Inhibition of Protein Synthesis by *N*-Methyl-*N*-nitrosourea *in vivo*

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1. The intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100 mg/kg) caused a partial inhibition of protein synthesis in several organs of the rat, the maximum effect occurring after 2–3 h. 2. In the liver the inhibition of protein synthesis was paralleled by a marked disaggregation of polyribosomes and an increase in ribosome monomers and ribosomal subunits. No significant breakdown of polyribosomes was found in adult rat brains although *N*-methyl-*N*-nitrosourea inhibited cerebral and hepatic protein synthesis to a similar extent. In weanling rats *N*-methyl-*N*-nitrosourea caused a shift in the cerebral polyribosome profile similar to but less marked than that in rat liver. 3. Reaction of polyribosomal RNA with *N*-[¹⁴C]methyl-*N*-nitrosourea *in vitro* did not lead to a disaggregation of polyribosomes although the amounts of 7-methylguanine produced were up to twenty times higher than those found after administration of sublethal doses *in vivo*. 4. It was concluded that changes in the polyribosome profile induced by *N*-methyl-*N*-nitrosourea may reflect the mechanism of inhibition of protein synthesis rather than being a direct consequence of the methylation of polyribosomal mRNA.

Investigations on the biological effects of *N*-methyl-*N*-nitrosourea *in vivo* have been focused on its carcinogenicity which, like that of other acyl-alkyl-nitrosamides (Druckrey *et al.*, 1967) is preferentially directed towards the nervous system. Repeated intravenous injections of *N*-methyl-*N*-nitrosourea in rats have been shown to induce selectively malignant gliomas of the central nervous system (Druckrey *et al.*, 1967). However, rats that survived a single dose of *N*-methyl-*N*-nitrosourea (60–90 mg/kg body wt.) developed tumours in a variety of tissues without any predilection for the nervous system (Druckrey *et al.*, 1967; Leaver *et al.*, 1969).

N-Methyl-*N*-nitrosourea is converted *in vivo* into an alkylating intermediate that reacts with various cell components. For example, methylation of nucleic acids has been demonstrated in several organs of the rat including the brain (Swann & Magee, 1968; Kleihues & Magee, 1973). Sublethal doses of *N*-methyl-*N*-nitrosourea cause an inhibition of DNA synthesis (Kleihues, 1969) that is paralleled by severe cytotoxic effects on proliferating cells (Leaver *et al.*, 1969). Topical application of *N*-methyl-*N*-nitrosourea, leading to higher concentrations in selected organs, e.g., brain (Hossmann & Kleihues, 1971) and bladder (Hicks & Wakefield, 1972) has been shown to damage non-dividing cells as well.

Experimental

Animals

Female Wistar rats (120–140 g or 10 days old) were used.

Chemicals

L-[1-¹⁴C]Leucine (specific radioactivity 58 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and dissolved in 0.9% NaCl. Anti-ferritin serum (rabbit) was supplied by Nutritional Biochemicals, Cleveland, Ohio, U.S.A. *N*-Methyl-*N*-nitrosourea and *N*-[¹⁴C]methyl-*N*-nitrosourea were prepared as described by Swann & Magee (1968). For injection, *N*-methyl-*N*-nitrosourea was dissolved in 3 mM-sodium citrate buffer (pH 5.6) containing 0.9% NaCl.

Incorporation studies

L-[1-¹⁴C]Leucine was injected under light ether anaesthesia into the femoral vein at different time-intervals after an intraperitoneal dose of *N*-methyl-*N*-nitrosourea (100 mg/kg). If not otherwise stated, animals were killed 30 min later. Organs were homogenized in ice-cold water, with an Ultra-Turrax blender (Jankel und Kunkel, Staufen/Brs.,

West Germany). After initial precipitation of the homogenate with an equal volume of 10% (w/v) trichloroacetic acid, 1 ml of the acid-soluble supernatant was taken for radioassay. The precipitate was further extracted with 5% (w/v) trichloroacetic acid at room temperature (once) and at 90°C (20 min and 10 min successively), washed with water, and extraction was continued with ethanol, chloroform-methanol (2:1, v/v), ethanol-ether (3:1, v/v) and ether (twice).

