higher than the control level, but nevertheless smaller than under the influence of NA and adrenalin. Similar results also were obtained by counting the number of damaged cells.

Previously it was shown by experiments *in vitro* [10] that loss of enzymes from the myocardium induced by NA is largely inhibited by the β-blocker metoprolol. Propranolol also had an inhibitory effect on leakage of enzymes induced by adrenalin [6]. Correlation was found between the loss of enzymes from the myocardium and the tissue concentration of cAMP, and also the ability of dibutyryl cAMP to induce loss of intracellular enzymes [4]. All this led to the assertion [7] that the cardiotoxic effect of catecholamines is exerted through excessive β-adrenergic stimulation.

IP is known to have a powerful β-stimulating action, and its contact with receptors leads to a sharp rise in the rate of formation of intracellular cAMP [8]. On the basis of investigations cited above [6, 7, 10] and the hypothesis of Opie [7] which generalizes their results, it can be concluded that IP must have a damaging action on the myocardium that is at least comparable with that of NA and adrenalin. The results of the present investigation contradict Opie's hypothesis, and the question of the mechanism of the cardiotoxic effect of catecholamines can evidently not be reduced simply to their influence of β-receptors.

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PHOSPHORYLATION OF RAT LIVER AND ZAJDELA HEPATOMA NUCLEAR MATRIX PROTEINS

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Proteins of the cell nucleus are phosphorylated to a much greater degree than cytoplasmic proteins [1]. The skeletal structure of the cell nucleus or nuclear matrix (NM) consists to the extent of 90-95% of proteins, it preserves the shape of the nucleus, and has high metabolic activity [15]. During phosphorylation of proteins in isolated nuclei of the regenerating rat liver, NM proteins incorporate label twice to three times more actively than total nuclear protein [4].

There is no information in the literature on the characteristics of phosphorylation of NM proteins with tumors. However, a study of protein phosphorylation in cell nuclei of Novikoff's hepatoma and the regenerating rat liver revealed four proteins phosphorylated only in the hepatoma and one only in the liver [14]. Essential differences also were observed in the protein composition of NM of the rat liver, hepatoma 27, and Zajdela's hepatoma [2, 5]. For instance one characteristic feature of tumors is that they contain polypeptides with

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TABLE 1. Phosphorylation of Proteins (in cpm/mg protein × 10^{-3}) of NM in Nucleus and in Isolated Preparation, Isolated by Different Methods (M ± m)

Parameter studied	Normal liver		Zajdela hepatoma	
	with inhibitors	without inhibitors	with inhibitors	without inhibitors
Phosphorylation of NM proteins in nucleus	60±15 (4)	10 (2)	100±20 (3)	20 (2)
Phosphorylation of proteins in isolated NM	80±10 (4)	60±10 (4)	120±25 (4)	90±20 (3)

Legend. Number of experiments shown in parentheses.

molecular weight of about 200 kilodaltons. The study of phosphorylation of nonhistone nuclear proteins in tumors is very interesting also in connection with data on phosphorylation substrates linked with activity of oncogenes.

The aim of this investigation was to study phosphorylation of NM proteins of the rat liver and Zajdela's hepatoma in isolated nuclei and in isolated preparations of NM. The spectra of proteins phosphorylated under different conditions in normal and tumor cells also were studied.

EXPERIMENTAL METHOD

Noninbred female albino rats weighing 100-150 g were used. Zajdela's ascites hepatoma was taken on the 5th day after inoculation. Nuclei and NM were isolated by the method described in [3] with some modifications: 200-500 mg (as protein) of nuclei was placed in 5 ml of medium containing 200 mM sucrose, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4, and 0.5% Triton X-100 (buffer I), mixed until homogeneous, and incubated at 4°C for 20 min. The residual nuclei were sedimented by centrifugation at 1000g for 10 min. The residue was washed with buffer II (the same as buffer I, but without detergent). The residue thus obtained was treated with 40 µg/ml of DNase I (from Worthington, USA) and 640 µg/ml of RNase A (from Reanal, Hungary), boiled for 10 min in medium containing 1 mM 5,5'-dithio-bis-nitrobenzoic acid (DTNB), 1 mM phenylmethylsulfonyl fluoride (PMSF) (from Serva, West Germany), 0.005 mM $ZnCl_2$, 0.01 mM Na_3VO_4, and 150 mM NaCl in buffer II for 1 h at 30°C. The DTNB, PMSF, and Na_3VO_4 were added to prevent proteolysis and dephosphorylation of the phosphorylated proteins, and the Zn^{++} as an inhibitor of protein phosphatases. To 5 ml of medium containing the nucleases 45 ml of ice-cold 2.2 M NaCl was added and the sample was kept at 0-4°C for 10 min. It was then centrifuged at 2800g for 30 min. The residue was suspended in 5 ml of the same medium as for the first treatment (containing nucleases), allowed to stand for 30 min at 30°C, after which 45 ml of buffer II was added and the sample was centrifuged for 10 min at 1000g. The residue was washed again with buffer II not containing Ca^{++}, and with $MgCl_2$ in a concentration of 5 mM. The second version of obtaining NM without phosphatase and proteolysis inhibitors was exactly the same as that described previously [3].

