at 3hr., the highest specific activity. It is known that some of the methyl label of dimethylnitrosamine will enter the C_1 unit metabolic pool (Brouwers & Emmelot, 1960) and therefore label the purine rings of the nucleic acids. However, ion-exchange chromatography of an acid hydrolysate of rat-liver nuclear RNA labelled by [^{14}C]dimethylnitrosamine *in vivo* showed that about

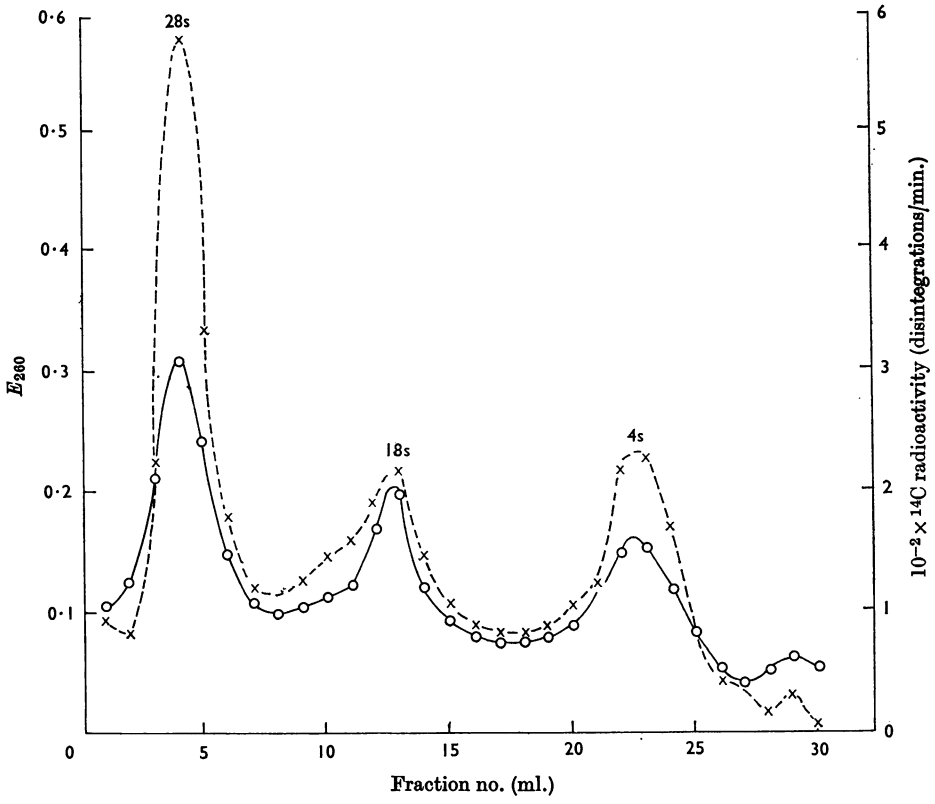

Fig. 5. Zonal centrifugation of nuclear RNA from livers of rats killed 5hr. after treatment with [^{14}C]dimethylnitrosamine (30mg./kg. body wt., 180μc). ○, E_{260}; ×, ^{14}C radioactivity.

63% of the radioactivity is in the form of 7-methylguanine (Fig. 4). Analysis of the labelled RNA from the different cellular fractions by zonal centrifugation revealed that there is labelling of the 28s, 18s and 4s peaks, and that with exception of the 28s nuclear peak the E_{260} profiles are closely followed by the radioactivity profiles. The nuclear 28s peak shows a somewhat higher specific activity (Figs. 5, 6 and 7).

DISCUSSION

The inhibition of hepatic protein synthesis produced by dimethylnitrosamine is paralleled by breakdown of the ribosomal aggregates, and some ideas have been proposed to explain these observations, including breakdown of messenger RNA in the cytoplasm (Mizrahi & de Vries, 1965; Villa-Treviño, 1965). The inhibition of amino acid incorporation seems to be located in the liver microsomes, since the amino acid-activating enzymes and transfer RNA of the treated animals are as active as those from control animals (Brouwers & Emmelot, 1960; Hultin *et al.* 1960). The microsomal fraction contains the ribosomal aggregate, which is the active complex in amino acid incorporation (Noll, Staehelin & Wettstein, 1963). This aggregate is composed mainly of messenger RNA and ribosomal units (73s particles), and therefore alteration in either component might explain the breakdown of this structure.

