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RADIOSENSITIVITY OF GLYCOLYTIC ENZYMES IN THE NUCLEUS*

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SUMMARY

1. Thymus-nuclei of mice and rats have been isolated during the first 12 h following 800-R whole-body irradiation and extracted with 0.14 M NaCl.

2. In the extracts total protein, lactate dehydrogenase (EC 1.1.1.27), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and aldolase (EC 4.1.2.7) have been assayed.

3. Within the first 2 h after irradiation, there is a decrease in the enzymic activity of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase down to 50–70 % of the controls; simultaneously, there is a depletion of protein.

4. After a short recovery period, the activities of lactate dehydrogenase and of aldolase decrease slightly to 50 % of the activities of their controls 12 h after irradiation. The nuclear glyceraldehyde-3-phosphate dehydrogenase, however, is completely inactivated 8 h after irradiation, whereas in the cytoplasm this enzyme is unaffected.

5. Studies on the supernatants obtained in the course of isolating the nuclei, and assays on nuclei isolated in non-aqueous media, suggest that the enzymes are released from the nucleus into the cytoplasm in an early post-irradiation period, as a result of damage to the nuclear membrane.

6. The glyceraldehyde-3-phosphate dehydrogenase is completely destroyed in the nucleus simultaneously with the death of the cell.

INTRODUCTION

One of the most striking effects of ionizing radiation on the metabolism of the cell is the inhibition of the synthesis of DNA. This effect has been reviewed by LAJTHA[1]. It can be assumed that the inhibition of the synthesis of DNA is due to the radiosensitivity of the phosphorylation of the nucleotides described by CREASEY AND STOCKEN[2]. In the nucleus, most of the energy is gained by glycolysis[3]. Therefore we have been interested in the radiation effect on nuclear glycolytic enzymes.

Thymus-nuclei have been chosen for the experiments as we have gained some experience[4–8] of the chemical and histological alterations induced in them by radiation. A lethal whole body-irradiation induces in the thymus distinct histological changes

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within the first 24 h. After a lag period of 2 h an increasing number of cells with pycnotic nuclei is seen. Some time (6 h) after irradiation, most of the cells have died. Phagocytosis of these dead cells starts at 12 h after irradiation[4]. These events have to be taken into consideration, if biochemical studies are made on the thymus after irradiation. The cells are still living 2 h after irradiation but die within the next few hours. Specific irradiation effects can only be seen during this time. Later, 6 h after irradiation, any biochemical changes must be attributed to the death of the cells.

ERNST[9–12] has shown a characteristic drop of nuclear albumins and globulins immediately after whole body-irradiation. At 2 h after irradiation, 50 % of the control values are observed. After a short period of recovery, there is a loss of nuclear proteins simultaneously with the death of the cell.

The saline extracts of the nucleus contain the glycolytic enzymes of the nucleus. We thought it possible that the enzymic activity would decrease after irradiation as well as the amount of total nitrogen. The activity of lactate dehydrogenase (EC 1.1.1.27), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and aldolase (EC 4.1.2.7) were determined in the nuclei of the thymus after whole body-irradiation. Results were obtained from nuclei isolated both from a solution of sucrose and from a non-aqueous medium.

METHODS

Animals and irradiation

Rats and mice (male and female) of our own inbred strains were used; the mice had an average weight of 20 ± 1 g; the rats, of 145 ± 15 g. The mice were irradiated with 150 kV X-rays (X-ray machine of C.H.F. Müller, Hamburg, 20 mA, filter, 0.43 mm Cu; HVL, 0.87 mm Cu; FD, 30 cm) and the rats with 200 kV X-rays (X-ray machine MG 300 of C.H.F. Müller, Hamburg, 15 mA; filter, 0.52 mm Cu; HVL, 1.2 mm Cu; FD, 30 cm). Details of the irradiation and animal breeding are given by HAGEN AND BRAUN[4].

