TRIS(2-HYDROXYETHYL)AMMONIUM ORTHOCRESOXYACETATE: A STIMULATOR OF REGENERATING HEPATOCYTES

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The aim of this investigation was to study the action of the preparation Cresacyne (CR), namely Tris(2-hydroxyethyl)ammonium orthocresoxyacetate, synthesized for the first time at the Irkutsk Institute of Organic Chemistry, on mitotic activity of regenerating hepatocytes, and also on the system of oxidation and coupling with energy metabolism in the liver.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 150-180 g. Two-thirds of the mass of the animals' liver was removed by the method of Higgins and Andersen. Identical control and experimental groups were formed from the hepatectomized rats. Immediately after the operation, animals of the experimental group received a single intraperitoneal injection of CR in a dose of 25 mg/kg. The injection was repeated 24 h later. The control rats were given an injection of a corresponding volume of physiological saline. The rats were sacrificed 48 h after the end of the operation.

Homogenates and mitochondria of hepatocytes were used as tissue preparations.

For electron-microscopic study pieces of liver measuring 5 × 5 mm were excised and fixed in a 4% solution of paraformaldehyde in Hanks' buffer (pH 7.3) for 3 h. The material were then minced to a size of 1 × 1 × 3 mm and washed in the same buffer. After dehydration in acetone, the tissue fragments were embedded in a mixture of Epon and Araldite resins. Sections were studied in the BS-613 microscope.

For the biochemical analyses the liver was cooled at 4°C for 2-3 min in isolation medium, containing 0.3 M sucrose and 10 mM Tris-HCl, pH 7.5. The concentration of cytochrome C was determined spectrophotometrically [3], for which purpose about 1 g of liver was homogenized (tissue:medium = 1:3). For oxidation of the cytochrome a medium consisting of 0.3 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, 240 μM 2,4-dinitrophenol (DNP), 3.2 mM amytal, 1 mM NADH, pH 7.5 was used; for reduction the medium was composed of 0.3 M sucrose, 10 mM Tris-HCl, 40 μM DNP, 5 mM succinate, 0.3 mM KCN, and 1 mM NADH, pH 7.5. Not more than 30 mg/ml of tissue was added to two cuvettes with a volume of 2 ml.

The rate of respiration of the mitochondria (MCh) of the hepatocytes was studied in the liver of rats of the other group by means of an oxygen electrode of the Clark type. The MCh preparations were isolated from the liver by the method in [2]. The suspension of MCh contained 80-100 mg protein in 1 ml.

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TABLE 1. Effect of Cresacyne on Rate of Respiration of Hepatocyte Mitochondria (nmoles O_2/min·mg protein) (M ± m, n = 10)

Section	Experimental conditions	Groups of animals		
		standard (intact)	control (untreated)	experiment (treated with CR)
A	V_0	6,6±0,3	7,2±0,3	7,1±0,4
	V_3	39,2±1,9	51,4±2,6	43,2±1,9
	V_4	16,3±0,9	36,7±2,3	19,8±1,2
	V_3/V_4	2,4±0,1	1,4±0,1	2,2±0,1
B	TMPD 400 μm	112,0±9,7	97,5±6,9	89,2±6,2
	TMPD 800 μm	162,0±12,3	172,0±10,1	148,0±4,7
	TMPD 800 M + cytochrome C	323,0±11,9	386,0±10,1	361,0±6,9
C	DNP	27,8±1,7	25,2±2,1	26,4±2,2
	DNP + amytal	4,1±0,5	3,9±0,4	4,2±0,5
	DNP + amytal + antimycin A	2,4±0,2	2,1±0,2	3,2±0,3
	DNP + amytal + antimycin A + NADH	4,0±0,5	5,8±0,5	9,2±0,9

Legend. Section A: V_0) initial rate of respiration of MCh in presence of glutamate and malate, V_3) respiration rate of MCh in state 3 after Chance, V_4) respiration rate in state 4 after Chance. Section B: incubation medium – 70 mM KCl, 5 mM Na_2HPO_4, 2 mM ascorbate, 20 μM DNP, 1.6 mM amytal, $8 \cdot 10^{-7}$ antimycin A, pH 7.5, additives – 400 μM TMPD (successively), 20 μM cytochrome C, protein 0.5 mg/ml. Section C: incubation medium – 70 mM KCl, 5 mM Na_2HPO_4, 4 mM glutamate, 1 mM malate, protein 2 mg/ml, additives – 20 μM DNP, 1.6 mM amytal, $8 \cdot 10^{-7}$ M antimycin A, 1 mM NADH.

The intensity of free-radical lipid peroxidation (LPO) was determined in the liver of rats of the third experimental group by the chemiluminescence method, on an apparatus described previously [1]. To initiate LPO, 1 ml of 10^{-2} M $FeSO_4$ was added to the liver homogenates. The liver tissue was homogenized in a Potter homogenizer and introduced into the measuring cell. The final volume of suspension in the cell was 10 ml. The incubation medium consisted of 105 mM KCl and 20 mM KH_2PO_4, at pH 7.4. The final concentration of the LPO initiator was 10^{-3} M. The intensity of chemiluminescence was calculated under conditions of an equal mass of homogenate in the cell. The ratio of the intensity of Fe^{2+}-induced luminescence to the spontaneous level of luminescence was used as the measure.

