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THE cGMP SYSTEM IN IRRADIATED ANIMALS

Changes in cGMP content and activities of guanylate cyclase and cyclic nucleotide phosphodiesterase

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Abstract

Changes in the functioning of the cGMP system of the thymocytes and liver of mice subjected to 8 Gy roentgen irradiation were found. Within one hour after irradiation an increase in the cGMP level in thymocytes was noted; two rises in the cGMP concentration in the liver were established, at 0.5 and 24 hours after irradiation. These changes in the cGMP level were correlated to an increase in the guanylate cyclase activities in the thymocytes and liver of the mice subjected to irradiation, and to a lesser extent to changes in the activities of cGMP phosphodiesterase in these tissues. A post-irradiational increase in the rat liver guanylate cyclase activity was also observed.

A decrease in cGMP phosphodiesterase activity in the liver of the irradiated mice was followed by a change in the enzymatic kinetics and an increase in cGMP phosphodiesterase thermolability. The post-irradiational rise in guanylate cyclase activity was produced by activation of the enzyme.

Cyclic guanosine-3′:5′-monophosphate (cGMP) was identified in biologic objects later than cAMP, and as a result the cGMP system has been studied considerably less thoroughly. Despite a rather incomplete knowledge of this system it is at present possible with sufficient certainty to assign it control functions in the regulation of such processes as secretion of lysosomal enzymes (8), as well as RNA synthesis (6), protein synthesis (20) and some other functions. The interest of radiation biologists regarding the mechanisms of irradiation-induced disturbances in these processes is evident. The possibility cannot be excluded, that release of lysosomal enzymes, changes in RNA and protein biosynthesis observed after irradiation of animals are related to a disordered function of the system of cyclic nucleotides, in particular the cGMP system. However, no data are available in the literature on the state of the cGMP system after irradiation. Therefore, the first necessary step is to investigate the action of irradiation on the cGMP system, which was the purpose of the present report.

The experiments showed post-irradiational changes in the cGMP system of the liver and thymocytes in mice irradiated with 8 Gy. The results were reported earlier in abstract form (19).

Materials and Methods

Male SHK mice weighing 18 to 20 g and male Wistar rats weighing 180 to 200 g were used. The animals were deprived of food 24 hours before the experiment, water was given ad libitum.

The animals were irradiated in plastic cages with 180 kV roentgen rays generated at 15 mA; filters used: 0.5 mm Cu and 1.0 mm Al; the dose rate was 1 Gy/min, the total dose being 8 Gy (mice) or 7 Gy (rats).

cGMP assay. The tissue under study or cells were homogenized in 20 volumes of 96% ethanol. The

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homogenate was centrifuged (6000×g, 10 min) and the supernatant was put on the column (0.4×5 cm) containing 0.5 g of dry aluminium oxide (neutral, activity II according to Brockman; 'Reanal', Hungary). cGMP was eluted from the column by 1.5 ml of water-ethanol mixture (2:1, v/v); dried eluate was dissolved in 1 ml of 50 mmol/l Tris-EDTA buffer (pH 7.5). The cGMP was determined in 100 µl aliquots by the radioimmunoassay using kits from Amersham International, England.

Determination of guanylate cyclase activity. Gyanylate cyclase activity was determined in the lysate of thymocytes and the homogenate of murine or rat livers. Thymocytes were lysed by adding 2 ml of 25 mmol/l Tris-HCl buffer, pH 7.6 (4°C) containing 1 mmol/l $MgSO_4$. Murine and rat livers were homogenized in 10 volumes of 25 mmol/l Tris-HCl buffer, pH 7.6 (4°C). Guanylate cyclase activity was determined in the incubation mixture (the final volume 75 µl): 30 mmol/l Tris-HCl buffer (pH 7.6), 5 mmol/l $MnCl_2$, 10 mmol/l caffeine, 5 mmol/l creatine phosphate, 10 µg (50 units/mg) of creatine phosphokinase, 100 µg of bovine serum albumin, 1 mmol/l GTP (the last four preparations were manufactured by 'Reanal', Hungary), 500000 cpm 8-[^{14}C]GTP ('UVVVR', Czechoslovakia), 1 mmol/l cGMP ('Sigma', USA), 1000 cpm 8-[^{3}H]cGMP (Amersham International, England) and 25 µl of guanylate cyclase preparation. Further details were described previously (15).

