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Inhibition of *in-vitro* amino acid incorporation by the carcinogen N-methyl N'-nitro N-nitrosoguanidine

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Abstract. The addition of the carcinogen, N-methyl N'-nitro N-nitrosoguanidine, to a cell-free system consisting of purified polysome and 'pH 5 enzyme' fraction resulted in a marked inhibition of incorporation of (^{14}C)-leucine into polypeptides. The extent of inhibition was remarkably high if the cell-free system contained limiting amount of 'pH 5 enzyme' fraction. Under this condition, the rate of inhibition was dependent on the concentration of carcinogen. Some component present in the 'pH 5 enzyme' fraction was inferred to be the susceptible factor, since the inhibition at low concentration of carcinogen could be reversed by increasing the amount of this fraction in the polysomal system. It was ascertained that tRNA was the primary target of carcinogenic action. Evidence suggested that functions attributed to tRNA such as aminoacylation and ribosomal transfer were both affected in a characteristic way by the action of the carcinogenic N-nitroso compound.

Keywords. N-methyl N'-nitro N-nitrosoguanidine; carcinogen: amino acid incorporation; tRNA.

Introduction

A number of studies indicate that a variety of carcinogenic N-nitroso compounds lead to marked impairment of *in-vivo* liver protein synthesis (Magee, 1958; Emmelot *et al*., 1962; Magee and Barnes, 1967; Stewart and Magee, 1972; Arnold and Alonoso, 1973; Kleihues and Magee, 1973; Hradec and Kolar, 1974; Chu and Nirvish, 1977). A few studies also reveal that administration of certain N-nitroso compounds to rats decreases the *in-vitro* amino acid incorporating activity of their cell-free liver preparations (Hutlin *et al*., 1960, Abakumova *et al*., 1974). This inhibition, reflecting the acute effect of N-nitroso compounds, is perhaps dependent on their prior metabolism which is a necessary step for carcinogenic activity (Magee *et al*., 1975; Lijinsky, 1976).

Our aim is to examine the role of chemical carcinogens at translational level and its relevance to carcinogenesis. N-methyl N'-nitro N-nitrosoguanidine (MNNG), a member of the N-nitroso group, is one useful compound for this purpose since it does not need metabolic activation for its action (Sugimura and Kwachi, 1973). Its mutagenic (Mandel and Greenberg, 1960) and carcinogenic (Druckery *et al*., 1966;

Schoental, 1966; Sugimura *et al*., 1966) activities have been well established. Like other N-nitroso compounds, MNNG has been shown to inhibit the synthesis of macromolecules such as DNA, RNA and protein in bacterial cells (Cerda-Olmedo and Hanawalt, 1967) as well as in mammalian cells (Anderson and Burdon, 1970). Impairment of function of the individual macromolecule modified by MNNG has also been documented (Chandra *et al*., 1967; Drahovsky and Wacker, 1975; Bagewadikar and Bhattacharya, 1977).

Being a direct acting carcinogen, its effect on several parameters of protein synthesis can be studied in *in-vitro* model system. Our preliminary observations in this direction showed that addition of low concentration of MNNG resulted in a marked inhibition of amino acid incorporation in a cell-free system containing limiting but not saturating level of S-30 fraction derived from rat liver (Bagewadikar and Bhattacharya, 1979). The present study has been initiated with a view to identifying the limiting factor(s) of the subcellular components which is susceptible to inhibition.

Materials and methods

Chemicals

L-amino acids were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. L-(U-^{14}C)-leucine (Sp. act. 198 mCi mmole^{-1}) was a product from Isotope Division, Bhabha Atomic Research Centre, Bombay. N-methyl N'-nitro N-nitrosoguanidine was obtained from Fluka AG, Buchs SG, Switzerland. All other chemicals were of Analytical grade.

Buffers

Buffer A contained 0.2 M sucrose, 0.1 M NH_4Cl, 5 mM Mg-acetate, 1 mM dithio-threitol, 0.25 mM EDTA, and 0.02 M tris-HCl, pH 7.5. Buffer B had the following composition: 0.3 M sucrose, 5 mM Mg-acetate, 1 mM dithiothreitol, 0.25 mM EDTA and 0.01 M tris-HCl, pH 7.5.

