Analysis of translation products synthesized in isolated rat hepatocytes treated with diethylnitrosamine[1]

A. Delpino, E. Mattei, R. Falcioni and U. Ferrini

Istituto Regina Elena per lo Studio e la Cura dei Tumori, Viale Regina Elena, 291, I-00161 Rome (Italy), 22 August 1983

Summary. Isolated rat hepatocytes were labeled with ^{35}S-methionine in the presence of 25 mM diethylnitrosamine (DENA). The intrinsically labeled proteins were analyzed by one- and two-dimensional gel electrophoresis and the fluorographic patterns were compared with those obtained from untreated hepatocytes. The results of short term experiments (2 h) show that, in the presence of 25 mM DENA, protein synthesis is inhibited by 50%. This reduction encompasses all protein species without selective inhibition of certain proteins.

Key words. Diethylnitrosamine; isolated hepatocytes; protein synthesis.

It is known that diethylnitrosamine (DENA), like other dialkylnitrosamines, must be N-deacylated by specific mixed function oxidases (present mainly in the liver) to generate the ultimate biologically active metabolite/s, responsible for the well known carcinogenic and toxic effects of this drug[2].

Previous experiments carried out in our laboratory have shown that isolated rat hepatocytes, by retaining their ability to enzymatically activate dialkylnitrosamines, represent a reliable experimental model for the study of the early effects of DENA in short-term experiments[3].

An early and severe protein synthesis inhibition takes place in isolated hepatocytes exposed to DENA[3], which is similar to the effect observed when this drug is injected into the animal. The DENA-promoted protein synthesis inhibition, however, is reversible and isolated hepatocytes, following a two-hour exposure to the drug, rapidly regain their initial protein synthetic rate when further incubated in a nitrosamine-free medium.

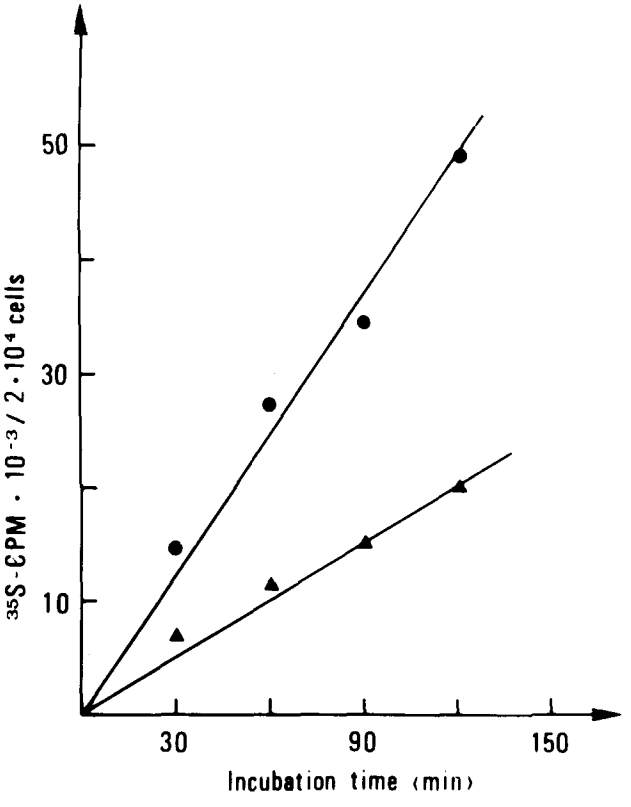

Figure 1. Time course of ^{35}S-methionine incorporation in control (●) and DENA-treated (▲) hepatocytes. Isolated rat hepatocytes were resuspended at a density of $1 \cdot 10^6$ cells/ml in Hanks' balanced salt solution, containing malate, unlabeled amino acids, vitamins, antibiotics and 100 μCi/ml of ^{35}S-methionine. In the treated sample, DENA was added at a final concentration of 25 mM. Cells were incubated at 37 °C for 120 min. Aliquots (20 μl, corresponding to 20,000 cells) were withdrawn every 30 min to determine TCA-insoluble radioactivity according to Mans and Novelli[6].