Determination of cGMP and cAMP phosphodiesterase activity. Phosphodiesterase activity was determined in the specimens of the murine liver and thymocytes obtained as described above. The final volume of the incubation mixture (75 µl) contained 60 mmol/l Tris-HCl buffer, pH 8.0; 5 mmol/l $MgSO_4$, 1 mmol/l 5'-GMP or 5'-AMP ('Reanal', Hungary), 0.05 µmol/l cGMP or 0.2 µmol/l cAMP ('Fluka', Switzerland), 20000 cpm [^{3}H]cGMP or [^{3}H]cAMP (Amersham International, England), and 25 µl of phosphodiesterase preparation. After 10-min incubation at 37°C the reaction was stopped, the products were separated and ^{3}H radioactivity was determined as described elsewhere (16). Subsequently phosphodiesterase activity was estimated with regard to the blank probe.

Other methods. Isolation of thymocytes was described earlier (12). Protein was determined by the microbiuret method (2). Statistical processing of the data was performed according to Student's t-test. The Figures show mean values ± SE.

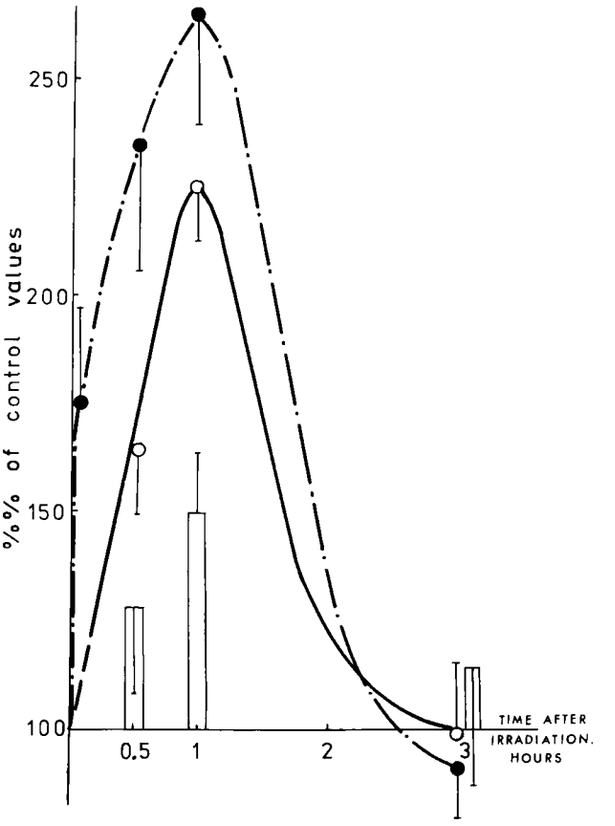

Fig. 1. Relative content of cGMP (●) and relative activities of guanylate cyclase (○) and cGMP phosphodiesterase (□) expressed in per cent of control (thymocytes of irradiated mice (8 Gy)).

Results

A distinct 1.8-fold increase of the thymic cGMP concentration immediately after 8 Gy irradiation (0–3 min) was observed. 'Sham' irradiation did not induce any alterations in the cGMP level (data not shown). The level of cGMP continued to rise and 0.5 or 1 hour later it exceeded normal values 2.3 and 2.7 times, respectively. Subsequently normalization of the cGMP level was observed (Fig. 1). Within 24 h after irradiation of mice two elevations of the cGMP level in the liver were noted after 0.5 h (1.6 times) and by the end of the 24 h period (1.5 times); (Fig. 2).

To obtain general characteristics of guanylate cyclase function following irradiation, studies of the enzyme activity were performed in cell-free systems (whole liver homogenate and lysate of thymocytes) which made it possible to determine overall changes in activities of both forms of the enzyme: membrane-bound and 'soluble'. Guanylate cyclase activity of thymocytes isolated 0.5 h after irradiation of

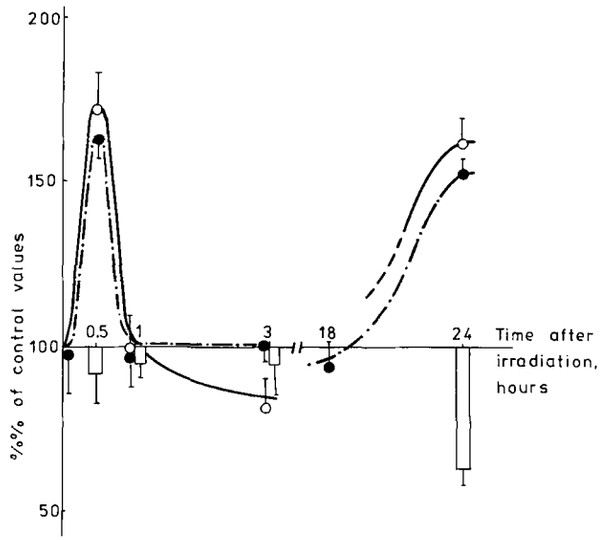

Fig. 2. Relative content of cGMP (●) and relative activities of guanylate cyclase (○) and cGMP phosphodiesterase (□) expressed in per cent of control liver of irradiated mice (8 Gy)).