Since LPO is connected with the cytochrome P-450-hydrolase system, the dynamics of cytochrome P-450 was studied in experiments on isolated microsomes. For this purpose, liver homogenates were prepared by the usual method in 0.15 M KCl in 0.02 M Tris-HCl buffer. The microsomal fraction was sedimented from the postmitochondrial supernatant (10,000*g*) on a centrifuge at 105,000*g* for 1 h. The isolated microsomes were washed in isolation medium and resedimented under the same conditions. The residues were resuspended in isolation medium and kept at –20°C for not more than 3 days.

Protein was determined by Lowry's method. Hepatocytes from intact rats, investigated by the same method, served as the standard. The results were subjected to statistical analysis by Student's method.

EXPERIMENTAL RESULTS

The morphological findings were as follows (Fig. 1). In the control the structure of the cells differed sharply from that in animals treated with CR, and the control series. In hepatocytes throughout the parenchyma marked edema of the cytoplasm was observed, accompanied by destruction. The process took place most actively in the lamellae. The lamellae of the destroyed organelles formed loose pseudomyelin bodies, scattered throughout the

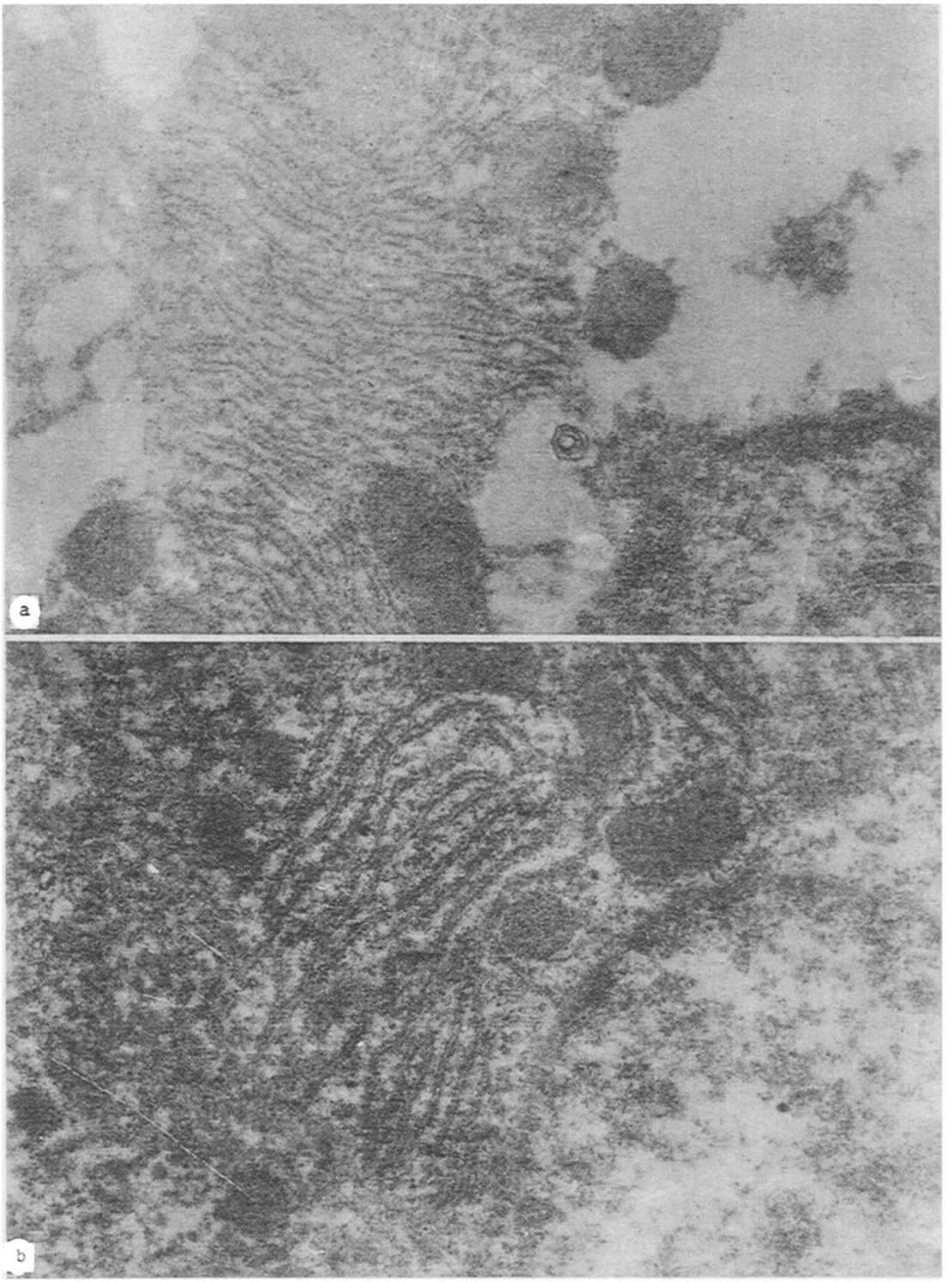

Fig. 1. Ultrastructure of parenchymatous liver cell: a) hepatocyte of experimental animal, b) of control animal. 10,000×.