In the present study we have characterized the translation products synthesized by isolated rat hepatocytes exposed to a DENA concentration which is able to reduce the overall protein synthesis by 50%, as compared to controls. The aim of the study was to verify whether the inhibitory activity of DENA is a specific or whether the synthesis of some proteins is specifically repressed or enhanced.

Materials and methods. Isolated hepatocytes were obtained from Sprague-Dawley male rats by the collagenase-hyaluronidase perfusion technique described by Seglen[4].

The hepatocytes were resuspended (1.10^6 cells/ml) in Hanks' balanced salt solution, supplemented with malate (5 mM), vitamins, antibiotics and unlabeled amino acids at a concentration of '4 × normal', as recommended by Seglen[5]. After the

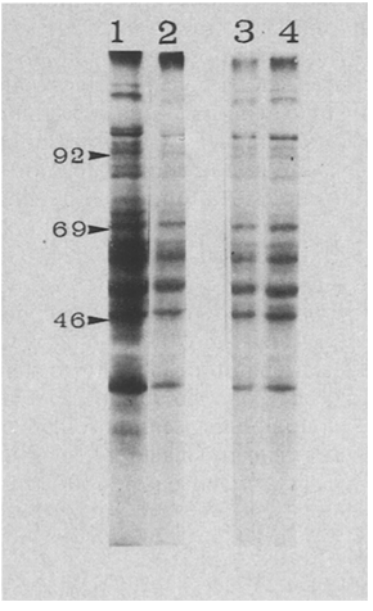

Figure 2. Electrophoretic analysis of ^{35}S-methionine labeled polypeptides synthesized in control and DENA-treated hepatocytes. Isolated hepatocytes were incubated at 37 °C for 120 min in the presence or absence of DENA, under the conditions described in the legend to figure 1. The hepatocytes were then lysed and aliquots of the labeled proteins were applied to a 7.5% polyacrylamide slab gel of 0.75 mm thickness and electrophoresed according to the buffer system and procedure described by Laemmli[7]. The gel slabs were fixed, embedded with PPO/DMSO, dried and exposed to a Fuji X-ray film at −70 °C for four days with intensifier screens. Equal amounts of protein (about 10 μg) were applied onto the first two lanes (Lane 1 = control sample; Lane 2 = treated sample). In contrast, equal amounts of radioactivity (about 25,000 cpm) were applied to the other two lanes (Lane 3 = control sample; Lane 4 = treated sample) to facilitate comparison of the relative rates of synthesis of the various polypeptides resolved in the gel. ^{14}C-methylated protein markers (phosphorylase B, mol.wt 92,500; bovine serum albumin, mol.wt 69,000; ovalbumin, mol.wt 46,000; carbonic anhydrase, mol.wt 30,000) were used to calibrate the relative mobility in the gel. Their positions, with the respective M_r in kdaltons, are indicated on the left of the figure.

addition of DENA (Merck-Schuchardt, Germany) to a final concentration of 25 mM and of 100 μCi/ml of ^{35}S-methionine (Amersham, England, s.a. > 800 Ci/mMol), the samples were incubated at 37 °C for 120 min.

To determine the time-course of methionine incorporation, aliquots from each sample were withdrawn at progressive time intervals, spotted onto Whatmann 3MM filter paper disks and processed according to Mans and Novelli[6].

For the one-dimensional electrophoretic separation of labeled proteins, the hepatocytes were lysed in a small volume of Tris-HCl 60 mM (pH 6.8) 2-mercaptoethanol 700 mM and 2% SDS. Aliquots were then electrophoresed in sodium dodecyl sulphate 7.5% polyacrylamide slab gels (SDS-PAGE) according to Laemmli[7]. Gels were stained with Coomassie blue or processed for fluorography[8].

For the two-dimensional analysis of the labeled proteins, the hepatocytes were lysed in a buffer containing 9.95 M urea, 4% NP-40, 100 mM dithiothreitol, ampholines (pH range 3–10) and 0.3% SDS as a protein solubilizing agent[9]. Aliquots containing approximately $0.5 \cdot 10^6$ cpm of TCA-precipitable material were electrofocused on 5% polyacrylamide-urea gels, containing ampholines as indicated. These gels were then subjected to a second dimension SDS-PAGE according to O'Farrell[10].