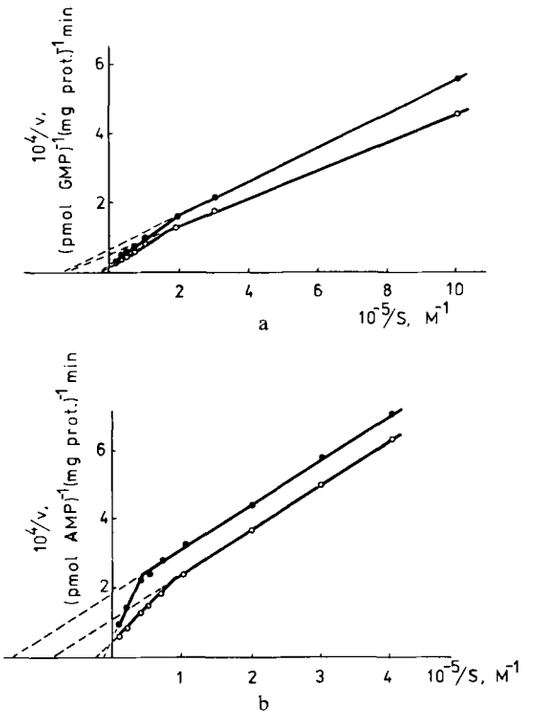

Fig. 3. Relationship between the rate of cGMP or cAMP hydrolysis (v) by the rat liver phosphodiesterase preparations and the cyclic nucleotide concentration (S). a) cGMP hydrolysis; b) cAMP hydrolysis. Unirradiated control (○), 24 hours after irradiation (●) (8 Gy). Kinetic characteristics of cGMP phosphodiesterase with high affinity to the substrate: (1) normal—K_M^1=8.1±1.3 μmol/l, V_{max}^1=202±19 pmol of GMP formed per mg protein per min; (2) after irradiation—K_M^1=7.6±1.1 μmol/l, V_{max}^1=152±23 ($p<0.05$) pmol of GMP formed per mg protein per min. cGMP phosphodiesterase with low affinity to the substrate: (1) normal—K_M^2=83.5±6.9 μmol/l, V_{max}^2=1266±118 pmol of GMP formed per mg protein per min; (2) after irradiation—K_M^2=91.4±10.2 μmol/l, V_{max}^2=1006±89 pmol of GMP formed per mg protein per min. The Hill coefficient (normal and after irradiation), n_H=0.85. Kinetic characteristics of cAMP phosphodiesterase: (1) normal—K_M^1=11.8±1.9 μmol/l, V_{max}^1=903±51 pmol of AMP formed per mg protein per min; K_M^2=38.5±3.0 μmol/l, V_{max}^2=2025±150 pmol of AMP formed per mg protein per min; n_H=0.90. (2) after irradiation—K_M^1=7.9±0.8 μmol/l, V_{max}^1=573±72 ($p<0.05$) pmol of AMP formed per mg protein per min; K_M^2=86±17.2 ($p<0.05$) μmol/l, V_{max}^2=1995±324 pmol of AMP formed per mg protein per min; n_H=0.55.

mice exceeded the activity of the non-irradiated controls 1.5 times. One hour later activity of the enzyme increased and was 220 per cent of the normal values, and then decreased to the control values (Fig. 1). Two rises of guanylate cyclase activity in the liver were found: 0.5 h after irradiation (1.7 times) and by the end of the first day (1.6 times; Fig. 2). It should be noted that similar changes in guanylate cyclase activity were detected by us in the liver homogenate of the rats irradiated in a dose of 7 Gy: after 0.5 h the activity was

$$48.5\pm3.9\frac{\text{pmol cGMP}}{\text{mg protein}\times\text{min}} \quad (p<0.05),$$

and after

$$24\text{ h }39.7\pm2.7\frac{\text{pmol cGMP}}{\text{mg protein}\times\text{min}} \quad (p<0.05),$$

the control activity being equal to

$$29.8\pm2.3\frac{\text{pmol cGMP}}{\text{mg protein}\times\text{min}}.$$

cGMP phosphodiesterase activity in thymocytes began to rise immediately after irradiation of mice and after 1 h it reached 150 per cent of the initial activity and then diminished (Fig. 1). In the liver the change of cGMP phosphodiesterase activity (a decrease by 40%) was found only by the end of the 24-h period after irradiation (Fig. 2).