For determination of the specific radioactivity, protein samples (8–12 mg) were weighed in scintillation vials. Hyamine hydroxide (0.5 ml) was added and incubated overnight at 60°C. Samples were counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer after the addition of 10 ml of a conventional toluene scintillator, quench correction being carried out by the automatic external-standardization method. For radioassay of the acid-soluble supernatant a dioxan-containing scintillator (Bray, 1960) was used.

Sucrose gradients

To determine the relative amount of monoribosomes, oligoribosomes and polyribosomes, organs were homogenized in 1 vol. (brain) or 2 vol. (liver) of TKM buffer (50 mM-Tris-HCl, 25 mM-KCl, 5 mM-MgCl₂, pH 7.5) containing 0.25 M-sucrose, by using a loose-fitting Teflon-glass homogenizer (750 rev./min, five strokes). To the liver homogenate 0.1 vol. of anti-ferritin serum was added (Drysdale & Munro, 1967). After centrifugation for 20 min at 10000g, sodium deoxycholate was added to the postmitochondrial supernatant to give a concentration of 1.3% (w/v). After dilution with 1 vol. of water, samples of 20–30 $E_{260}^{1\text{cm}}$ units were layered over a linear sucrose gradient (10–40%, w/v) which was prepared in the same TKM buffer. Liver samples were centrifuged in the Spinco SW 25.1 rotor (28 ml, 25000 rev./min, 3 h, 3°C) and brain samples in the SW 40 rotor (12 ml, 30000 rev./min, 3 h, 3°C).

In some experiments, exponential sucrose gradients and a buffer of different ionic strength were used to separate ribosomal subunits as well. Organs were homogenized in a medium containing 10 mM-Tris, 0.25 M-KCl and 2 mM-MgCl₂ (adjusted to pH 7.4 with HCl). Gradients were prepared in the same buffer, as described by Morgan *et al.* (1971), and centrifuged in the Spinco SW 40 rotor (40000 rev./min, 195 min, 3°C).

Gradients were removed by suction from the bottom of the centrifuge tubes and passed through the flow cell of a Uvicord analyser (LKB Produkter, Stockholm, Sweden). Extinction at 255 nm was continuously recorded after logarithmic conversion of transmission values. All steps were carried out at 1–3°C.

Alkylation by *N*-methyl-*N*-nitrosourea of polyribosomal RNA *in vitro*

The postmitochondrial supernatant (prepared in TKM buffer as described above) from the pooled brains of six untreated rats was made 1.3% with respect to deoxycholate and the pH adjusted to 8.4 with 1 M-Tris. *N*-[¹⁴C]Methyl-*N*-nitrosourea (347 μCi/mmol) was added to portions of the supernatant to give concentrations of 1.6, 3.2, 6.4 and 12.8 mM respectively, and each was incubated for 3 min at 37°C. Under these conditions, the half-life of *N*-methyl-*N*-nitrosourea is approx. 1.4 min. The polyribosome profile was then analysed on a linear sucrose gradient (SW 40 rotor) as described above. To determine the extent of alkylation, the remainder of the supernatant was centrifuged in the Spinco SW 56 Ti rotor for 1 h at 33200g. The microsome pellet was resuspended and precipitated with 5% (w/v) trichloroacetic acid. RNA was extracted by incubation in 5% (w/v) trichloroacetic acid for 20 min at 85°C. The supernatant fraction was made 1 M in HCl and further hydrolysed for 1 h at 100°C. The extent of alkylation of polyribosomal RNA at the N-7 position of guanine was determined after ion-exchange chromatography on Dowex 50 (H⁺ form) as described previously (Swann & Magee, 1968; Kleihues & Magee, 1973).

Results

In a preliminary experiment, the specific radioactivity of total proteins from liver, blood serum and brain was determined at 15, 30 and 60 min after injection of L-[1-¹⁴C]leucine (Fig. 1). In brain and liver, maximum specific radioactivity was reached after about 30 min. Serum proteins, mainly produced in the liver, showed a delayed increase in specific radioactivity. In the following incorporation studies, a time-interval of 30 min after application of labelled leucine was used.

The intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100 mg/kg) caused an inhibition of the incorporation of [¹⁴C]leucine into proteins of several organs of the rat (Fig. 2a). The maximum effect was present after 2–3 h. At this time, the specific radioactivity of liver, serum and brain proteins was decreased by 30–45% ($P < 0.001$). Concurrent with the decrease in [¹⁴C]leucine incorporation there was an increase in the total ¹⁴C radioactivity in the acid-soluble pool (Fig. 2b). Again, the maximum effect was present after 3 h, and at 9 h values had returned to the control value. The marked changes in the amount of ¹⁴C radioactivity in blood serum seemed to indicate that the effect of *N*-methyl-*N*-nitrosourea on the incorporation of [¹⁴C]leucine into proteins might not be restricted to liver and brain. This was

Fig. 1. Incorporation of L-[1- 14 C]leucine into proteins of liver, blood serum and brain

Adult rats received an intravenous injection of L-[1- 14 C]leucine (13.3 μ Ci/kg) and were killed after different time-intervals. Isolation of proteins and determination of specific radioactivities were as described in the text. Each point is the mean of five experiments $\pm$ S.D.; ●, liver; ■, brain; ▲, serum.

confirmed by additional experiments on several rat tissues (Table 1).

Analysis of the postmitochondrial supernatant from rat liver on linear sucrose gradients (5mM-MgCl₂, 25mM-KCl) revealed that *N*-methyl-*N*-nitrosourea causes a disaggregation of polyribosomes (Fig. 3). The concomitant increase in monomeric ribosomes coincided with the time-course of inhibition of [14 C]leucine incorporation into liver proteins. At 9h after application of *N*-methyl-*N*-nitrosourea, the relative amount of polyribosomes was somewhat higher than in control animals. Similar gradient analyses on the pooled brains of *N*-methyl-*N*-nitrosourea-treated rats showed no significant changes in the polyribosome profile.

In the weanling rats, however, *N*-methyl-*N*-nitrosourea caused a breakdown of polyribosomes both in liver and brain (Fig. 4) but in the latter organ the effect was considerably less marked (Fig. 4b). Preparation of the postmitochondrial supernatant in a buffer of low Mg²⁺ concentration revealed that in animals treated with *N*-methyl-*N*-nitrosourea the breakdown of polyribosomes is paralleled by an increase in ribosomal subunits and monoribosomes (Fig. 4). Disaggregation of polyribosomes by mild ribonuclease treatment *in vitro*, on the other hand, led to an increase in monoribosomes and oligoribosomes whereas the amount of ribosomal subunits remained unchanged (Fig. 5).

Fig. 2. Effect of *N*-methyl-*N*-nitrosourea on the incorporation of L-[1- 14 C]leucine into total proteins of liver, serum and brain

At different time-intervals after an intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100mg/kg), adult rats received an intravenous injection of L-[1- 14 C]leucine (13.3 μ Ci/kg) and were killed 30min later. Each point is the mean of five experiments $\pm$ S.D.; ●, liver; ■, brain; ▲, serum. (a) Specific radioactivity (d.p.m./mg) of proteins. For each organ control values were set at 100%. The changes observed at 1.5 and 3h were statistically significant ($P < 0.001$). (b) Total 14 C radioactivity in the acid soluble supernatant (expressed as d.p.m./mg wet wt. $\pm$ S.D.).

The incubation *in vitro* (37°C, 3min) of the postmitochondrial supernatant from adult rat brain led to a partial breakdown of polyribosomes but this effect was not enhanced by the addition of *N*-methyl-*N*-nitrosourea (1.6–12.8mM) to the incubation mixture (Fig. 6). The latter treatment resulted in a methylation at the N-7 position in 0.4–3.2% of the guanine moieties in polyribosomal RNA.

Table 1. *Effect of N-methyl-N-nitrosourea on the incorporation of L-[1-¹⁴C]leucine into proteins of several organs of the rat*

Adult rats received an intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100mg/kg). Control animals were injected with a similar volume of 0.9% NaCl. L-[1-¹⁴C]Leucine (12.5 μ Ci/kg) was administered intravenously 2½ h later and animals were killed after a further 30 min. Isolation of proteins and determination of specific radioactivities were as described in the text. Results are the mean of five experiments $\pm$ s.d.

