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THE PHOSPHORYLATION OF RAT LIVER RIBOSOMES FOLLOWING ADMINISTRATION OF DIMETHYLNITROSAMINE

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Abstract—Following a single dose of dimethylnitrosamine (DMNA) the phosphorylation of rat liver ribosomes was strongly enhanced. The incorporation of [^{32}P]phosphate into the 40 S subunit was stimulated 5- and 9-fold 2 and 5 hr, respectively, after administration of the drug. The phosphorylation of the large ribosomal subunit was not affected. The enhanced phosphorylation of the small subunit was due to a 20-fold stimulated incorporation of phosphate into ribosomal protein S6, the only one affected by DMNA.

One of the most prominent lesions of hepatocellular metabolism induced by the carcinogen dimethylnitrosamine (DMNA) concerns the synthesis of proteins in the liver cell [1]. Early effects of the drug are (1) a severe inhibition of the incorporation of amino acids into total liver proteins *in vivo* and *in vitro* [2–6], which is paralleled by (2) a disaggregation of polyribosomes and a concomitant increase in monomers [4, 7–12], (3) detachment of ribosomes from the membrane of the endoplasmic reticulum [13–15] and (4) a reduction in number of ribosomes per cell [1, 9]. As yet the mechanism by which DMNA or its alkylating metabolite produces the multiple and probably interrelated effects on the protein synthesizing machinery is not fully understood. However, some possibilities such as chain scission of mRNA due to the methylation of the secondary phosphate groups [4, 9, 16, 17], inhibition of mRNA synthesis and an inactivation of the ribosomal particles *per se* [9] have been made unlikely [3, 12, 17–20]. Several reports suggest that DMNA interferes with the formation of the initiation complex [2, 3, 8]. A review has also recently been published on the alkylation of cellular targets by nitroso compounds [36]. In view of the proposed regulatory functions of postsynthetic modifications of ribosomal proteins [21], by reversible phosphorylation which has been shown to occur *in vivo* on the seryl-residues of rat liver ribosomal protein S6 [22], we studied the effect of DMNA on the state of liver ribosomal protein phosphorylation. It was found that in addition to the above mentioned DMNA-induced changes of protein synthesis a prompt and strong stimulation of the phosphorylation of small subunit protein S 6 occurs.

MATERIALS AND METHODS

Treatment of rats. Male Sprague–Dawley rats (280–310 g body wt) which had free access to food and water were i.p. injected between 08.00–09.00 with 30 mg/kg body wt of dimethylnitrosamine (Merck-Schuchardt, Munich) freshly dissolved in 2 ml of 0.154 M saline. Control rats received the same volume of saline alone. Two and 5 hr later 0.8 mCi of carrier-free [^{32}P]orthophosphoric acid (New England Nuclear, Boston, MA) were administered i.p. to the animals which were sacrificed 30 min thereafter. The liver was quickly removed and chilled in ice-cold buffer. All experiments were performed in triplicate.

Preparation of ribosomes, ribosomal subunits and ribosomal protein. Ribosomes were isolated from the liver [23], their subunits prepared [24] and the proteins from the subunits extracted [25, 26]. Before extraction the 60 S subunits were recentrifuged under dissociating conditions to remove small amounts of contaminating 40 S subparticles. The specific radioactivity of the ribosomal particles (cpm/E_{260}) and of the ribosomal protein (cpm/μg r-protein) was determined as described before [22]. The concentration of protein was measured according to Lowry *et al.* [27] using bovine serum albumin as a standard.

Two-dimensional polyacrylamide gel electrophoresis and autoradiography of ribosomal proteins. 540 μg of extracted 40 S ribosomal proteins were separated by two-dimensional electrophoresis as described [22]. For autoradiography the dried gels were exposed for 8–10 days to Kodak Kodirex X-ray film. The radioactivity of protein S6 was estimated after separation of identical amounts of 40 S proteins from control and treated rat liver. The area of S6 was excised, the gel dissolved in a mixture of H_2O_2–perchloric acid and counted [28].