Analyses

The content of protein was determined according to BEISENHERZ *et al.*[14]; the content of DNA according to WEBB AND LEVY[15]. The enzymic activity was recorded as μmoles of substrate metabolized/h at 25°. Total activity was calculated to 1 g of the wet weight, specific activity to 1 mg of the protein of the fraction analysed. The extracts were used as the source of enzyme without further treatment. The activity of glyceraldehyde-3-phosphate dehydrogenase and of lactate dehydrogenase was assayed by the method of DELBRÜCK *et al.*[16]; that of aldolase was assayed by the method of SCHOLZ *et al.*[17]. The standard deviation σ_M is calculated according to

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

Isolation of the nuclei from a non-aqueous medium

The nuclei were isolated from a non-aqueous medium by the method of BEHRENS[12]. The thymus glands of rats were used in these experiments, as 3–4 g of tissue are needed for the isolation procedure (approx. 12 rat glands). A mixture of carbon tetrachloride and petroleum ether of increasing specific gravity (1.345–1.355) was used. About 200 mg of nuclei are prepared from 4 g of wet tissue. They will be defined as "dry

nuclei". The cytoplasmic contamination of each sample was examined by microscopic observation after staining with May Grünwald-Giemsa stain. Particular attention was paid to the reticular membranes attached to the nuclei. Contaminated samples were discarded. A further criterion of cytoplasmic contamination is the DNA-content of the nuclei; 26.0 ± 1.2 % was found to be an average value, *i.e.*, similar to the DNA content of the nuclei reported by SIEBERT[3].

Isolation of the nuclei in 0.25 M sucrose

Most of the experiments were carried out using this method. The thymus glands of 10–20 mice (0.6–1.2 g of tissue) were sampled, cooled to 0° and weighed. The following procedure was carried out in the cold room at +1°. The tissue (1 part) was homogenized with 10 parts of 0.25 M sucrose in a glass tissue grinder, similar to the DOUNCE homogenizer[18]. The homogenate was centrifuged at 1500 × *g*. The sediment was washed three times with 0.25 M sucrose. During the first washing, 0.18 M $CaCl_2$ was added to a final concentration of 0.0018 M. After the 4th sedimentation in sucrose, the nuclei were extracted with 0.14 M NaCl + 0.002 M EDTA for 30 min and were then centrifuged for 20 min at 20000 × *g*. This extraction procedure was repeated twice (5 min each time). The supernatants resulting from the washings with sucrose (indicated as S, I, II, III) and those from the saline extractions (4, 5 and 6) were brought to constant volume and analysed separately.

Cytoplasmic contamination of the nuclei isolated from sucrose solution

According to SIEBERT[3], the ratio of the content of DNA in the homogenate to that in the isolated nuclei can be used as a criterion for cytoplasmic contamination. The data analysed in our preparations are given in Table I. They show an increase in the concentration of DNA from 1 to 3.12. SIEBERT[3], isolating nuclei from liver, found a ratio of 1:10. As the thymus contains mostly lymphocytes, poor in cytoplasm, one would expect a lower ratio than that in the liver to be found.

TABLE I

ANALYSIS OF HOMOGENATE, AND OF ISOLATED NUCLEI, OF THYMUS

	Homogenate	*Nuclei*
Protein (mg/g)	262.0	75.0
DNA (mg/g)	49.0	43.7
DNA/Protein	0.187	0.582

The best criterion for having obtained nuclei uncontaminated with other cell particles, would be the absence of cytoplasmic proteins or enzymes in the supernatant of the sucrose washings of nuclear suspension. The nuclear suspension was divided into two parts after the third washing with sucrose. One part was washed further with sucrose, the other one was extracted with saline. The supernatants were analysed for protein and lactate dehydrogenase. The results are given in Table II.

Only 1 % of the total lactate dehydrogenase of the tissue was found in the sucrose-supernatants of the sucrose washings after the third washing (in Fractions IV, V and VI) and this may belong to the cytoplasm. In the corresponding saline extracts

TABLE II

PROTEIN AND LACTATE DEHYDROGENASE ACTIVITY IN THE SUPERNATANT IN THE COURSE OF SUCCESSIVE WASHINGS OF THYMUS NUCLEI

(Normal mice, $n = 5$–14; $\pm\sigma_M$.)