	Incorporation (d.p.m./mg)		Inhibition (%)	Probability (by Student's <i>t</i> test)
	Control	Treated		
Spleen	185 $\pm$ 8	154 $\pm$ 26	16	<i>P</i> < 0.05
Kidney	290 $\pm$ 33	204 $\pm$ 14	30	<i>P</i> < 0.001
Intestine	599 $\pm$ 57	392 $\pm$ 47	35	<i>P</i> < 0.001
Bone marrow	443 $\pm$ 37	277 $\pm$ 57	37	<i>P</i> < 0.001
Muscle	27 $\pm$ 6	14 $\pm$ 3	48	<i>P</i> < 0.005

Fig. 3. *N*-Methyl-*N*-nitrosourea-induced changes of the polyribosome profile from rat liver

Adult rats received an intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100mg/kg) and were killed (b) 3h or (c) 9h later; (a) control. The postmitochondrial supernatant (in 5mM-MgCl₂, 25mM-KCl) of the pooled livers of three rats was analysed on a linear sucrose gradient (SW 25.1 rotor, 25000 rev./min, 3h). Sedimentation was from the right to the left. Monoribosomes (1) and oligoribosomes (containing 2–6 ribosomes) are indicated.

Discussion

The present investigation shows that sublethal doses of *N*-methyl-*N*-nitrosourea cause an inhibition of protein biosynthesis in the rat. When changes in the precursor pool, indicated by the increase in acid-soluble radioactivity, are taken into account, the incorporation of [¹⁴C]leucine into total proteins of liver and brain was decreased by more than 50%. After systemic administration, *N*-methyl-*N*-nitrosourea is rapidly distributed throughout all organs of the rat (Kleihues & Patzschke, 1971) and no enzymic activation seems to be required for its breakdown *in vivo*. Accordingly, *N*-methyl-*N*-nitrosourea was shown to react with nucleic acids of several organs of the rat (Swann & Magee, 1968; Kleihues & Magee, 1973). This is consistent with the finding that the effect of *N*-methyl-*N*-nitrosourea on protein

synthesis was present in all organs investigated, although the degree of inhibition varied to some extent (Table 1).

The early onset of inhibition with a maximum effect at 2–3h (Fig. 2) may be explained by the rapid conversion of *N*-methyl-*N*-nitrosourea into biologically active metabolites *in vivo* (Swann, 1968). In liver and brain the incorporation of [¹⁴C]leucine returned to the normal within 9h (Fig. 2).

Dis-aggregation of polyribosomes has been observed to parallel the inhibition of protein synthesis by dimethylnitrosamine (Villa-Trevino, 1967) and methylazoxymethanol acetate (Shank, 1968) and this effect was also found in the rat liver after injection of *N*-methyl-*N*-nitrosourea (Fig. 3). The possible significance of the emergence of monomeric ribosomes after dimethylnitrosamine application has

Fig. 4. Effect of *N*-methyl-*N*-nitrosourea on the polyribosome profiles from liver and brain of weanling rats

Animals (10 days old) received an intraperitoneal injection of *N*-methyl-*N*-nitrosourea (100mg/kg) and were killed 3h later. The postmitochondrial supernatant (in 2mM-MgCl₂-0.25M-KCl) of the pooled organs of four rats was analysed on an exponential sucrose gradient as described in Fig. 5. (a) Liver; (b) brain; (i) control; (ii) +*N*-methyl-*N*-nitrosourea. Small (S) and large (L) ribosomal subunits are indicated.

been discussed by various authors (Kriek & Emmelot, 1963; Magee & Barnes, 1967; Villa-Trevino, 1967; Magee & Swann, 1969; Vernie *et al.*, 1971). The disaggregation of polyribosomes could either be the

Fig. 5. Effect of ribonuclease on the polyribosome profile from rat brain

The postmitochondrial supernatant (2mM-MgCl₂, 0.25M-KCl) of the pooled brains of three adult rats was analysed on an exponential sucrose gradient. (a) Control; (b) a sample of the supernatant that had previously been treated with pancreatic ribonuclease (1μg/ml) for 30min at 0°C. Centrifugation was carried out in the SW 40 rotor (40000rev./min, 195min). Small (S) and large (L) ribosomal subunits, monoribosomes (1), oligoribosomes (2-4), and polyribosomes (P) are indicated.

