Early effects of dimethylnitrosamine on the initiation of protein synthesis in mouse liver

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1. Dimethylnitrosamine (37.5 mg/kg body wt.) was administered to mice by a single intraperitoneal injection, and the early effects on protein synthesis and related functions were studied in a liver S-30 system. 2. The incorporation of [^{14}C]leucine into protein decreased rapidly after dimethylnitrosamine administration. The effect was associated with a decreased ability of the system to utilize methionyl-tRNA^{fMet} and formyl-methionyl-tRNA^{fMet} for 80S ribosomal initiation-complex formation (primary initiation), and a loss of poly(A)-containing RNA from the postmicrosomal fraction. All the three effects developed simultaneously, and were clearly demonstrable within 15 min. 3. Initiation-complex formation in the polyribosomal fraction (re-initiation) was decreased to the same extent as the primary initiation, indicating that the initiation defect was not a result of the decrease in free mRNA. 4. The inhibition of initiation was only manifest at the joining of the 40S pre-initiation complex to 60S ribosomal subunits. It was not a result of methionyl-tRNA^{fMet} deacylation. The functions between the formation of the methionyl-tRNA^{fMet}-containing 80S ribosomal complex and the first translocation on the ribosome were not involved, since the incorporation of formylmethionine into *N*-terminal polypeptides decreased to the same extent as the 80S initiation-complex formation. 5. Inhibitors of protein synthesis (cycloheximide and pactamycin) decreased poly(A)-containing RNA in the postmicrosomal fraction in a similar way to dimethylnitrosamine.

The hepatotoxin DMNA inhibits liver protein synthesis (Magee, 1958; Hultin *et al.*, 1960; Brouwers & Emmelot, 1960; Mizrahi & Emmelot, 1964; Stewart & Magee, 1971), and causes a rapid loss of ribosomes from polyribosomal aggregates, leading to an accumulation of inactive single ribosomes (Mizrahi & Emmelot, 1964; Vernie *et al.*, 1971; Williams & Hultin, 1973). During the development of these effects there is no marked decrease in the activity of isolated ribosomes in translating artificial or physiological mRNA *in vitro* (Williams & Hultin, 1973; Delpino & Ferrini, 1973, 1976, 1977; Nygård & Hultin, 1975). The defect in protein synthesis has therefore been ascribed to damage either to the mRNA or to the mRNA-dependent mechanism of protein-chain initiation (Mizrahi & Emmelot, 1964; Villa-Trevino, 1967; Williams & Hultin, 1973).

Although liver mRNA is methylated by DMNA

(Nygård & Hultin, 1975; Pegg & Jackson, 1976), no loss of messenger activity could be demonstrated in an assay system *in vitro* (Nygård & Hultin, 1975). On the other hand, attempts to account for the depression of protein synthesis by a lower activity of isolated components of the initiation system were also unsuccessful (Nygård & Hultin, 1975, 1978; Delpino & Ferrini, 1977; Pegg, 1977).

Working with a more integrated system, based on the S-30 fraction (Austin & Kay, 1975) of mouse liver homogenates, we observed a DMNA-induced deficiency of the system in forming 80S ribosomal initiation complexes (Nygård & Hultin, 1979). The effect was associated with a loss of poly(A)-containing RNA from the postmicrosomal fraction. These observations raised several questions pertaining to the causes and possible interdependence of the two effects. Their relationship to the inhibition of protein synthesis also remained to be clarified.

The present experiments deal with some of these questions. The results indicate that the DMNA-

Abbreviation used: DMNA, dimethylnitrosamine.

induced initiation defect in the S-30 system develops concomitantly with the inhibition of protein synthesis. The data are therefore compatible with a causal relationship. The defect involves both primary initiation of 80S ribosomes and re-initiation of polyribosomes, and is confined to the joining of the 40S pre-initiation complex to 60S ribosomal subunits. The experiments suggest that the decrease in postmicrosomal mRNA is not a cause, but rather an indirect consequence, of the inhibited protein synthesis.

Experimental

Materials

Chemicals. DMNA was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. [^3H]Poly(U) (30494 c.p.m./ μg) was from Miles Laboratories, Slough, Bucks., U.K. L-[^{14}C]Leucine (330 Ci/mol) and L-[^{35}S]methionine (8 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Glass-fibre filters (GF/C) were from Whatman Biochemicals, Maidstone, Kent, U.K. Phosphocreatine, creatine kinase (EC 2.7.3.2), ATP, GTP, dithiothreitol, amino acids, cycloheximide and *Escherichia coli* aminoacyl-tRNA synthetases (ligase sub-class EC 6.1.1) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Calcium leucovorin was from Lederle Laboratories Division, Pearl River, NY, U.S.A. Pactamycin was from Upjohn Co., Kalamazoo, MI, U.S.A. Triton X-100 was obtained from Rohm and Haas, Philadelphia, PA, U.S.A.