Fraction	*Lactate dehydrogenase (μmoles/h/g)*		*Protein (mg/g)*		*Specific activity*	
	Sucrose	*0.14 M NaCl*	*Sucrose*	*0.14 M NaCl*	*Sucrose*	*0.14 M NaCl*
Supernatant	9000 ± 1210		75.5 ± 3.86			
Sucrose I	4070 ± 640		44.3 ± 6.9			
Sucrose II	528 ± 73		17.3 ± 2.56			
Sucrose III	233 ± 31.4	201 ± 19.3	8.7 ± 0.95	8.14 ± 0.55		
Sucrose IV respectively NaCl (4)	73.2 ± 16.0	752 ± 47.7	4.9 ± 0.34	13.8 ± 0.88	14.95	54.5
Sucrose V respectively NaCl (5)	27.2 ± 11.6	190 ± 20.4	2.84 ± 0.42	7.97 ± 0.66	9.61	23.9
Sucrose VI respectively NaCl (6)		34.8 ± 3.3	2.4 ± 0.4	3.55 ± 0.17	0	9.9
Total (4–6)	100.4 ± 25.6	976.8 ± 51.4	10.14 ± 0.90	25.32 ± 0.87		

TABLE III

PROTEIN AND LACTATE DEHYDROGENASE ACTIVITY IN THE SUPERNATANT OF SUCROSE WASHINGS OF THYMUS NUCLEI BEFORE, AND AFTER, 60 MIN OF CENTRIFUGING AT 20000 × *g*

Given value is an average of 2 experiments.

Fraction	*Protein (mg/g)*			*Lactate dehydrogenase (μmoles/h/g)*	
	Total	*In the supernatant*	*Percent sedimentable*	*Total*	*In the supernatant*
III	10.1	6.6	34.6	226.0	226.0
IV	6.4	3.3	48.4	90.4	90.4
V	5.7	3.3	42.1	—	—
VI	4.2	2.1	50.0	—	—

(Fractions 4, 5 and 6) 10 times more lactate dehydrogenase is measured. We neglected, in the following experiments, the amount of enzyme obtained by further sucrose washings and considered the enzymic activity in Fractions 4, 5 and 6 as being the total activity in the nucleus.

The data on the proteins gained from the same experiments are not as satisfactory as the enzymic data mentioned above. The sucrose-supernatant beyond the third washing procedure contains considerable amounts of protein, but the enzymic activity is small. It can be assumed, that the protein in these fractions is partly protein of the nuclear membrane or of the endoplasmic reticulum. Support for this assumption lies in the observation that 50% of the proteins in the sucrose Supernatants III–VI are sedimented like microsomes. The activity of lactate dehydrogenase, however, is unchanged after centrifugation with 20000 × *g* (Table III).

HOGEBOOM *et al.*[19] suggested adding calcium ions to the nuclei to prevent leakage through the nuclear membrane. During the isolation of thymus nuclei there is, after the second washing without Ca^{2+}, a swelling of the sediment making the further sedimentation more and more difficult. An addition of 0.0018 M Ca^{2+} to the sucrose solution completely prevents the swelling, but complicates the extraction of protein from the nuclei (Table IV). If Ca^{2+} ions are added to the sucrose of each washing procedure, the extraction of nuclei with physiological saline needs two or three steps. If Ca^{2+} is added only to the first sucrose, the traces of Ca^{2+} in the next sucrose washings are sufficient to prevent the swelling; furthermore, a nearly complete saline extraction of the nuclei can be obtained in a single step.