The analysis of the kinetic characteristics of the cGMP phosphodiesterase preparations isolated from murine livers 24 h after irradiation made it possible to detect a statistically significant decrease of V_{max} of the enzyme with a high affinity to cGMP (V_{max}^1). Affinity of the enzyme to the substrate (K_M^1 and K_M^2) as well as a 'negative cooperativity' of the enzyme to the substrate virtually did not change after irradiation (Fig. 3 a).

While studying thermoinactivation of the cGMP phosphodiesterase preparations isolated from the livers of intact and irradiated mice an increase of thermolability of the enzyme was found. The kinet-

ics of thermoinactivation in both cases corresponded to kinetics of the first-order reactions, but the rate constant (k_{obsd}) of the enzyme inactivation from the liver of the irradiated mice was 4 times that of the control: $(4.60\pm0.18)\times10^{-2}$ min^{-1} as compared with $(1.15\pm0.01)\times10^{-2}$ min^{-1} in the control (Fig. 4a).

The post-irradiational changes in kinetic characteristics and thermostability of enzymatic hydrolysis of cGMP and cAMP can be compared. The maximum rate of cAMP hydrolysis (V^1_{max}) for high affinity cAMP phosphodiesterase decreased, while K^2_M of the enzyme with low substrate affinity increased (affinity to cAMP becomes lower) (Fig. 3b). Such changes in V^1_{max} and K^2_M lead to the fact that the largest deviations from the control values are observed for intermediate substrate concentrations. Irradiation amplifies the enzyme's 'negative cooperativity', resulting in a decrease of the Hill's coefficient from $n_H=0.90$ in the control to $n_H=0.55$ after the treatment. Thermostability studies have shown that normal cAMP phosphodiesterase is inactivated as a linear function of time (Fig. 4b), i.e. follows the first order kinetics with $k_{obsd}=(3.36\pm0.11)\times10^{-2}$ min^{-1}. Irradiation decreased thermostability, and the function of inactivation vs. time became nonlinear. This function may be accounted for either by a reaction with the order greater than 1, or by inactivation of two populations of enzyme molecules (parallel reactions). The latter situation was demonstrated by PICHARD et coll. (13) for cyclic nucleotide phosphodiesterase from human blood platelets and by BEVERS et coll. (3) for the enzyme from rat livers.

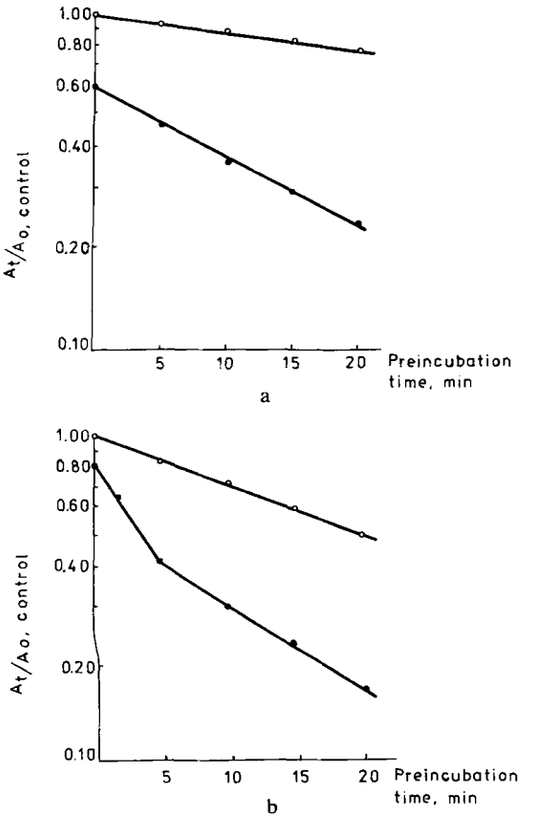

Fig. 4. Kinetics of thermoinactivation of phosphodiesterase activity from the mouse liver. a) cGMP hydrolysis, b) cAMP hydrolysis. Unirradiated control (○), 24 hours after irradiation (●) (8 Gy). The enzyme preparations were preincubated for the given time at 50°C, thereafter phosphodiesterase activity was determined. The quotient obtained when the activity (A_t) determined in the preparations after incubation at 50°C for t min was divided by the activity ($A_{o,control}$) of the control preparation not incubated at 50°C (t=0) was calculated. Mean values of the results of three experiments are given in the plot.