Results. Figure 1 shows the time course of ^{35}S-methionine incorporation into hot TCA-insoluble material by isolated rat hepatocytes incubated at 37 °C in the presence or absence of DENA at a final concentration of 25 mM. In the control sample the incorporation rate is linear for up to 2 h of incubation. In the presence of DENA, however, the incorporation of labeled methionine is still linear but proceeds at a reduced rate and the value attained at the end of the incorporation is reduced by approximately 50% as compared to the control sample. Dye exclusion test indicates that the amount of viable hepatocytes does not significantly decrease following DENA exposure.

The one-dimensional electrophoretic patterns of labeled proteins synthesized by isolated hepatocytes during 120 min of incubation in the presence or absence of DENA are shown in figure 2. The fluorograms show that both the control and the treated hepatocytes are able to synthesize a large number of proteins, ranging from less than $2 \cdot 10^4$ to more than $1 \cdot 10^5$ daltons. The fact that in the treated sample the protein pattern shows no shift towards lower molecular species excludes the possibility that the reduced labeling rate occurring during

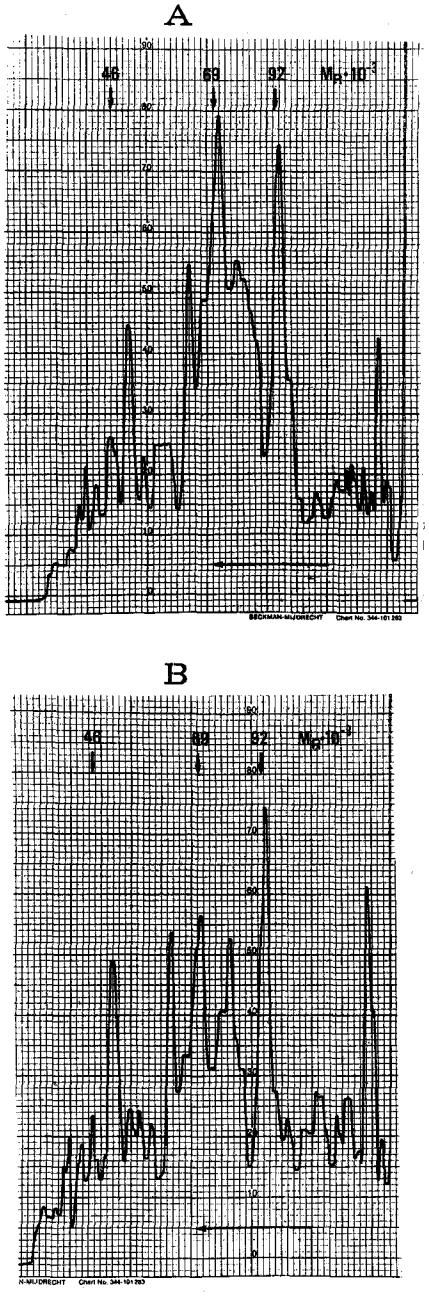

Figure 3A and 3B. Densitometric scan of fluorograms from control (panel A) and DENA-treated (panel B) hepatocytes. Spectrophotometric scanning of the fluorograms shown in figure 2, lanes 3 and 4, were performed by the gel scan attachment of model 35 Beckman spectrophotometer (wavelength = 580 nm). At the top of each panel the positions of ^{14}C-methylated protein markers (phosphorylase B, mol.wt 92,500; bovine serum albumin, mol.wt 69,000; ovoalbumin, mol.wt 46,000) are indicated, with the respective M_r in kdaltons.