TABLE IV

INFLUENCE OF CALCIUM ADDITION TO THE SUCROSE IN THE COURSE OF ISOLATION OF NUCLEI

Fraction	*Protein (mg/g)*		*Lactate dehydrogenase (μmoles/h/g)*	
	0.0018 M Ca^{2+} in S, I, II, III	*Ca^{2+} only in I*	*0.0018 M Ca^{2+} in S, I, II, III*	*Ca^{2+} only in I*
Sucrose III	6.0	6.3	188.0	311.0
NaCl-EDTA (4)	12.2	25.0	439.0	620.0
NaCl-EDTA (5)	10.0	3.1	201.0	99.5
NaCl-EDTA (6)	3.0	0.3	47.7	18.7

RESULTS

The enzymic activity assayed in the nuclei is shown in Table V.

Most of the analyses were carried out with the sucrose-washed nuclei of mice. The enzymic activities of rat thymus are much the same as those of mice excepting with respect to lactate dehydrogenase-activity. Also, the specific activity of dry nuclei are in agreement with those of nuclei from sucrose and also with the values reported by SIEBERT[3] for dry liver nuclei.

The specific activity of dry nuclei is in agreement with that of nuclei from sucrose and with the values reported by SIEBERT[3] for dry liver nuclei. The calculation of the specific activity in nuclei from sucrose depends on the fraction determined. As shown in Table II, the enzymes are extracted easier than the total proteins. Therefore, the specific activity in the Fraction 4 is higher than in 5 or 6. In order to simulate as

TABLE V

ACTIVITY OF GLYCOLYTIC ENZYMES IN THE NUCLEUS

The activity (E) in μmoles/h at 25°.

	Mouse-thymus nuclei isolated from sucrose (number of experiments)	*Rat-thymus nuclei isolated in sucrose*	*Rat-thymus dry nuclei*	*Rat-liver dry nuclei* SIEBERT[3]
Lactate dehydrogenase E/g	1018.0 ± 80.9 (20)	3320.0	33 200.0	8400.0
E/mg of protein	50.6 ± 3.48	236.0	230.0	169.0
Glyceraldehyde-3-phosphate dehydrogenase E/g	195.7 ± 21.6 (10)	177.0	2 260.0	1260.0
E/mg of protein	13.2 ± 1.11	12.8	15.7	19.1
Aldolase E/mg	20.5 ± 1.55 (10)	14.5	123.0	96.0
E/mg of protein	0.96 ± 0.09	1.2	0.86	1.4

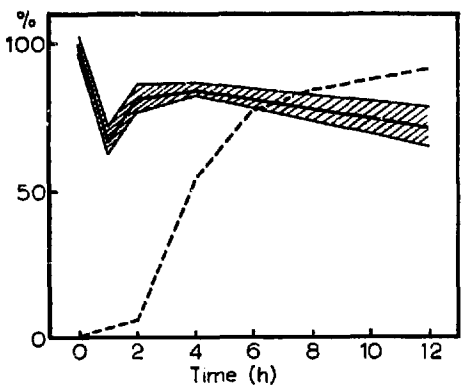

Fig. 1. Protein in Fractions 4–6 of the thymus nucleus after 800-R whole body irradiation. ▨ mg of protein/g thymus $\pm\sigma_M$; – – – –, percent pycnotic nuclei in the section.

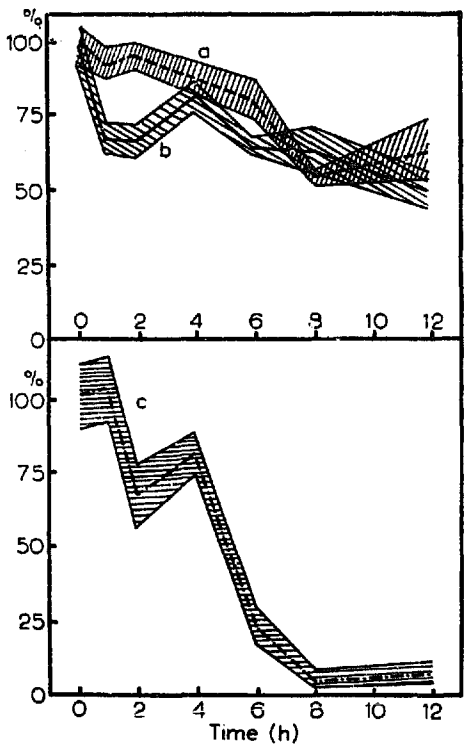

Fig. 2. Total activity of the glycolytic enzymes in the nucleus of thymus after 800-R whole body irradiation (percent of the control $\pm\sigma_M$). a, aldolase; b, lactate dehydrogenase; c, glyceraldehyde-3-phosphate dehydrogenase.

closely as possible the conditions inside the nucleus of the cell, we calculated only the specific activity of Fraction 4.