An alteration in the ribosomal unit can be tentatively discarded because ribosomes from dimethylnitrosamine-treated animals, when supplied with a synthetic messenger (polyuridylic acid), incorporate amino acids at rates above the control levels (Mizrahi & Emmelot, 1964; Mager, Bornstein & Halbreich, 1965). Inhibition of synthesis of messenger RNA is also unlikely since there is no decrease in the incorporation of orotate into nuclear RNA at a time when there is 30% inhibition of amino acid incorporation into liver proteins (Table 1 and Fig. 1). The inhibition of protein synthesis might therefore result from accelerated destruction or possibly from defective function of the messenger RNA.

The evidence that nuclear RNA is methylated in

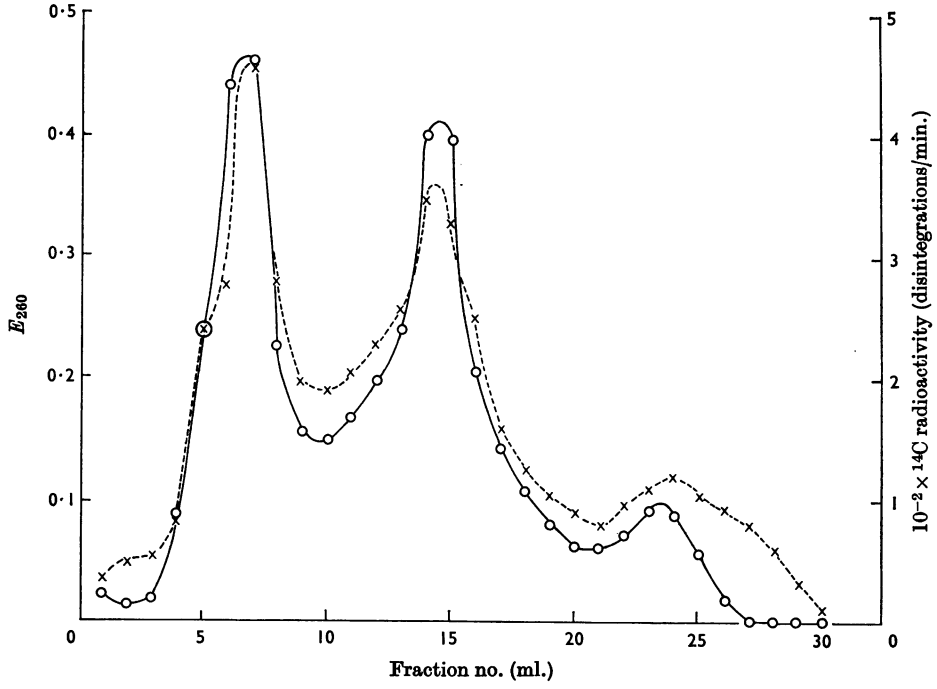

Fig. 6. Zonal centrifugation of microsomal RNA from livers of rats killed 5hr. after treatment with [^{14}C]-dimethylnitrosamine (30mg./kg. body wt., 180μc). ○, E_{260}; ×, ^{14}C radioactivity.