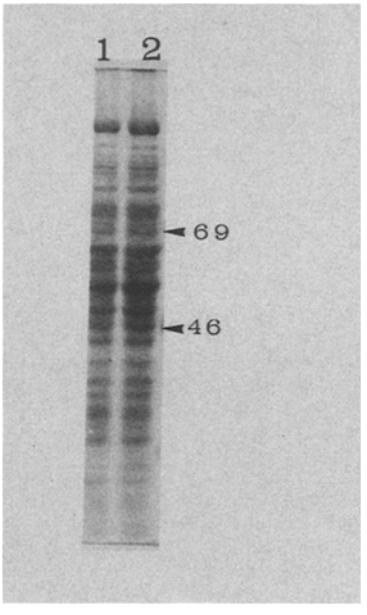

Figure 4. Coomassie brilliant blue R-250 stained gels showing total proteins from control (lane 1) and DENA-treated (lane 2) hepatocytes. Isolated hepatocytes were incubated at 37 °C for 120 min in the presence or absence of DENA, in the conditions described in the legends to figures 1 and 2 but omitting the radioactive precursor. The hepatocytes were then lysed in 'Laemmli sample buffer' and aliquots of the solubilized proteins (about 40 μg) were analyzed by SDS-PAGE[7]. Gels were stained with 0.5% Coomassie brilliant blue in 30% isopropyl alcohol 10% acetic acid. On the right, the position of the two molecular weight markers (bovine serum albumin, mol.wt 69,000; ovoalbumin, mol.wt 46,000) is indicated.

DENA exposure is the result of a proteolytic breakdown of neosynthesized proteins. A comparison of the fluorographic patterns also demonstrates that the same molecular species are synthesized in untreated and DENA-exposed hepatocytes. The relative amount of some proteins, however, appears to differ as indicated by the different intensities of the corresponding bands. These findings can be better observed in the densitometric scan of the fluorograms reported in figures 3A and 3B. It is also noteworthy that in both the control and in the treated cells all the prominent bands, apparent as labeled proteins in the fluorographic patterns, are also present as relevant bands in the corresponding Coomassie blue-stained slab gels (fig. 4). When comparing the patterns of the proteins synthesized by the isolated hepatocytes prepared from different animals, minor differences were found. However, when a comparison was made between DENA-treated and control hepatocytes prepared from an individual animal, a close similarity in the protein synthetic pattern was consistently observed (data not shown).