Animal tissues

Male Wistar rats weighing about 150 g were used. These were sacrificed by decapitation and livers were removed quickly, washed and rinsed in ice-cold buffer A. All subsequent operations were carried out.at 4°C.

Liver polysomes

The method of Flavey and Staehlin (1970) was used with suitable modification. A 40% (wt/vol) homogenate of livers was prepared in buffer A using a glass-teflon homogenizer. The homogenate was centrifuged for 10 min at 30,000 g, the upper two-thirds of the post-mitochondrial supernatant fluid was removed and treated with sodium deoxycholate at a final concentration of 1.3%. Following this treatment the solution was layered over a discontinuous gradient consisting of 1 M and 0.5 M sucrose solutions prepared in buffer A without sucrose. The gradient was centrifuged for 3 h at 105,000 *g,* the pellets were quickly rinsed with distilled water and then dissolved in buffer A without sucrose at a concentration of about 100 A_{260nm} unit ml^{-1}. The polysomes thus obtained were stored in small portions at –80°C.

Liver 'pH 5 enzyme' fraction

The rat liver pH 5 enzyme fraction was prepared according to Falvey and Staehlin (1970).

Incorporation of (^{14}C)-leucine into polysomal protein

The experiment was carried out using certain modifications of the methods employed by Griffin *et al.* (1964) and Falvey and Staehlin (1970). The incubation mixture (200 µ1) contained tris-HCl, pH 7.5, 4 µmol; NH_4Cl, 30 µmol; Mg-acetate, 0.8 µmol; dithiothreitol, 0.2 µmol; ATP, 0.2 µmol; GTP, 0.08 µmol; creatine phosphate, 1 µmol; creatine Phosphokinase, 10 µg; l-(U-^{14}C)-leucine, 0.5 µCi; polysome, 6 $A_{260\ nm}$ units; 'pH 5 enzyme' protein, as specified; and 19 non-labelled amino acids, 5 n mol each. When required, MNNG in distilled water was added to give a final concentration as specified. The reaction mixture was incubated for 30 min at 37°C. Aliquots of 50 µ1 were then spotted on Whatman No. 3 MM filter paper discs (24 mm) which were washed several times with cold 10% trichloroacetic acid (TCA). The discs were then put in 10% TCA and heated to 90°C for 10 min. After a further wash with cold 5% TCA and two washes with ethanol: ether (1:1) and ether, the discs were air dried and the radioactivity was determined.

Analytical procedure

Protein was determined by the method of Lowry *et al.*, (1951). RNA was estimated from A_{260nm} using $A^{1\%}_{1cm}$ value of 230. The radioactivity of the samples were determined in Bray's solution using a Beckman model LS-100 liquid scintillation spectrometer.

Results

In our standard assay maximum incorporation of (^{14}C)-leucine into polysomal protein (8.7×10^4cpm mg^{-1} RNA) was observed when the reaction mixture contained 6 A_{260nm} unit of polysome and 1 mg of 'pH 5 enzyme' protein. These concentrations were considered to be optimum. The pH 5 supernatant was not required in our study since all the soluble factors were either bound to polysomes or were present in the 'pH 5 enzyme' fraction. At limiting concentration of either 'pH 5 enzymes' or polysomes, the incorporation was considerably reduced. Since we were interested in identifying the factor(s) susceptible to inhibition by MNNG, most of our initial studies were conducted with limiting concentration of either one or the other component required for *in-vitro* amino acid incorporation.

Table 1 shows the inhibition by MNNG of (^{14}C)-leucine incorporation into polysomal protein under different conditions. Under optimum condition, i.e., without any limiting factor in the reaction mixture, significant inhibition was observed only at a higher concentration of MNNG. When the 'pH 5 enzyme' fraction was limiting (0.2 mg protein), however, significant level of inhibition was apparent even at a lower concentration of MNNG. At optimum concentrations of both polysomes and 'pH 5 enzymes' or at limiting polysome concentration (1 A_{260nm} unit), the magnitude of inhibition, as compared to that at limiting 'pH 5 enzymes' concentration, was much less with 0.05 mM or with 0.2 mM and relatively

Table 1. Inhibition of incorporation of (^{14}C)-leucine by MNNG.