RESULTS

An early effect of the administration of DMNA to rats is the stimulation of the incorporation of [^{32}P]phosphate into liver ribosomes. It is shown in Fig. 1 that the sp. act. of the ribosomes was elevated by nearly 100 and 300 % 2 and 5 hr, respectively, after giving the hepatotoxic agent. After separation of the ribosomal subunits it became evident that DMNA stimulated the phosphorylation of the small and not of the large subunit. The radioactivity of the 40 S

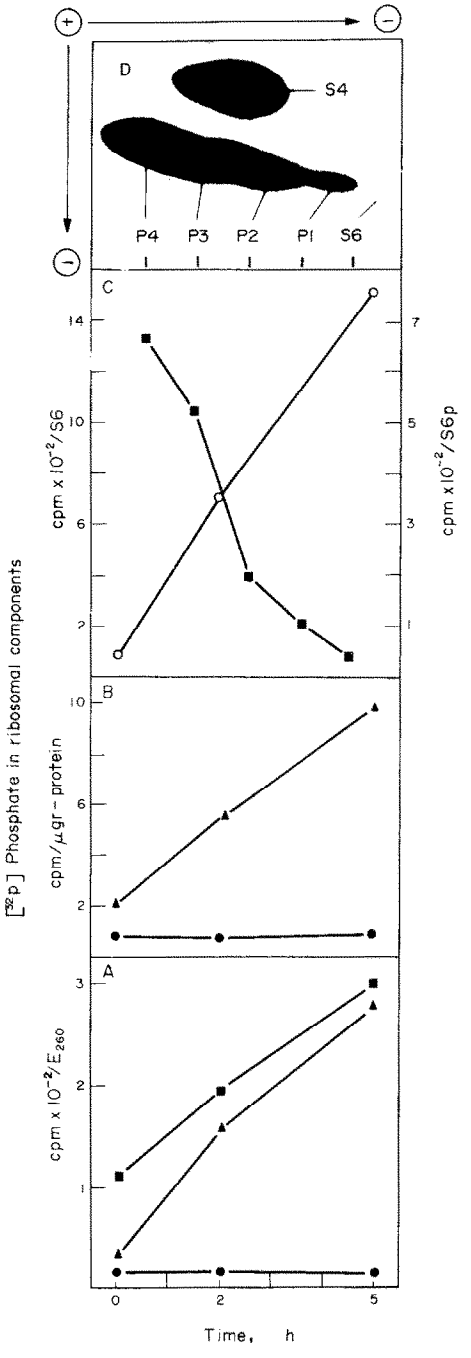

Fig. 1. The effect of DMNA on the incorporation *in vivo* of [^{32}P]phosphate into ribosomal components. Rats were i.p. injected with 30 mg/kg body wt of DMNA and received 2 and 5 hr later 0.8 mCi [^{32}P]orthophosphoric acid 30 min before exitus. Control rats received saline instead of DMNA. Ribosomes and ribosomal subunits were isolated from the liver and their specific radioactivity (cpm/E_{260}) determined (A, ■——■ 80 S; ▲——▲ 40 S; ●——● 60 S). The sp. act. of ribosomal subunit proteins (cpm/μg r-protein) was measured (B, ▲——▲ 40 S; ●——● 60 S). The radioactivity associated with protein S 6 was estimated after separation of identical amounts (540 μg) of total 40 S ribosomal proteins by two-dimensional gel electrophoresis. The area of S6 was cut out, the gel dissolved and counted (C, ○——○ S 6). For the determination of the radioactivity in the phosphorylated derivatives (S6p) of protein S6 the stained area of this protein was sliced from the cathode to the anode in the portions P_1–P_4 as indicated in (D) and counted as above (C, ■——■ S6, $S6p_{1-4}$). In (D) the region of the two-dimensional electrophoretogram containing S4 and S6p is enlarged and the phosphorylated portions P_1–P_4 (which were seen on the gel but could not be reproduced by the photograph) are indicated

subparticle increased 5- and 9-fold 2 and 5 hr, respectively, after initiation of liver injury (Fig. 1A). The DMNA-induced nearly 5-fold increase in the incorporation of [^{32}P]phosphate into the 40 S ribosomal proteins is represented in Fig. 1B. Again, the sp. act. of the 60 S ribosomal proteins was not affected by the treatment.