Animals. Male mice of the Naval Medical Research Institute strain were used without being starved. Groups of three animals received an intraperitoneal injection of DMNA (37.5 mg/kg body wt.) in 0.154 M-NaCl. Controls were given an equal volume of 0.154 M-NaCl. Cycloheximide (10 mg/kg body wt.) and pactamycin (10 mg/kg body wt.) were given intraperitoneally in 0.154 M-NaCl.

Methods

Liver subcellular fractionation. The livers from the animals of each experimental group were minced, homogenized and centrifuged as previously described (Nygård & Hultin, 1979).

For protein-synthesis and initiation experiments the 30000 g_{av} supernatant (S-30 fraction) was passed through a Sephadex G-25 column as described previously (Austin & Kay, 1975; Nygård & Hultin, 1979).

For poly(A) analysis the S-30 fraction was centrifuged at 165 000 g_{av} for 60 min to give the postmicrosomal supernatant.

Preparation of [^{35}S]methionyl-tRNA fMet and formyl[^{35}S]methionyl-tRNA fMet . Rat liver tRNA was prepared as described by Stanley (1974), and charged with [^{35}S]methionine by the use of *E. coli* aminoacyl-tRNA synthetases. For the preparation of formyl[^{35}S]methionyl-tRNA fMet , calcium leucovorin was included in the reaction mixture (Stanley, 1974). The charged [^{35}S]methionyl-tRNA fMet and formyl[^{35}S]methionyl-tRNA fMet were further purified by Sepharose-4B chromatography as described by Nygård & Hultin (1976).

Protein synthesis and initiation-complex formation. The incorporation of [^{14}C]leucine into protein was determined as described previously (Austin & Kay, 1975; Nygård & Hultin, 1979). The 50 μl reaction mixtures contained 75 mM-KCl, 2.5 mM-magnesium acetate, 25 mM-Tris/HCl, pH 7.6, 1 mM-dithiothreitol, 1 mM-ATP, 0.2 mM-GTP, 4 mM-phosphocreatine, 50 μg of creatine kinase, 4 μM -L-[^{14}C]leucine, 20 μM of each L-amino acid (except leucine) and 25 μl of the gel-filtered S-30 fraction. Incubation was for 5 min at 37°C.

For the incorporation of formyl[^{35}S]methionine into the N-terminal position of newly initiated polypeptides, amino acids were omitted from the reaction mixtures. After preincubation for different periods at 20°C as indicated, 3.5 μg of formyl-[^{35}S]methionyl-tRNA fMet (2.6×10^4 c.p.m.) was added and the incubation continued for 5 min. Then a mixture of 20 amino acids each at 20 μM final concentration was added, and the incubation was continued for 20 min at 20°C.

For the initiation experiments, 12.4 μg of [^{35}S]methionyl-tRNA fMet (22.3×10^4 c.p.m.) or 8.8 μg of formyl[^{35}S]methionyl-tRNA fMet (14.9×10^4 c.p.m.) was substituted for the amino acid mixture. Incubation was for 2 min at 37°C in final volumes of 0.6 ml. After incubation, 0.1 vol. of 10% (v/v) Triton X-100 was added, and the reaction mixtures were analysed by centrifugation for 2 h at 201 000 g_{av} in 11 ml linear 0.29–1.20 M-sucrose gradients containing 75 mM-KCl, 8 mM-magnesium acetate and 20 mM-Tris/HCl, pH 7.6. The gradients were monitored at 260 nm. Consecutive 0.42 ml fractions were collected, precipitated with N-cetyl-N,N,N-trimethylammonium bromide and collected on glass-fibre filters (Darnbrough *et al.*, 1973).

Poly(A) analysis. RNA was prepared from the postmicrosomal fraction by phenol extraction (Brawerman *et al.*, 1972), precipitated with ethanol and hybridized to [^3H]poly(U) as described by Nilsson & Hultin (1975).

Protein, RNA and radioactivity determinations. Protein was determined as described by Bradford (1976), with bovine serum albumin as standard. RNA determinations were as described previously (Hultin *et al.*, 1969). Radioactivity was measured in an Intertechnique SL 36 liquid-scintillation counter

at an efficiency of 30% for ^3H , 65% for ^{14}C and 70% for ^{35}S .