The phosphorylation medium [13] contained 5 mM dithiothreitol, 0.1% NP-40, 5 mM $MgCl_2$, 30 mM NaCl, and 30 mM Tris-HCl, pH 7.4; 10-20 µBq [$^{32}P\gamma$]-ATP (Tashkent Radioisotopes Factory) per 10 mg of protein (50 µm calculated on the date of manufacture) was added to the same medium. The phosphorylation reaction, conducted in isolated nuclei at 30°C, was stopped by the addition of 10 volumes of ice-cold buffer I, after which NM were isolated as described above. Control nuclei were incubated before isolation of NM in the same phosphorylation medium but not containing ATP.

During phosphorylation in isolated NM the reaction was stopped by addition of 2 volumes of 15% TCA containing 1 mM cold ATP (from Reanal) and 5 mM pyrophosphate; the residue was washed twice with acetone and then dissolved in application buffer for electrophoresis.

Electrophoresis in the presence of sodium dodecylsulfate [8] was carried out in slabs of 5-20% polyacrylamide gel gradient. The gel was dried on an instrument from Pharmacia Fine Chemicals (Sweden). Autoradiographs were obtained on RT-1 film. The gel was stained with Coomassie R-250 in the presence of formaldehyde [12]. Samples for measurement of radioactivity were applied to Whatman 3 mM paper, dried, and washed with terminating solution,

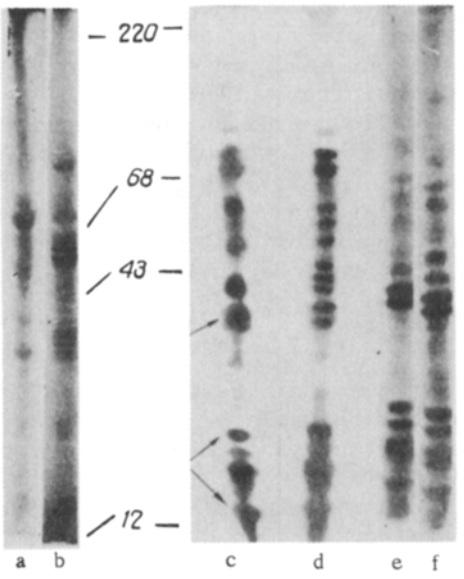

Fig. 1

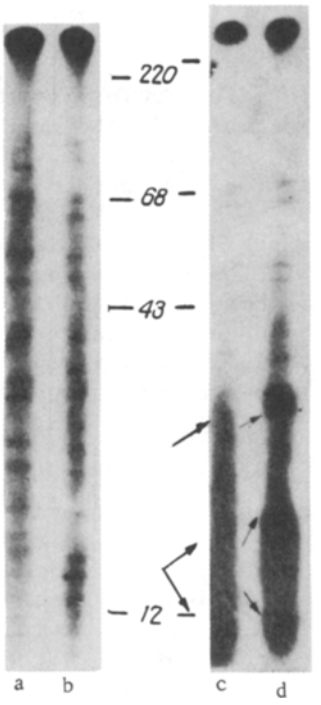

Fig. 2

Fig. 1. Electrophoresis of NM proteins of rat liver and Zajdela hepatoma. a,c,d) Liver; b, e, f) Zajdela's hepatoma. a, b) Isolation without proteolysis and phosphorolysis inhibitors; c, d, e, f) isolation with proteolysis and phosphorolysis inhibitors; c, e) phosphorylation carried out in isolated nuclei; a, b, d, f) phosphorylation carried out in preparations of isolated NM. Arrows indicate protein bands present in NM preparations if isolated in presence of inhibitors. Numbers denote molecular weight standards. Stained with Coomassie R-250.