Limiting factor	Percent inhibition at MNNG concentration (mM)		
	0.05	0.2	0.5
None	16	19	55
pH 5 enzyme	34	57	85
Polysome	19	33	75

Incubation mixture without any limiting factor contained 6 A_{260nm} unit polysome and 1.0 mg 'pH 5 enzyme' protein. Limiting concentration of 'pH 5 enzyme' was 0.2 mg protein and that of polysome was 1.0 A_{260nm} unit. Other conditions are as described in the text.

less with 0.5 mM MNNG. In other words, it required more MNNG to achieve 50% inhibition under optimum or limiting polysome conditions than under limiting 'pH 5 enzyme' condition. The inhibition of incorporation of (^{14}C)-leucine into polysomal protein under limiting 'pH 5 enzyme' condition was dose dependent, showing lower incorporation with increasing concentrations of added MNNG.

These results are shown in figure 1. Preincubation of 'pH 5 enzyme' fraction with 0.5 mM MNNG completely inhibited its ability to mediate (^{14}C)-leucine

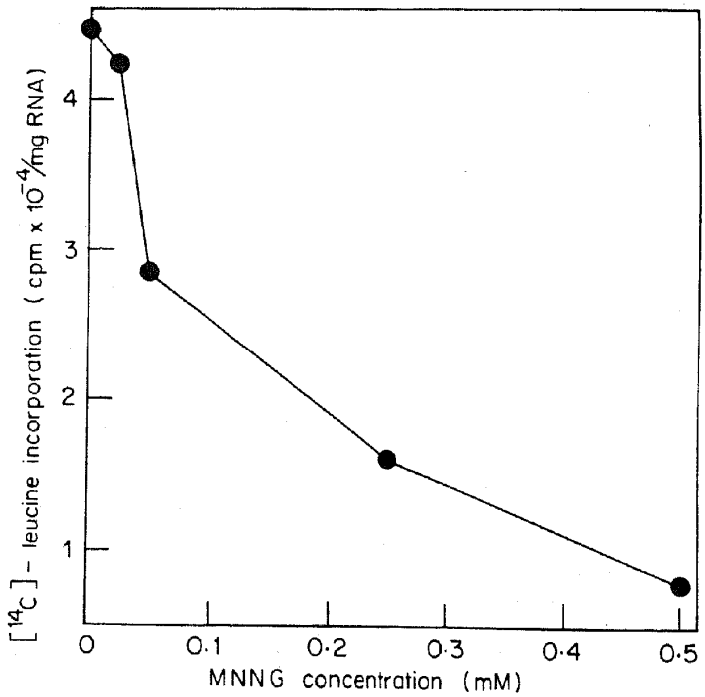

Figure 1. Effect of different concentrations of MNNG on the incorporation of (^{14}C)-leucine into polysomal protein.

Each incubation mixture contained 0.25 mg 'pH 5 enzyme' protein (limiting) and 6 A_{260nm} unit polysome. Other conditions are as described in the text.

incorporation using normal polysomes. The polysomes, on the other hand, retained more than 50% of the activity after similar treatment.

Analogous to our previous results with S-30 fraction (Bagewadikar and Bhattacharya, 1979), we have made a similar observation with the 'pH 5 enzyme' fraction. The extent of inhibition at a given concentration of MNNG was found to be inversely proportional to the amount of 'pH 5 enzyme' fraction in the assay medium. Thus the inhibition was maximum when (^{14}C)-leucine incorporation was measured with a low amount of 'pH 5 enzyme' protein. At 0.2mM MNNG, with 0.2 mg 'pH 5 enzyme' protein, where the inhibition of (^{14}C)-leucine incorporation was to the extent of 55%, a near complete reversal of activity (92% of control) could be achieved when the amount of 'pH 5 enzyme' protein was increased to a saturating level (1.6 mg protein).These results are depicted in figure 2. Under the condition of limiting amount of 'pH 5 enzyme' protein (0.2 mg), addition of exogenous tRNA, a