Results

Time-dependence of the effects of DMNA on protein synthesis and initiation-complex formation

At a dosage of 37.5 mg/kg body wt., DMNA rapidly inhibited mouse liver protein synthesis as measured in the S-30 system (Fig. 1). Already after 15 min of DMNA treatment the incorporation of ^{14}C leucine into protein was decreased by approx. 30%.

The activity of the S-30 preparations in initiation-complex formation was analysed under the same conditions. As was shown previously (Nygård & Hultin, 1979), the amount of ^{35}S methionyl-tRNA^{Met} bound to 40S ribosomal subunits (pre-initiation complex) was not diminished, but slightly increased, after DMNA treatment (results not shown). Among the more rapidly sedimenting structures, 30% of the labelled methionyl-tRNA^{Met} was associated with 80S ribosomal monomers (primary initiation), and 70% with ribosomal oligomers and polyribosomes (re-initiation). As shown in Fig. 2(a), the formation of 80S initiation complexes containing ^{35}S methionyl-tRNA^{Met} was inhibited by DMNA treatment to the same extent, and with closely the same time course, as the incorporation of ^{14}C leucine into protein. Thus, after 15 min there

was a 30% decrease in the binding of ^{35}S methionyl-tRNA^{Met} to the 80S fraction.

Within the fractions of ribosomal oligomers and polymers, the number of potential initiator sites per

Fig. 1. Protein synthesis in the mouse liver S-30 system after different periods of DMNA treatment

The 50 μl reaction mixtures contained 75 mM-KCl, 2.5 mM-magnesium acetate, 25 mM-Tris/HCl, pH 7.6, 1 mM-dithiothreitol, 1 mM-ATP, 0.2 mM-GTP, 4 mM-phosphocreatine, 50 μg of creatine kinase, 4 μM -L- ^{14}C leucine, 20 μM of each L-amino acid (except leucine) and 25 μl of the gel-filtered S-30 fraction. Incubation was for 5 min at 37°C. DMNA was administered at 37.5 mg/kg body wt. The incorporation of ^{14}C leucine in the S-30 system derived from control mice was 11.2×10^4 c.p.m./mg of RNA (100%).

Fig. 2. Effect of different periods of DMNA treatment on primary initiation and re-initiation in the mouse liver S-30 system

Incubation was as described in Fig. 1 with the following modifications. Amino acids were replaced by 12.4 μg of ^{35}S methionyl-tRNA^{Met} (22.3×10^4 c.p.m.). The incubation mixtures (0.6 ml) were incubated at 37°C for 2 min. After incubation, 66 μl of 10% (v/v) Triton X-100 was added, and the reaction mixtures were analysed on 11 ml 0.29–1.20 M-sucrose gradients containing 75 mM-KCl, 8 mM-magnesium acetate and 20 mM-Tris/HCl, pH 7.6. The gradients were centrifuged for 2 h at 201 000 g_{av} . Gradient fractions were precipitated with *N*-cetyl-*NNN*-trimethylammonium bromide. DMNA dosage was 37.5 mg/kg body wt. (a) Binding of ^{35}S methionyl-tRNA^{Met} to 80S ribosomal monomers (primary initiation). (b, c) Binding of ^{35}S methionyl-tRNA^{Met} to ribosomal dimers and trimers respectively (re-initiation). Control values (100%) were 30.9×10^4 c.p.m./mg of total RNA (a), 5.2×10^4 (b) and 4.4×10^4 (c) c.p.m./ A_{260} unit.

A_{260} unit of ribosomes will decrease with increasing number of mRNA-linked ribosomes per aggregate. However, within each class of aggregate the ratio should be constant, irrespective of the pretreatment of the animals. In Figs. 2(b) and 2(c), the binding of methionyl-tRNA^{fMet} to ribosomal dimers and trimers has been calculated per A_{260} unit, i.e. at comparable mRNA availability. After DMNA treatment the binding was inhibited to the same extent as the concomitant binding to the 80S ribosomes (Fig. 2a). Again, the time course of the decrease was closely similar to that observed for [¹⁴C]leucine incorporation (Fig. 1).

The inhibitory effects of DMNA as analysed in the S-30 system were strictly additive. When equal volumes of S-30 preparations from DMNA-treated and control mice were included in the system, the inhibition of initiation-complex formation was close to 50% of that obtained in the homogeneous system derived from DMNA-treated animals (results not shown).

The experiments indicate that the deficient initiation-complex formation in the S-30 system after DMNA administration develops simultaneously with the inhibition of protein synthesis. The data are therefore compatible with a causal relationship. The equal depression of primary initiation and re-initiation suggests that mRNA shortage is not the limiting factor.