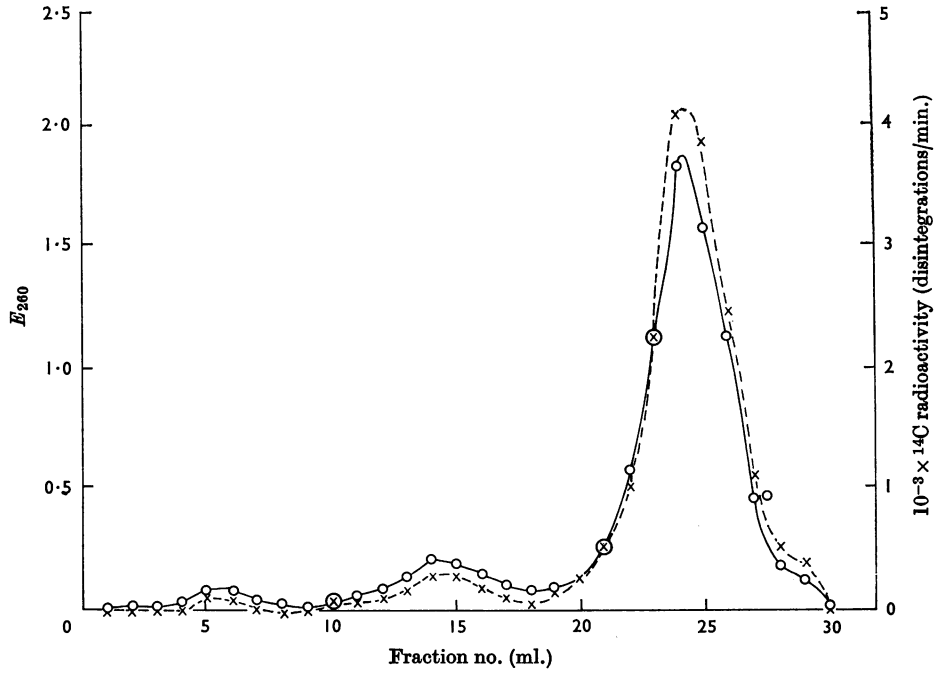

Fig. 7. Zonal centrifugation of transfer RNA from livers of rats killed 5hr. after treatment with [^{14}C]dimethylnitrosamine (30mg./kg. body wt., 180μc). ○, E_{260}; ×, ^{14}C radioactivity.

the livers of rats treated with [^{14}C]dimethylnitrosamine (Fig. 4), and the finding of incorporation of radioactivity in the 28s, 18s and 4s peaks (Fig. 5), suggests that the messenger RNA could also have been methylated. Similar conclusions might be drawn for the cytoplasmic messenger RNA, since the peaks of the microsomal and transfer RNA also all show a similar degree of labelling (Figs. 6 and 7). However, direct evidence is not available, owing to the low amounts and heterogeneity of rat-liver messenger RNA (Henshaw, Revel & Hiatt, 1965). The postulated defect in the messenger RNA might therefore result from its methylation. Kriek & Emmelot (1963) have reported that methylation of RNA makes it less stable at neutral pH, which might favour an increased rate of breakdown in the cell. On the other hand, Brookes & Lawley (1966) maintain that alkylated RNA is stable in neutral aqueous solution (see discussion by Lawley, 1961). The possibility that the methylated messenger RNA might be defective functionally is supported by the work of Wilhelm & Ludlum (1966), who have shown that methylated co-polymers of UMP and GMP have a markedly decreased template activity for polypeptide synthesis *in vitro* owing to steric effects of the 7-methyl groups.

Enzymic systems that methylate RNA have been described in plants, bacteria and mammals (Borek & Srinivasan, 1966). These methylations occur mainly in transfer RNA and to a smaller extent in microsomal RNA. Several methylated bases have been reported, including trace amounts of 7-methylguanine (Dunn, 1963). It is thought that enzymic methylation of messenger RNA does not normally occur (Srinivasan & Borek, 1964); thus it is possible that small degrees of methylation by an unphysiological methylating agent might exert relatively large biological effects including the observed inhibition of protein synthesis.

The sequence of events after giving dimethylnitrosamine is similar to that observed in carbon tetrachloride poisoning. The early inhibition of amino acid incorporation, accompanied by a parallel breakdown of the ribosomal aggregate not preceded by an inhibition of RNA synthesis, and the response to added synthetic messenger RNA *in vitro* (Mager *et al.* 1965; Smuckler & Benditt, 1965; Webb, Blobel & Potter, 1966), can be seen after administration of both hepatotoxins. These two compounds also produce liver necrosis. The inhibition of protein synthesis by ethionine is different. It is preceded by an inhibition of RNA synthesis and can be prevented or reversed by methionine or adenine administration (Villa-Treviño *et al.* 1962, 1964). The effect of ethionine is also different from that of dimethylnitrosamine and carbon tetrachloride, in the sense that it does not produce liver necrosis.

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