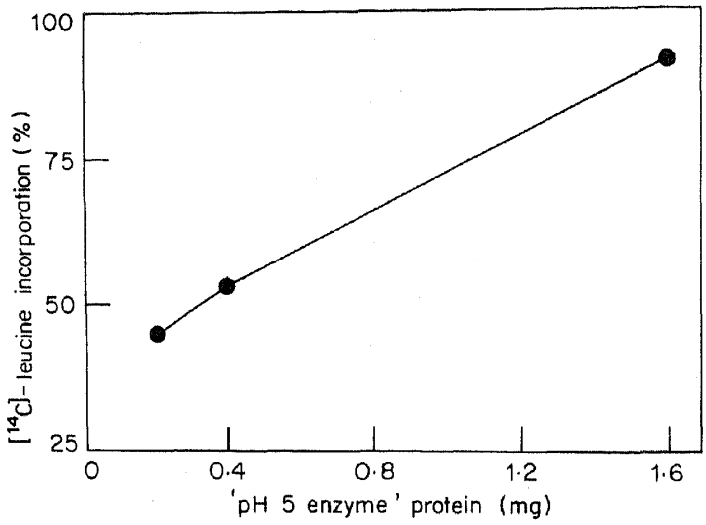

Figure 2. Reversal of inhibition by MNNG of incorporation of (^{14}C)-leucine with increasing concentrations of 'pH 5 enzyme' protein.

Each incubation mixture contained 6 A_{260nm} unit polysome and varying concentrations of 'pH 5 enzyme' protein. The concentration of MNNG was 0.2 mM. Other conditions are same as described in the text.

component of 'pH 5 enzyme' fraction, also partially reversed the inhibition induced by 0.25 mM MNNG (table 2). In this case the inhibition of 73% was brought down to a level of 21%, affording protection by 0.4 A_{260nm} unit of tRNA to the extent of 52%. Addition of more tRNA did not result in further reversal, and lower concentration was not effective.

In order to find out if the inhibition was at the level of transfer of aminoacyl-tRNA to ribosome, we conducted the following experiment. The 'pH 5 enzyme' fraction (0.8 mg protein) was incubated for 10 min at 37°C with (^{14}C)-leucine, non-labelled amino acids and other co-factors. This was performed to allow the aminoacylation reaction to be completed. After this reaction, more co-factors

Table 2. Reversal of MNNG inhibition of incorporation of (^{14}C)-leucine by exogenous tRNA

tRNA added (A_{260nm} unit)	Inhibition (%)
None	73
0.4	21

Incubation mixture contained 0.2 mg 'pH 5 enzyme' protein and 6 A_{260nm} unit polysome. MNNG concentration was 0.25 mM. Other conditions are as described in the text.

were added followed by addition of $6A_{260nm}$ unit of polysomes and the mixture was inbutated at 37°C in the presence of 0.5 mM MNNG. Control sample was identical but had no MNNG. Incorporation of (^{14}C)-leucine was then measured at intervals. The results as presented in figure 3 showed that the incorporation of (^{14}C)-leucyl-tRNA preformed in the 'pH 5 enzyme' fraction proceeded well under normal experimental condition, while it was strongly inhibited in the presence of MNNG.

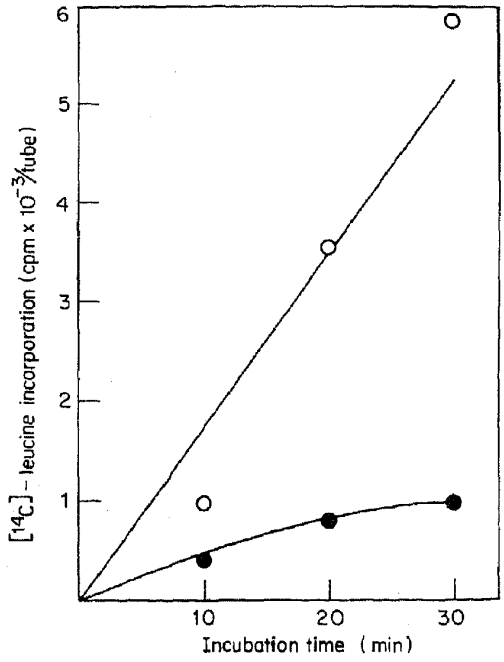

Figure 3. Effect of MNNG on the incorporation in the polysomal protein of (^{14}C)-leucyl-tRNA preformed in the 'pH 5 enzyme' fraction. Conditions are as described in the text. O, no MNNG; ●, in presence of 0.5 mM MNNG.