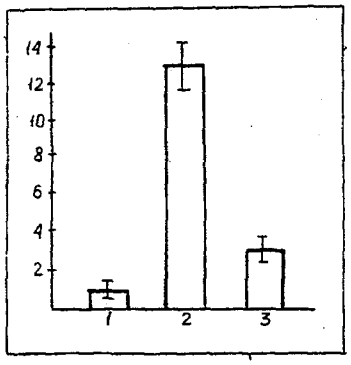

Fig. 2. Intensity of Fe^{2+}-induced chemiluminescence in rat liver homogenates. Abscissa, intensity of very weak luminescence (in relative units): 1) Intact animals; 2) control (untreated) rats; 3) rats treated with CR.

cytoplasm. Granular osmiophilic masses were observed in the central part of these bodies. Areas with an intact rough endoplasmic reticulum and small mitochondria could be seen among the pale and strongly hydrated zones. On the surface of the tubules of the reticulum there were many ribosomes. The number of free ribosomes was reduced. Separate fat droplets of average size and also granular destructive masses could be distinguished in the cytoplasm.

In the group of animals receiving CR most liver cells showed no signs of destruction. Occasionally single fat vacuoles of average size were seen or three to five small fat droplets, corresponding in size to MCh, were grouped together. The rough endoplasmic reticulum in the hepatocytes formed several spherical complexes. Tubules of the reticulum in a complex were arranged more closely together, their lumen was narrow, and they were covered with numerous ribosomes. Many circular MCh were distributed uniformly throughout the cytoplasm. Lengthened MCh were occasionally found. The number of liposomes was smaller than in the standard, and some hepatocytes had strongly hydrated cytoplasm. Signs of destruction of membranous organelles were seen in these same cells. Rarefaction and discontinuity of the tubules of the endoplasmic reticulum could be seen. No disturbances of the structure of MCh or of the nuclear apparatus were observed in such hepatocytes. The total number of cells in different phases of mitosis in the standard cells was 0.62 ± 0.03 ‰ of the total number of cells. In the control this figure was 0.71 ± 0.04 ‰, but among rats receiving CR, it reached 1.28 ± 0.05 ‰ compared with the standard ($p < 0.01$), which also significantly ($p < 0.05$) exceeded this parameter in the control animals. The quota of cells in the metaphase and anaphase stage amounted to 41.2 ± 2% of the total number of cells in the state of mitosis in the standard rats, in the control this figure was 44.2%, whereas among animals receiving CR it was 64 ± 3% ($p < 0.05$). The mitotic index of the hepatocytes during the metaphase and anaphase period in intact rats was 9.2 ± 1.7, whereas among the control rats it was 10.5 ± 2.1. In rats receiving CR the mitotic index rose above the control level to 22 ± 2.1 ($p < 0.01$).

The results of the polarographic investigations of mitochondrial respiratory function are given in Table 1. They show that administration of CR led to a significant decrease in the velocity of oxygen transport in MCh of the hepatocytes, and also to a significant change in their respiratory quotient, compared both with the control and with the standard, i.e., with the uncoupling effect, which depends on activity of mitochondrial phospholipase A_2. To verify the effect of CR on this enzyme we studied the action of CR on oxidation of endogenous NADH in hepatocyte MCh via the external pathway. Under the influence of CR the velocity of oxidation of NADH via the external pathway increased significantly.

Comparison of cytochrome oxidase activity in hepatocytes of groups of rats receiving and not receiving CR showed that the velocity of ascorbate oxidation was reduced in MCh of animals receiving CR in the presence of saturating concentrations of TMPD (N′,N′,N,N-tetramethyl-p-phenylenediamine hydrochloride) (Table 1).

The study of the intensity of LPO in rat liver homogenates revealed a raised level of very weak luminescence among hepatectomized animals not receiving CR, whereas in the group of rats treated with CR the level of luminescence was significantly lower (Fig. 2), i.e., CR possessed an antioxidant action. The level of cytochrome P-450 in the liver microsomes of the standard rats was 10.4 ± 0.5 and in the control rats 11.9 ± 0.6 nmoles/g liver, whereas in rats receiving CR it reached 25.6 ± 1.9 nmoles/g liver, or in other words the cytochrome P-450 concentration in liver microsomes of rats receiving CR also was significantly raised. This effect is an indicator of inhibition of cytochrome oxidases under the influence of CR. Consequently, we are right in assuming that CR is an inducer of cytochromes C and P-450.

Activation of anabolic processes in hepatocytes by Cresacyne is thus connected with its ability to modify processes of biological oxidation, through its complex antioxidant and, evidently, its stabilizing effect on hepatocyte membranes and organelles.

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