Limited proteolysis (e.g. with tripsin (10)) may change the thermostability of phosphodiesterase molecules and their affinity to substrate. As is known, irradiation stimulates proteolytic activity in some tissues (4). In the present experiment the addition of 0.1 mmol/l phenylmethylsulfonyl fluoride (inhibitor of serine proteases) to the isolation and incubation media did not influence kinetic parameters and thermostability of the enzyme. Thus, the change of phosphodiesterase activity following irradiation is probably caused by intracellular processes, and if proteolytic enzymes participate in them, their action takes place rather inside the cells of the irradiated organism than in the course of preparative isolation of the enzyme.

Irradiation of mice (8 Gy) and rats (7 Gy) produced an increase of guanylate cyclase activity in the liver of both species of animals 0.5 and 24 hours after the action of ionizing radiation (see above). Guanylate cyclase is known to exist in low active and high active forms. Transition into a high active form takes place under the action of activators of an oxidative nature. Reducing agents (e.g. dithiothreitol) interfere with activation transforming all enzyme molecules into a low active form (11, 15). Hence, if after addition of dithiothreitol to the preparations from control and irradiated mice the guanylate cyclase activity in these preparations are equal, a conclusion may be drawn about activation of the enzyme following irradiation, that is, about an increase in the share of the enzyme which is in a high active form. The experiments performed (Fig. 5) confirmed this supposition.

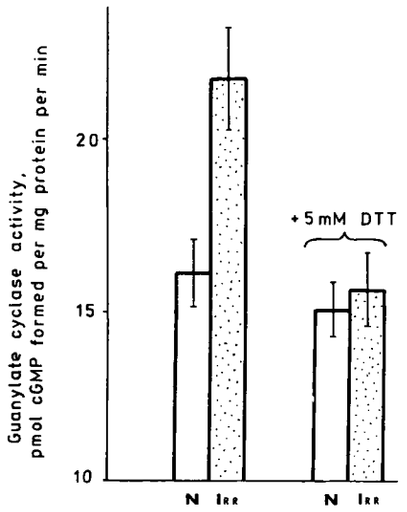

Fig. 5. Guanylate cyclase activity of the liver homogenates of normal and irradiated mice in the presence of dithiotreitol (DDT). N—normal, Irr—0.5 hour after irradiation (8 Gy). Time of incubation—5 min, protein content in the incubation mixture—30–35 mg/ml. The data of 3 experiments are given.

Discussion

The experimental data suggest that the changes revealed in the cGMP concentrations in the liver and thymocytes of the irradiated mice were caused by disorders in the function of the enzymes controlling the cGMP steady-state concentration: guanylate cyclase and cGMP phosphodiesterase. This suggestion becomes especially obvious and illustrative if the changes of the enzymes' activities are compared with changes in the cGMP level (Figs 1, 2). The justifiability of such comparison is based on the following considerations: 1) within the first three hours after irradiation of mice (8 Gy) no significant changes occur in the composition and characteristics of suspensions of isolated thymocytes (12); it is also well established that within the first 24 hours the mouse liver cell composition undergoes no changes at such doses of irradiation; 2) the concentrations of the enzymes' substrates used are close to those actually existing in the cell (14). As can be seen from Figs 1 and 2, rises in the tissular cGMP level were attended by increase of guanylate cyclase activity in these tissues which sometimes was accompanied by a decrease of phosphodiesterase activity. In all cases when intensity of cGMP production exceeded the rate of enzymatic hydrolysis of this nucleotide, an increase of cGMP concentration was noted.

Thus, already soon after irradiation of mice with 8 Gy it was possible to detect a disorder in the cGMP system functioning in the thymocytes and liver of the animals.