Discussion

Our results clearly show that MNNG, in a dose-dependent fashion, inhibits incorporation of (^{14}C)-leucine in an *in-vitro* protein synthesizing system utilizing rat

liver polysome and 'pH 5 enzyme' fraction. The inhibition is markedly significant at a limiting concentration of 'pH 5 enzyme' protein but not at limiting concentration of polysomes or when both the components are present in optimum concentrations. Further, the inhibition of incorporation of (^{14}C)-leucine into polysomal protein at a low concentration of MNNG is gradually reversed with the system being increasingly saturated with respect to 'pH 5 enzyme' protein. The 'pH 5 enzyme' fraction, in contrast to polysomal fraction, is also more susceptible to inactivation when preincubated with MNNG. These evidences as well as our earlier results (Bagewadikar and Bhattacharya, 1979) suggest that the limiting factor(s) susceptible to inhibition is present in one or more components of the 'pH 5 enzyme' fraction. The main components involved in protein synthesis of this fraction are aminoacyl-tRNA synthetases and tRNA molecules. In as much as the amino-acyl-tRNA synthetases are not affected by MNNG (Bagewadikar and Bhattacharya, 1977), it is reasonable to infer that the tRNA molecules are susceptible to attack by MNNG. Our earlier observations have revealed that tRNA has great propensity for modification by MNNG (Bagewadikar and Bhattacharya, 1977). Similar observations have been noted with other carcinogens (Farber and Magee, 1960; Magee and Farber, 1962; Weinstein, 1971; Blobstein, *et al.,* 1975; Gurtoo and Dave, 1975; Pietropaolo and Weinstein, 1975; Daoud and Griffin, 1976). We have also demonstrated here that, under suitable condition, addition of exogenous tRNA to the *in-vitro* amino acid incorporation system can reverse the inhibitory effect of MNNG. All these evidences indicate that MNNG as a carcinogen alters the characteristics of tRNA, through certain modification, in such a way that its ability to function normally in protein synthesis is impaired.

Having fairly etstablished that tRNA is the primary target of attack by MNNG, it is necessary to find out at what level of protein synthesis MNNG acts. It appears likely that the aminoacylation step is primarily affected. Clear evidence in support of this has been obtained earlier (Bagewadikar and Bhattacharya,1977).In addition, it is also seen from the results presented in figure 3 that the transfer of aminoacyl-tRNA to ribosomes is prevented in the presence of MNNG. This experiment, however, is of preliminary nature. We need more proof in this respect by studying the factor-dependent transfer of (^{14}C)-leucyl-tRNA to ribosome.

Various studies have pointed to multiple mode of action of chemical carcinogens in protein synthesis. Grab *et al.* (1979) showed that methylazoxymethanol, a methylating carcinogen, inhibited protein synthesis in rat liver. The site of its action was shown to be at the polysome level, and that both free and membrane bound polysomes were affected. Dissociation of polysome was found to be the primary mechanism by which several different carcinogens inhibited protein synthesis (Sidransky *et al.,* 1977; Murthy and Verney, 1977; Sidransky and Verney, 1978). Other effects involving different components were also observed (Hutlin, 1960; Terawaki and Greenberg, 1965; Craddock, 1969). It is evident from our observations that the inhibition of protein synthesis by MNNG is mediated through modification of tRNA molecules, impairing both aminoacylation and transfer steps. This modification involves hypermethylation (Bagewadikar and Bhattacharya, 1977),since MNNG imparts its biological action through methylation (Sugimura and Kwachi, 1973).

The modification of tRNA may be related to suppressed protein synthesis, but its relevance to carcinogensis cannot easily be established. It can be viewed, however, that altered tRNA may lead to a functionally altered protein which otherwise may participate in controlling the perpetual growth of cells.

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