ERNST[9–11] demonstrated the characteristic drop in concentration of nuclear proteins in the thymus of rats. Similar results are obtained with the thymus nuclei of mice (Fig. 1).

There is a decrease of protein concentration immediately after irradiation, followed by a short period of apparent recovery. A final decrease at hour 4 begins when most of the cells die. For comparison the number of pycnotic nuclei, reported in an earlier paper[4], are given. It can be seen that the significant changes occur before the cells have died.

The total activity of the glycolytic enzymes in the first hours after irradiation is

shown in Fig. 2. Each point of these curves represents the average value of 6–10 experiments and its standard deviation. The activity of the lactate dehydrogenase behaves in the same way as the amount of total protein: Immediately after irradiation a decrease is noticed, then a short increase, and later again a decrease of the activity. The activity of aldolase, however, doesn't show this initial fall; it decreases only slightly. The glyceraldehyde-3-phosphate dehydrogenase seems to be the most sensitive enzyme. In the second hour a loss of activity is found, then a short increase, and thereafter a decrease down to zero. Only traces of activity can be measured 8 h after irradiation.

The specific activity is given in Table VI. The values of lactate dehydrogenase and of aldolase don't change very much; only the activity of glyceraldehyde-3-phosphate dehydrogenase decreases distinctly 6 h after irradiation.

Comparing these results with the histological observations, it can be seen that there are two phases of the loss of glycolytic enzymes in the nucleus. In the first 2 h, where no sign of an irreversible damage can be seen, there is a decrease of the lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase in the nucleus, due to the release of protein and enzyme into the cytoplasm. In the second phase, simultaneously with the death of the cells, there is a complete inactivation of the rest of the glyceraldehyde-3-phosphate dehydrogenase. It should be pointed out that the glyceraldehyde-3-phosphate dehydrogenase is destroyed only in the nucleus, not in the cytoplasm (Table VII).

TABLE VI

SPECIFIC ACTIVITY OF GLYCOLYTIC ENZYMES IN THE NUCLEAR FRACTION 4 AFTER 800-R WHOLE BODY IRRADIATION

Activity in μmoles/h/mg of protein.

	Lactate dehydrogenase	*Glyceraldehyde-3-phosphate dehydrogenase*	*Aldolase*	*Number of experiments*
Control	50.6 ± 3.48	13.20 ± 1.11	0.96 ± 0.09	20
1 h	45.4 ± 4.08	18.55 ± 2.10	1.31 ± 0.05	12
2 h	40.3 ± 3.72	9.82 ± 1.21	1.24 ± 0.08	12
4 h	49.4 ± 3.48	11.40 ± 0.82		5
6 h	41.9 ± 2.29	3.78 ± 1.12	1.02 ± 0.04	3
8 h	43.4 ± 5.95	0.70 ± 0.56	0.78 ± 0.06	4
12 h	37.6 ± 3.09	1.26 ± 0.70	0.83 ± 0.02	6

We assume that the effect of the first phase of radiation on the glycolytic enzymes in the nucleus is due to damage of the nuclear membrane. However, do the enzymes leave the nucleus in the living animal or are they washed out of the damaged nucleus in the course of isolation? To answer this question, the enzymic activity of each supernatant was studied in the course of isolation. The nuclei from normal thymus, and from a thymus taken 1 h after irradiation, were used (Figs. 3 and 4; 5 experiments each). If the effect of radiation is due to the washing out of the nuclear enzymes during isolation, the supernatants of the irradiated thymus homogenates should have more activity than the supernatants of the normal ones. According to our measurements, however, we found less activity in these fractions. We think, therefore, that the enzymes leave the nucleus in the living animal.