Methionyl-tRNA deacylation as a possible cause of initiation deficiency

The experimental data indicate that the inhibitory effect of DMNA on protein-chain initiation involves primarily the joining of the methionyl-tRNA-containing pre-initiation complex to 60S ribosomal subunits (Nygård & Hultin, 1979). In the course of the joining reaction, the bound methionyl-tRNA^{fMet} may be transiently exposed to methionyl-tRNA deacylase (Nygård & Hultin, 1977). A slight increase in the activity of this enzyme has been observed in liver microsomal extracts from DMNA-treated rats (Nygård & Hultin, 1978). In addition, methionyl-tRNA deacylation is involved in the haemin-controlled regulation of protein synthesis in reticulocytes (Gross, 1979).

To elucidate the possible role of methionyl-tRNA deacylation in the DMNA-induced initiation deficiency, advantage was taken of the fact that formylated initiator tRNA, formylmethionyl-tRNA^{fMet}, is resistant to the deacylase (Morrissey & Hardesty, 1972; Gupta & Aerni, 1973; Gross, 1979). As is shown in Table 1, the utilization of formylmethionyl-tRNA^{fMet} for 80S initiation-complex formation in the S-30 system was inhibited by DMNA treatment *in vivo* to nearly the same extent as the utilization of methionyl-tRNA^{fMet}. It is

Table 1. *Effect of DMNA treatment on the utilization of methionyl-tRNA^{fMet} and formylmethionyl-tRNA^{fMet} for 80S initiation-complex formation*

Incubation was as described in Fig. 2 except that, in experiments b, [³⁵S]methionyl-tRNA^{fMet} was replaced by 8.8 µg of formyl[³⁵S]methionyl-tRNA^{fMet} (14.9×10^4 c.p.m.). DMNA dosage was 37.5 mg/kg body wt.

Type of tRNA ^{fMet}	DMNA treatment (min)	Radioactivity in 80S fraction	
		($10^{-3} \times$ c.p.m./mg of RNA)	(% of control)
(a) Met-tRNA ^{fMet}	—	30.9	100
	60	13.6	44
	120	9.7	31
(b) fMet-tRNA ^{fMet}	—	25.7	100
	60	13.1	51
	120	11.1	43

concluded that methionyl-tRNA deacylation is of little, if any, importance in the initiation deficiency.

Synthesis of N-terminal peptides in the S-30 system

Sedimentation analysis of initiation-complex formation as described above may reflect the stability of the labelled complexes during centrifugation rather than the efficiency of the system in protein-chain initiation. An alternative, more direct, approach seemed motivated not only by this consideration, but also by the suggestion (Schoecketman & Perry, 1972) that the movement of the ribosome from the initiator site on the messenger may constitute an additional, limiting step in the initiation.

In the experiment shown in Fig. 3, formyl-[³⁵S]methionyl-tRNA^{fMet} was used for initiation-complex formation and subsequent N-terminal labelling of the initiated peptides *in vitro*. The presence of the formyl group would prevent the labelled N-terminal sequence from being detached during the further processing of the growing polypeptide (Housman *et al.*, 1970). The experiment confirms the deficiency in initiation-complex formation indicated by the sedimentation analyses (Fig. 2). In combination, the two sets of data seem to preclude the possibility of a DMNA-induced inhibition of peptide synthesis beyond the stage of initiation-complex formation. It is notable that a preincubation of the S-30 system for different periods failed to aggravate the DMNA-induced inhibition. The rate of decline of the initiating activity was approximately the same in the S-30 system from DMNA-treated and control animals (Fig. 3). It is concluded that the observed effects of

Fig. 3. Effect of DMNA on the incorporation of formyl-methionyl-tRNA^{fMet} into N-terminal polypeptides by the S-30 system

Preincubation, in final volumes of 50 μ l, was as described in Fig. 2, except for the omission of [³⁵S]methionyl-tRNA^{fMet}. At the times indicated, 3.5 μ g of formyl[³⁵S]methionyl-tRNA^{fMet} was added. After 5 min at 20°C a mixture of 20 L-amino acids was added, to final concentrations of 20 μ M. The other components of the translation system were as indicated in Fig. 1. The incubation was continued for 20 min at 20°C. ●, Control. ▲, DMNA treatment for 60 min at 37.5 mg/kg body wt.

Fig. 4. Poly(A) content of the postmicrosomal supernatant after DMNA treatment

Liver S-30 fractions from DMNA-treated (37.5 mg/kg body wt.) and control mice were centrifuged for 60 min at 166 000 g_{av} . RNA was extracted from the postmicrosomal supernatants and hybridized to [³H]poly(U). The specific poly(A) content in the supernatant of control animals was 1.2 μ g of poly(A)/mg of RNA (100%). The total RNA content of the fraction was not altered by the DMNA treatment.