It is known that cyclic nucleotide phosphodiesterase has several forms which are capable of hydrolyzing either cAMP alone or cGMP alone or both nucleotides (18, 21). The availability of data on enzymatic hydrolysis of both cAMP and cGMP by phosphodiesterase preparations isolated from the irradiated animals permits a general evaluation of post-irradiation changes in the activities of cyclic nucleotide phosphodiesterase. The rate of cAMP hydrolysis in the liver starts to fall soon after irradiation of the mice, reaching a minimum after 24 hours (17) whereas enzymatic hydrolysis of cGMP by preparations isolated from the livers of the mice irradiated with the same dose (8 Gy) did not differ in the first three hours, from the control, and decreased first after 24 hours (the present paper). The kinetic characteristics of enzymatic hydrolysis of cAMP by the preparations isolated from the liver 24 hours after irradiation of the mice also appear to be different: irradiation causes enhancement of 'negative cooperativity' for cAMP (Fig. 3 b) and exerts no influence on 'negative cooperativity' for cGMP (Fig. 3 a). Thermostability of enzymic hydrolysis of both cyclic nucleotides following irradiation decreases; however the kinetics of the process of thermoinactivation proves to be different (compare Fig. 4 a and b). Thus, a combination of all these data shows that irradiation of mice affects in different ways the forms of phosphodiesterase hydrolyzing various cyclic nucleotides. It is quite possible that the main post-irradiational changes occur in those forms of phosphodiesterase that comprise the main part of the enzymatic activity which hydrolyze only one substrate (cAMP and cGMP).

In the search for mechanisms of the post-irradiation increase of guanylate cyclase activity (at least for early periods after irradiation) the interest is directed towards the nature of substances activating guanylate cyclase after irradiation, and the mechanism of activation. It is at the present time known that all activators of guanylate cyclase studied (most of them are abiogenic substances) possess oxidative properties (11). It is obvious that candidates for the role of activators of guanylate cyclase in vivo must meet the following requirements: 1) they must be biogenic substances, 2) be present in any cell, and 3) be oxidants. In particular, products of lipid peroxidation fulfil these requirements. Earlier, we have

demonstrated formation of guanylate cyclase activators during lipid peroxidation in biologic membranes. These activators are carbonyl compounds and not only those which occur during lipoperoxidation but also those forming at enzymatic oxidative deamination of biogenic amines (15). Thus, activation of the processes which lead to the formation of carbonyl compounds capable of activating guanylate cyclase may cause an increase in the enzymatic production of cGMP. Let us consider it in terms of post-irradiational changes in metabolism.

In early phases after irradiation of animals (in the first minutes and hours) activation of lipid peroxidation is observed, then another rise of lipoperoxidation intensity occurs reaching a maximum on the 1st to 2nd day after irradiation (4, 5, 22). In early phases after irradiation a considerable release of biogenic amines is also observed which, naturally, subsequently undergoes enzymatic oxidative deamination; it results in the formation of aldehydes which later are enzymatically oxidized and excreted (4). Thus, irradiation of the organism leads to the appearance of guanylate cyclase activators in tissue cells. Based on the data, we suppose that irradiation-induced intensification of lipid peroxidation and release of biogenic amines producing an increase of the level of carbonyls in cells is one of the causes of post-irradiational activation of guanylate cyclase.

It must be presumed that dysfunction of the regulatory system entails disturbances in the processes which the system regulates. Without covering in detail the entire spectrum of the processes regulated by cyclic GMP, we will consider in greater detail only one of them as a possible example: release of lysosomal enzymes. Post-irradiational activation of the release of lysosomal enzymes is a well-known phenomenon observed already in the first hours following irradiation (4).

It has been shown in many objects including white blood cells and hepatocytes that an increase in the intracellular content of cGMP results in release of lysosomal enzymes. Cyclic AMP exerts a contrary action (1, 7, 8). The change in the rate of release of lysosomal enzymes produced by cyclic nucleotides is explained by the authors by various effects of cAMP and cGMP on microtubules. The present experiments revealed an early increase in the cGMP concentration in the thymocytes and liver of the irradiated mice. It should be noted that an increase in the cGMP concentration in thymocytes is accompanied by a decrease in the cAMP content, and a rise in the cGMP concentration in the liver occurs with the cAMP level constant (12, 17). It is natural to suppose a relationship between the changes in the levels of cyclic nucleotides and changes in the rate of the process which is controlled by them, i.e. release of lysosomal enzymes. It is obvious, however, that for quantitative assessment of the contribution of cyclic nucleotides to this irradiation phenomenon as well as to other processes controlled by them, special experiments are necessary.

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