This idea is supported by the fact that the immediate loss of nuclear enzymes is also observed when the nuclei are prepared in a non-aqueous medium according to BEHRENS. In this method an exchange of proteins and enzymes during the preparation is not possible. Table VIII shows the values before, and 1 h after, irradiation.

TABLE VII

ENZYMIC ACTIVITY IN THE NUCLEUS AND IN THE HOMOGENATE OF THE THYMUS AFTER 800-R WHOLE BODY IRRADIATION

Total activity (E/g of tissue).

		Homogenate	*Nucleus*
Lactate dehydrogenase	Control	15 700	1018
	8 h	11 900	640
	12 h	11 900	502
Glyceraldehyde-3-phosphate dehydrogenase	Control	3 700	196
	8 h	3 500	8
	12 h	3 260	14
Aldolase	Control	178	20.5
	8 h	160	11.0
	12 h	151	13.0

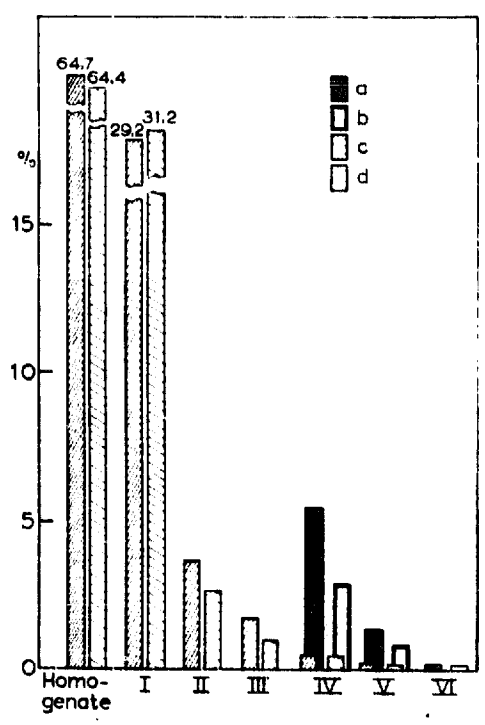

Fig. 3. Lactate dehydrogenase in the supernatant in the course of washings of nuclei. Percentage of the total activity. a, 0.14 M NaCl (control); b, 0.14 M NaCl (1 h post irradiation); c, 0.25 M sucrose (control); d, 0.25 M sucrose (1 h post irradiation).

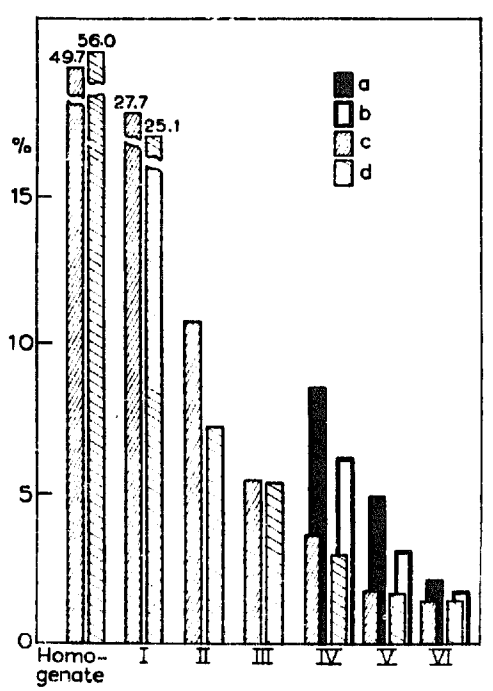

Fig. 4. Protein in the supernatants in the course of washings of nuclei. Percentage of the total protein: a, 0.14 M NaCl (control); b, 0.14 M NaCl (1 h post irradiation); c, 0.25 M sucrose (control); d, 0.25 M sucrose (1 h post irradiation).