Fig. 2. Autoradiographs of NM proteins of rat liver and Zajdela hepatoma. a, b) Liver; c, d) Zajdela's hepatoma. a, c) Phosphorylation in preparation of cell nucleus; b, d) phosphorylation in NM preparations. Arrows indicate protein bands phosphorylated differently in preparations of Zajdela's hepatoma and liver. Numbers indicate molecular weight standards.

stopping the reaction (see above), twice with 5% TCA, and once with 96% ethanol. Radioactivity was measured in ZhS-20 toluene scintillator on an Intertechnique SL-30 counter. Protein was determined by the method in [11].

EXPERIMENTAL RESULTS

As may be seen in Fig. 1 the polypeptide profile of NM after staining with Coomassie R-250 and the phosphorylation reaction differed significantly in cases when inhibitors of proteolysis and phosphorolysis were used. Proteins with molecular weights of 29-31 and 12-19 kilodaltons, the quantity of which rose sharply if inhibitors were used during proteins in a system with sodium dodecylsulfate corresponds to that of histones, proteins not characteristic of NM [15] but, perhaps, remaining in the composition of the preparations since vanadium compounds, which are inhibitors of metabolism of phosphorus-containing compounds [10], may inhibit nucleases, as has been shown for RNase [9]. DTNB also modifies the composition and increases the yield of NM a little [2]. Furthermore, in the course of the experiments it was observed that Zn^{++} considerably reduces the solubility of NM preparations in the application buffer for electrophoresis, suggesting that it increases aggregation of nuclear proteins.

Comparison of the gels after electrophorsis of NM proteins phosphorylated in the nucleus or in isolated NM preparations revealed no appreciable differences after staining with Coomassie R-250 (Fig. 1).

It will be clear from Table 1 that if phosphorylation was carried out in the matrix itself the effect of a change in the conditions of isolation of NM on incorporation of label into the proteins of this structure was negligible in both liver and Zajdela's hepatoma. If, however, phosphorylation was done in NM isolated without inhibitors, still in the nucleus, the NM lost most of its label. Without the use of inhibitors of proteolysis and phosphorolysis, it would therefore be impossible to compare proteins of NM phosphorylated by different methods. Phosphorylation of total protein of NM in the isolated matrix, in the presence of proteolysis and phosphorolysis inhibitors, gave values a little higher than those obtained with phosphorylation under the same conditions in isolated nuclei in both liver and Zajdela's hepatome (Table 1). Some label is thus lost in any event during isolation of NM, even when phosphorylation in the nucleus was carried out in the presence of inhibitors.

The NM preparation from the tumor, whatever the method of isolation, incorporated label more actively than the corresponding preparation from normal liver.

Labeled phosphorus was incorporated into the same protein bands in liver NM when phosphorylation was carried out both in the nuclei and in the isolated preparation (Fig. 2a, b). The small difference observed in Zajdela's hepatoma on autoradiographs of proteins of NM phosphorylated in the nucleus and in the isolated matrix is more likely to be due to proteolysis of some proteins during isolation than to any difference in phsophorylation. For instance, during phosphorylation in the isolated NM (Fig. 2d) bands of intensively phosphorylated proteins with molecular weights of 29-31 and 11-19 kilodaltons were distinguished. This state of affairs is evidence in support of the histone nature of these proteins, for phosphorylation of histones is characteristic of proliferating cells [7]. Meanwhile, during phosphorylation in the nucleus (Fig. 2c) an indistinct band is visible in this region becoming stronger toward the low molecular weight end, indicating proteolysis of phosphorylated proteins.

During phosphorulation in the isolated NM in both liver and hepatoma, the same proteins are evidently phosphorylated as in the composition of the cell nucleus. This suggests that considerable protein kinase activity is associated with NM. The fact that NM isolated without proteolysis and phosphorolysis inhibitors differs only insignificantly in its level of phosphorylation from the preparation isolated with these inhibitors indicates that this activity is firmly bound with proteins of the matrix and has not been introduced due to modification of the method of isolation.

Meanwhile proteinases hydrolyzing phosphorylated proteins are evidently located mainly in the nucleoplasm and are separated from NM during isolation of the latter. These proteinases, incidentally, are much more active in cell nuclei of Zajdela's hepatoma than in rat liver cell nuclei.

The essential differences between phosphorylation of NM proteins from ascites hepatoma and that of liver NM proteins are the higher total level of phosphorylation and the ability of the tumor NM preparation to phosphorylate endogenous histones associated with it.

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