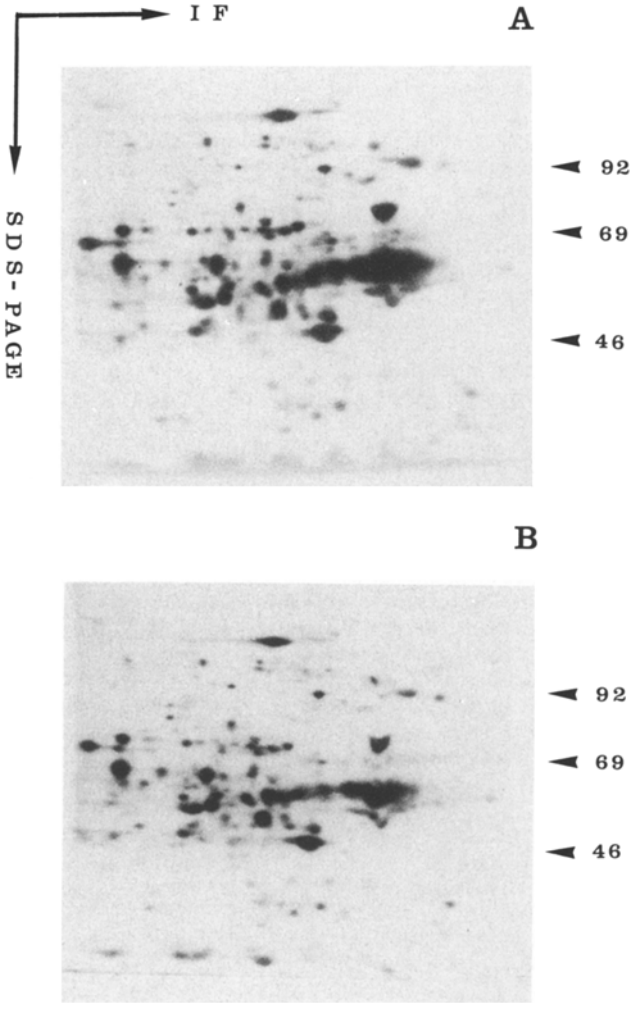

Figure 5. Two-dimensional analysis of translation products synthesized in control (upper panel) and in DENA-exposed (lower panel) hepatocytes. Isolated hepatocytes were labeled with ^{35}S-methionine for 120 min in the presence or absence of DENA (at a final concentration of 25 mM). Cell lysis and solubilization of labelled proteins were performed in a buffer containing 0.3% SDS, as described in 'Methods'. Aliquots containing about $0.5.10^6$ cpm of TCA-insoluble material, corresponding to 130 µg of proteins for the control sample and to 210 µg for the treated one, were resolved by a two-dimensional gel system (electrofocusing/electrophoresis) according to O'Farrell[10]. ^{14}C-methylated molecular weight markers (phosphorylase B, mol.wt 92,500; bovine serum albumin, mol.wt 69,000; ovoalbumin, mol.wt 46,000) were added to preformed wells on slab gels used for the second dimension. Their positions and respective M_r in kdaltons are indicated on the right side of each panel.

The molecular complexity of the translation products synthesized in isolated hepatocytes was further analyzed by a two-dimensional separation technique (electrofocusing/electrophoresis), able to resolve the translation products into a greater number of single components. When comparing the fluorograms presented in figure 5, it is evident that, even at this level of resolution, the patterns of the ^{35}S-labeled products in the control and in the DENA-treated hepatocytes are almost identical. In fact, all of the radioactive spots observed in the control cells can also be seen in the DENA-treated samples. However, careful examination of the gels shows that after DENA treatment several minor spots are either more or less intense than the corresponding spots in the control sample.

Discussion. It has already been demonstrated that a dose-dependent protein synthesis inhibition takes place in isolated rat hepatocytes incubated with DENA[3]. The data reported here show that the translation products synthesized in isolated hepatocytes, treated with a DENA concentration that reduces the overall protein synthetic rate to 50% as compared to the controls, do not differ from those synthesized in untreated hepatocytes. There appears to be only a slight difference in the relative rate of synthesis of some proteins between the control and the treated cells, as demonstrated by the quantitative differences in the respective protein synthetic patterns observed in the one-dimensional SDS-PAGE as well as in the two-dimensional gel system. These quantitative changes may be related to the fact that the competition between the various classes of messengers is magnified when protein synthesis is restricted[11]. Thus, the proteins synthesized by 'weak' messengers are relatively more inhibited than those coded for 'strong' messengers. It seems, then, that in the absence of any selective effect on the protein synthesis the early response of isolated hepatocytes to DENA treatment corresponds to a nonspecific toxic action exerted on the protein synthetic machinery. Although we cannot exclude that some changes in gene regulation occur during incubation with DENA (as described in the early stages of treatment with aflatoxin B_1[12] or with thioacetamide[13]), this effect does not seem to be relevant in the experimental conditions employed.

It would be interesting to ascertain if specific qualitative changes in protein synthesis take place when the hepatocytes, after the initial toxic damage, are allowed to recover their initial synthetic capacity. In this regard, studies are currently in progress to further clarify these possible modifications.

1 This work was supported by CNR (Project 'Control of Neoplastic Growth') grant No. 810132696 and partially by AIRC.

2 Magee, P.N., and Barnes, J.M., Adv. Cancer Res. *10* (1967) 163.
3 Mattei, E., Delpino, A., and Ferrini, U., Experientia *35* (1979) 1213.
4 Seglen, P.O., Exp. Cell Res. *82* (1973) 391.
5 Seglen, P.O., Biochim. biophys. Acta *442* (1976) 391.
6 Mans, E., and Novelli, G., Archs Biochem. Biophys. *94* (1961) 48.
7 Laemmli, U.K., Nature, Lond. *227* (1970) 680.
8 Bonner, W.M., and Laskey, R.A., Eur. J. Biochem. *46* (1974) 83.
9 Garrels, J.L., J. biol. Chem. *254* (1979) 373.
10 O'Farrell, P., J. biol. Chem. *250* (1975) 4007.
11 Lodish, H.F., A. Rev. Biochem. *45* (1976) 39.
12 Emeh, J.K., Niranjan, B.G., Bath, N.K., and Avadhani, N.G., Carcinogenesis *2* (1981) 373.
13 Chakrabartty, P.K., and Schneider, W.C., Cancer Res. *38* (1978) 2043.

0014-4754/84/121377-03$1.50 + 0.20/0