TABLE VIII

ACTIVITY OF GLYCOLYTIC ENZYMES IN DRY NUCLEI OF THYMUS AFTER IRRADIATION

Average values $\pm\sigma_M$ of 4–5 experiments each, with dry nuclei.

	Control	*1 h after 800 R*	*Percent of the control*
Protein (mg/g)	144 ± 5.6	125 ± 11	86.8
Total activity			
Lactate dehydrogenase	33 200 ± 5120	27 500 ± 5440	82.8
Glyceraldehyde-3-phosphate dehydrogenase	2 260 ± 460	1 550 ± 355	68.6
Aldolase	123 ± 28	118 ± 33	96.0
Specific activity			
Lactate dehydrogenase	230	220	
Glyceraldehyde-3-phosphate dehydrogenase	15.7	12.4	
Aldolase	0.855	0.944	

DISCUSSION

One effect of irradiation is the release of glycolytic enzymes through the nuclear membrane into the cytoplasm, the other is the inactivation of the glyceraldehyde-3-phosphate dehydrogenase within the nucleus.

Two other changes in the composition of the nucleus after irradiation have been reported. According to CREASEY[20] catalase activity in the nuclei of the cells of the thymus decreases after whole body irradiation. At 1 h after irradiation with 1000 R, only 50 % of the activity remains in the nucleus compared with that of the control. Further, the nucleus of the lymphoma cell loses RNAase after irradiation with 2000 R *in vitro*[21]. The authors suggest that the RNAase is bound to microsome-like particles in the nucleus and is released by X-rays into the soluble fraction in the nucleus. Once released, the enzyme diffuse through the nuclear membrane into the cytoplasm. Apparently, according to the authors, the effect of radiation is localized in the internal structure of the nucleus, whereas the nuclear membrane permits free diffusion of proteins and enzymes.

Our measurements on the glycolytic enzymes suggest also damage to the nuclear membrane. These enzymes are found in the nuclear sap and only a leakage of the nuclear membrane will lead to the release of enzymes into the cytoplasm. Therefore we assume that the whole nuclear structure is weakened after irradiation, resulting in a higher permeability of membranes and a loosening of bonds. A similar phenomenon has been described in an earlier paper[6]: There is a distinct weakening of the bonds between DNA and protein 2 h after irradiation. The immediate effect of radiation on the nuclear membrane may induce this instability of the nucleoprotein.

SCHÜMMELFELDER *et al.*[22] described swelling of the nuclei after high local doses (60 kR) of X-ray to the cerebellum of mice, leading to pycnosis. WENDT[23] irradiated fibroblasts with 600–1000 R. Within 30 min, he saw the swelling of the nuclei after release of nuclear sap droplets into the cytoplasm. These histological observations may correspond to our biochemical measurements. After the release of proteins, there is a change in the swelling capacity of the nucleoproteins.

The release of nuclear proteins into the cytoplasm is not a phenomenon of necrobiosis. It can be seen also in the nuclei of liver and brain[11], where no cell death occurs

in the early postirradiation period. Here the damage on nuclear membranes is restored completely, whereas in thymus nuclei the recovery period has just started, but is interrupted by cell death.

As the specific activity of the enzymes is unchanged during the first hours after irradiation, there is no immediate radiation effect on the glycolytic enzymes in the nucleus itself. It is still open, however, if the release of nuclear enzymes into the cytoplasm causes a damage to the energy metabolism in the nucleus.

The glyceraldehyde-3-phosphate dehydrogenase is completely destroyed in the nucleus at the moment the cells die. As in the cytoplasm the enzyme is unaffected and the two other enzymes tested are still present in the pycnotic nucleus, it is assumed that the inactivation of the glyceraldehyde-3-phosphate dehydrogenase is connected with changes in the nuclear structure, which occur at the moment of pycnosis. This observation is in agreement with the general experience that glyceraldehyde-3-phosphate dehydrogenase is more sensitive to external effects than other glycolytic enzymes[24].

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