cause or the consequence of the lower rate of protein synthesis. It has been suggested that the methylation *in vivo* of polyribosomal mRNA may cause an instability of the messenger leading to mRNA strand breaks with consecutive breakdown of polyribosomes and inhibition of protein synthesis (Villa-Trevino, 1967; Lundeen *et al.*, 1971). Degradation of RNA was shown to occur after reaction with diazomethane (Kriek & Emmelot, 1963) and *N*-methyl-*N*-nitrosourea (Lawley *et al.*, 1971) *in vitro* and has been attributed to the methylation of phosphodiester groups (see Lawley & Shah, 1972). It seems, however, doubtful whether the extent of this reaction after application of dimethylnitrosamine or *N*-methyl-*N*-nitrosourea *in vivo* is quantitatively sufficient to explain the marked disaggregation of polyribosomes (Magee & Swann, 1969).

The methylation of polyribosomal RNA from rat brain by *N*-methyl-*N*-nitrosourea *in vitro* did not affect the polyribosome profile (Fig. 6), although the amount of 7-methylguanine produced was up to 20 times higher than the concentration found after application of the median lethal dose *in vivo* (Kleihues & Magee, 1973). The view that the dis-aggregation of polyribosomes is not a direct consequence of mRNA methylation is further supported by the analysis of the postmitochondrial supernatant at low Mg²⁺ and high salt concentrations on exponential sucrose

Fig. 6. Alkylation by *N*-methyl-*N*-nitrosourea of polyribosomal RNA *in vitro*

The postmitochondrial supernatant from the pooled brains of six adult rats (5mM-MgCl₂, 25mM-KCl) was adjusted to pH 8.4 and kept at 0°C (shaded area), incubated for 3 min at 37°C (—), or incubated for 3 min at 37°C in the presence of *N*-[¹⁴C]methyl-*N*-nitrosourea (.....). The concentration of *N*-[¹⁴C]methyl-*N*-nitrosourea was 6.4mM, leading to the methylation at the N-7 position in 1.6% of the guanine moieties. The supernatant was subsequently analysed on a linear sucrose gradient as described.

gradients. Instability of mRNA as a result of strand scissions should have an effect on the polyribosome profile similar to that of mild ribonuclease treatment, which causes a loss of heavy polyribosomes and an increase in oligoribosomes and monomers, whereas the number of ribosomal subunits remains unchanged (Fig. 5). Treatment with *N*-methyl-*N*-nitrosourea *in vivo*, however, led to an increase in ribosomal subunits and monomers without a concomitant increase in oligoribosomes (Fig. 4).

The accumulation of subunits suggests that in rat liver *N*-methyl-*N*-nitrosourea interferes predominantly with steps involved in the initiation of new peptide chains. Initiation could be inhibited by a decrease in mRNA synthesis but this seems unlikely since *N*-methyl-*N*-nitrosourea was shown to have no effect on RNA synthesis in rat liver and kidney (Kleihues, 1972). Analyses of the postmitochondrial supernatant from adult rat brains did not reveal a

dis-aggregation of polyribosomes although *N*-methyl-*N*-nitrosourea inhibited cerebral and hepatic protein synthesis to a similar extent (Fig. 2). This would indicate that in adult rats the mechanism of inhibition of protein synthesis is not identical in liver and brain.

The increase in rat liver of ribosome monomers (Fig. 3) is more difficult to interpret since these could either represent ribosomes of the 'run-off' type or functional ribosomes capable of taking part in protein synthesis. Ribosomes of the first type emerge from chain termination. They seem to undergo a fall in the sedimentation coefficient (from 77S to 59S) before they dissociate into their subunits (Bielka *et al.*, 1968) and show a high degree of poly(U)-dependent phenylalanine incorporation *in vitro* (Hunter & Korner, 1969; Bont *et al.*, 1971). It is therefore noteworthy that Vernie *et al.* (1971) found an increased number of 77S ribosomes after application of dimethylnitrosamine *in vivo*. The observation that liver microsomal fractions from animals treated with dimethylnitrosamine (Mizrahi & Emmelot, 1964; Mager *et al.*, 1965) or *N*-methyl-*N*-nitrosourea (P. Kleihues & B. W. Stewart, unpublished work) show an increased stimulation by poly(U) of phenylalanine incorporation *in vitro* also indicates that the breakdown of polyribosomes leads to an accumulation of non-programmed monomers.

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