DMNA on protein-chain initiation were not the result of factors operating after cell disintegration.

Postmicrosomal poly(A)-containing RNA and the inhibition of protein synthesis

DMNA-induced inhibition of protein synthesis in mouse liver is associated with a loss of poly(A)-

Table 2. Effect of protein-synthesis inhibitors on post-microsomal poly(A)-containing RNA

The animals were given an intraperitoneal injection of DMNA (37.5 mg/kg body wt.), cycloheximide (10 mg/kg body wt.) and pactamycin (10 mg/kg body wt.) 60 min before death as indicated. In the combination experiments DMNA was given 30 min before decapitation. Hybridization with [³H]poly(U) was as in Fig. 4. Results are mean values $\pm$ S.D. from four experiments.

Treatment	Poly(A)	
	(μ g/mg of RNA)	(% of control)
Control	1.32 ± 0.05	100
DMNA	0.69 ± 0.09	52
Cycloheximide	0.78 ± 0.06	59
Cycloheximide + DMNA	0.59 ± 0.04	45
Pactamycin	0.62 ± 0.03	47
Pactamycin + DMNA	0.51 ± 0.01	39

containing RNA from the postmicrosomal fraction (Nygård & Hultin, 1979). The poly(A) determinations shown in Fig. 4 were carried out in connection with the experiments shown in Figs. 1 and 2, and indicate that these effects of DMNA have a closely similar rate of progression.

The possible relationship between the decrease in postmicrosomal poly(A)-containing RNA and the inhibition of protein synthesis is further illustrated in Table 2. In these experiments mice were treated with the specific inhibitor of protein synthesis, cycloheximide and pactamycin, alone or in combination with DMNA. Both inhibitors produced a loss of postmicrosomal poly(A)-containing RNA, similar and additive to that observed with DMNA.

Discussion

The DMNA-induced inhibition of liver protein synthesis is characterized by a rapid accumulation of unprogrammed ribosomal monomers (Mizrahi & Emmelot, 1964; Vernie *et al.*, 1971), derived from polyribosomes of decreasing size, in which the mRNA gradually becomes less densely occupied with ribosomes (Nygård & Hultin, 1979). This suggests a general retardation in the rate of protein-chain initiation in the affected cells, rather than a random loss of polyribosomes through inactivation by direct chemical attack. This interpretation is supported by the fact that during the early phases of inhibition no evidence has been obtained for a persistent inactivation of isolated cell components involved in the initiation reaction *in vitro* (Nygård & Hultin, 1975, 1978).

The S-30 system preserves some basic functions of the DMNA-treated liver, which are easily lost during further fractionation. The present experiments show that the DMNA-induced inhibition of protein-chain initiation, demonstrable in this system, develops concurrently with the decrease in endogenous amino acid incorporation into protein. Most probably, it reflects the mechanism *in vivo* leading to less efficient mRNA utilization on the polyribosomes. This is consistent with the general character of the inhibition, as shown by the equal depression of primary initiation and re-initiation. The inhibitory effects in the S-30 system clearly represent a situation *in vivo*. When mixed S-30 fractions from DMNA-treated and control animals were used, neither compensatory nor repressing mechanisms were found to operate in the system in the course of the assay.

The mechanism of DMNA-induced initiation deficiency differs from the well-known regulatory mechanisms involving haemin-controlled repressor (Farell *et al.*, 1977) and interferon (Kerr *et al.*, 1976). The formation of the 40S pre-initiation complex was not affected. As is shown in the accompanying paper (Nygård & Hultin, 1981), there was no activation of mRNA-degrading endonucleases. The inhibition was only observed at the mRNA-dependent joining of the 40S pre-initiation complex to the 60S ribosomal subunits. Methionyl-tRNA^{Met} deacylation (Gross, 1979) was not involved.

The rapid decrease in postmicrosomal poly(A)-containing RNA is of interest. This material, which accounts for about 15% of the poly(A) content of the S-30 fraction (Nygård & Hultin, 1979) apparently represents a significant part of the free, informosome-like mRNA of the liver cells. Although mRNA is a limiting factor in the S-30 system (Nygård & Hultin, 1979), our experiments clearly disfavour the possibility that the DMNA-induced depression of protein synthesis is mediated through the decrease in postmicrosomal mRNA. The experiments with cycloheximide and pactamycin show that the postmicrosomal poly(A)-containing RNA responds in a similar way to specific inhibitors of protein synthesis. This raises the possibility of a rapid, compensatory mobilization of stored mRNA in different situations of impaired protein synthesis.

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