To study number and location of ribosomal phosphoproteins the 40 S subunit proteins isolated from liver injured for 2 and 5 hr by DMNA were analysed by two-dimensional gel electrophoresis and autoradiography. Figure 2 demonstrates that DMNA affected the position and shape of only a single protein (S6, according to the nomenclature of Sherton and Wool [29]; the electrophoretic pattern of the other proteins remained unchanged. Two hr after administration of DMNA S6 was shifted anodically due to the occurrence of its negative charged phosphorylated derivatives (S6p). Five hr after initiation of liver damage this process was even more pronounced (Fig. 2C). Autoradiography of the proteins of the small subunit revealed that only protein S6 was phosphorylated; no additional phosphoprotein could be detected under the influence of DMNA (Fig. 2D).

The time-dependent stimulation of the phosphorylation of this protein is shown in Fig. 1C. Associated radioactivity of S6 was augmented nearly 10- and 20-fold 2 and 5 hr, respectively, after application of the compound. The distribution of radioactivity within protein S6 was not uniform but showed a gradient increasing from the cathode to the anode end (Fig. 1C, D; Fig. 2D).

In other experiments the number of phosphorylated derivatives of S6 occurring under the influence of DMNA was determined. In addition to S6 a maximum of four distinct spots (P_1–P_4) could be identified 3 and 5 hr after application of the agent (Fig. 3).

DISCUSSION

DMNA-induced liver cell injury and alteration of protein synthesis involves an enhanced phosphorylation of protein S6 in the 40 S ribosomal subunit. The functional significance of this type of postsynthetic modification of the small subunit is difficult to assess at present since the role of protein S6 and of its phosphorylated derivatives in the mechanism of peptide chain synthesis is still obscure.

Previous studies have shown that the activity of cell sap factors necessary for protein synthesis is not affected by DMNA [3, 5, 6, 20]. Therefore it is tempting to attribute the described inhibition of peptide chain initiation [2, 3, 8] or even some other drug-induced aberrations of hepatic protein synthesis to the structural modification of this ribosomal

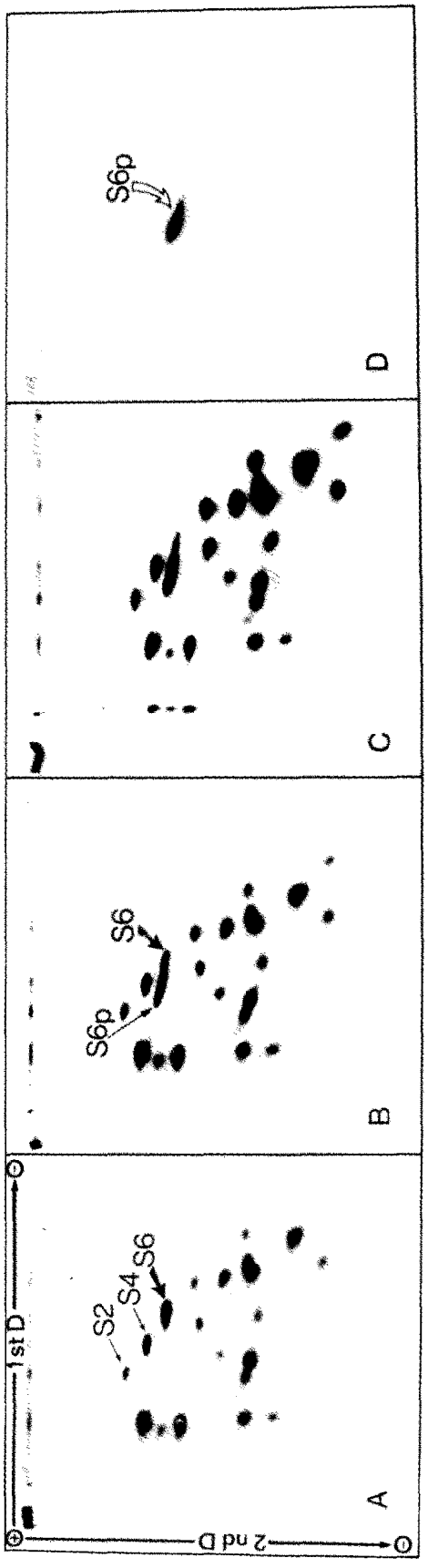

Fig. 2. Two-dimensional electrophoretograms and autoradiogram of ribosomal proteins from the 40 S subunit. The rats were pretreated as described in Fig. 1. Some 540 μg of 40 S ribosomal proteins isolated from normal and DMNA-injured rat liver were separated by two-dimensional gel electrophoresis. Electrophoretograms are from control rat liver (A), from liver treated for 2 hr (B) and 5 hr (C) with DMNA. In (D) the autoradiogram of C is shown. The position of protein S6 and of its phosphorylated derivatives S6p is indicated.

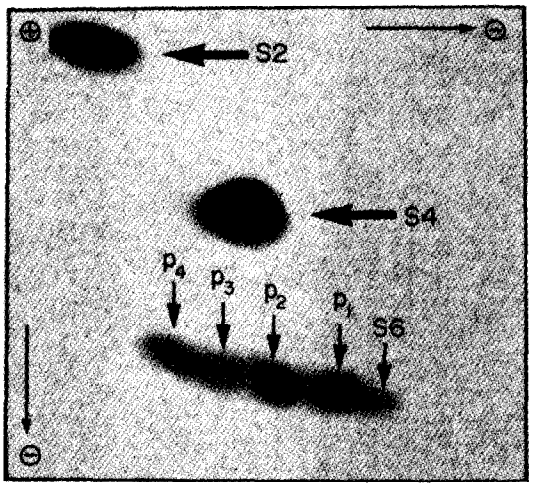

Fig. 3. Number and electrophoretic positions of the phosphorylated derivatives of protein S6 in liver injured by DMNA. The rat was treated as described in Fig. 1 and sacrificed 3.5 hr after application of DMNA. The 40 S ribosomal proteins isolated from the liver were analyzed by two-dimensional electrophoresis. The region showing S2, S4, S6 and its four derivatives (P_1–P_4) is enlarged.

protein. However, the functional activity with synthetic templates of monoribosomes and polyribosomes isolated from DMNA-damaged liver is not impaired [3, 9, 17, 20] and the minor defect found in the reassociation of 40 S and 60 S subunits into 80 S ribosomes is equally distributed among both subparticles [8]. It seems unlikely that S6p is involved in a very fundamental step, for instance in the quantitative regulation of overall protein synthesis by shutting out the 40 S subparticles from participation in the ribosome cycle. This assumption is in accord with the failure to show any functional differences between phosphorylated and non-phosphorylated rat liver ribosomes *in vitro* [30] and a correlation between the translational activity of ribosomes and the degree of ribosomal protein phosphorylation [22, 31, 32]. It is conceivable that S6 and S6p have influence on the selection of specific mRNAs by the 40 S subunit and thus on the qualitative regulation of protein synthesis on the translational level. Since an enhanced phosphorylation of S6 has been observed also during the development of D-galactosamine- and thioacetamide-induced liver injury [33, 34] and an imbalance of hepatocellular protein synthesis has been suggested to play a key role in the pathogenesis of liver cell death [35] the modification of S6 might provide a mechanism with possible significance in this process.

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