The Effect of Ionizing Radiation on Nuclear Phosphorylation in the Radio-sensitive Tissues of the Rat

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Little success has so far attended the many attempts to establish the activation or inactivation of an enzyme as an early step in the consequences of exposure *in vivo* to ionizing radiation. This may be due to the fact that attention has been focused mainly on the cytoplasm or the whole tissue. It occurred to us therefore that, since nuclear function is markedly disturbed, a comparison of the enzymic complement of the nucleus in radio-sensitive (i.e. those tissues which show early histological degeneration after exposure to sub-lethal irradiation) and non-sensitive tissues might provide a clue to the primary biochemical lesion.

About thirty nuclear enzymes have been described but, of those we have so far examined, only one system differentiates between the two kinds of tissue. This system is the one discovered by Osawa, Allfrey & Mirsky (1957), who showed that, in calf thymus, adenine, guanine and uridine mononucleotides were bound to the nucleus and that when the isolated nuclei were shaken aerobically in a sucrose medium at 0° these mononucleotides were phosphorylated to the corresponding triphosphates. We have found that nuclear generation of labile phosphate takes place in other radio-sensitive tissues, but not in the non-sensitive ones, and is adversely affected by irradiation either *in vivo* or *in vitro*. A preliminary account of these findings has already been reported (Creasey & Stocken, 1958).

EXPERIMENTAL

Materials and methods

Preparation of nuclear suspensions. Three different media have been used for the isolation and incubation of nuclei, the choice depending mainly on the appearance of the preparation under phase-contrast microscopy. The criteria were an absence of granularity in the nucleus, no adherent material and only trivial contamination with whole cells. Medium A: 0·25 M-sucrose, 3·3 mM-$CaCl_2$, 5 mM-triethanolamine hydrochloride adjusted with NaOH to give a pH of 7·1 (Allfrey, Mirsky & Osawa, 1957). Medium B: the same as A except that the $CaCl_2$ concentration was 1·8 mM (Hogeboom, Schneider & Striebich, 1952). The addition of triethanolamine buffer to A and B was a modification of the original formulae. Medium C: 45 mM-glucose, 85 mM-KCl, 8·5 mM-NaCl, 2·5 mM-$CaCl_2$, 2·5 mM-$MgCl_2$, 5 mM-triethanolamine hydrochloride adjusted to pH 7·2 with KOH (Barnes, Esnouf & Stocken, 1956).

Homogenizers of the Philpot & Stanier (1956) pattern were employed and appropriate differences between the diameters of the plunger and tube discovered for each tissue. Liver and brain required 0·004 in., spleen, lymph node, intestinal mucosa, thymus gland and pancreas 0·003 in. and bone marrow and kidney 0·002 in. Connective tissue was removed from liver, kidney, brain, thymus, spleen and pancreas by means of a preliminary extrusion through a stainless-steel mincer similar to that supplied by Broyeur de Fischer à Main, Jouan, Paris. One part of tissue was homogenized in four parts of medium and the suspension filtered through fine nylon cloth. The filtrate was diluted twenty-fold and then centrifuged in a Baro-Gyro centrifuge by a rapid acceleration to 3600 *g* (40 sec.), followed by an equally rapid arrest. The nuclear sediment was transferred to a homogenizing tube, resuspended in 10–20 parts of medium by means of a plunger with a diameter 0·01 in. less than the tube and centrifuged as before and the process repeated if necessary. The nuclei were suspended finally in 7 ml. of the medium. All operations were conducted in the cold room at 0–2°.

Blood cells were removed from liver preparations by perfusion of the organ *in situ* (Hogeboom *et al.* 1952) first with normal saline (aq. 0·9% NaCl) at room temperature and then with ice-cold medium B. From spleen the erythrocytes were removed by a modification in the method of centrifuging; the spleen homogenate was filtered and the filtrate diluted with two volumes of medium and spun at a mean force of 440 *g* for 2 min. in the Model 2 G.E.C. International Centrifuge. The overall time from start to rest was 3 min. or slightly less. In these circumstances the majority of the red cells remained in the supernatant and complete separation was effected by resuspending and spinning twice more. This process also gave satisfactory results with the other tissues, but in general we employed the first method because of the saving in time.

Bone marrow was obtained from the long bones. The ends of the bones were sawn off, a close-fitting hypodermic needle was inserted into the cavity and the marrow ejected into ice-cold medium by means of compressed air.

The 'intestinal mucosa' used in these experiments was the material obtained by scraping the wall of the small intestine after it had been washed out with water. Lymph nodes were taken randomly from the cervical, lumbar and inguinal regions, and no experiments with nodes of a particular location were undertaken.

Incubation and assay of labile phosphate. A sample (3 ml.) of the nuclear suspension was pipetted into 1 ml. of 2 M-$HClO_4$ and the remainder shaken gently in a 50 ml. conical flask at 0–2° for a convenient time. This was

Table 1. *Rate of formation of labile phosphate in nuclear suspensions shaken aerobically at 0°*

All tissues except liver and brain were prepared in 0·25 M-sucrose, 3·3 mM-$CaCl_2$, 5 mM-triethanolamine hydrochloride buffer (pH 7·1). For liver and brain the $CaCl_2$ concentration was 1·8 mM. The formation of labile phosphate was measured over 5–10 min. for bone marrow and 30–40 min. for other tissues.

Tissue	No. of experiments	Rate of formation of labile phosphate (μg. of P/min./mg. of DNA phosphorus)	Range of rates
Spleen	11	0·71±0·024	0·57–0·73
Thymus gland	8	0·31±0·028	0·23–0·37
Lymph node	6	1·83±0·18	1·3–2·4
Bone marrow	6	8·04±1·13	5·8–10·4
'Intestinal mucosa'	3	0·31	0·21–0·37
Liver	6	0	—
Kidney	2	0	—
Brain	4	0	—
Pancreas	2	0	—

usually 30–40 min. except with bone marrow, when 5–10 min. was sufficient. At the end of the incubation period a second 3 ml. sample was precipitated with 1 ml. of 2 M-$HClO_4$ and the remainder used for estimating deoxyribonucleic acid (DNA) and microscopical examination. The $HClO_4$ extracts were centrifuged, the residues extracted twice with 2 ml. of 0·5 M-$HClO_4$ and the combined supernatants made up to 10 ml. Samples of 4 ml. were taken for analysis. Acid-labile phosphate was obtained before and after incubation from the difference between the values of inorganic phosphate in the original samples and those which had been heated with 1 ml. of 5 N-HCl at 100° for 10 min. Inorganic phosphate was determined by the method of Berenblum & Chain (1938).

Animals. Male and female animals of the laboratory strain of Wistar rats (wt. 80–120 g.) were used. They were decapitated and the organs rapidly removed and placed in ice-cold medium.

Irradiation. X-radiation was carried out *in vivo* at the M.R.C. Radiobiological Research Unit, Harwell. The characteristics of the radiation were: half-value layer 1·2 mm. Cu, peak voltage 250 kv at 14 mA. The rats received 67 r. of total body radiation/min. Irradiation *in vitro* was effected by means of two 250 mc radium sources. The dose rate was either 2·8 or 43·6 r./min. as measured by oxidation of $FeSO_4$ according to the method of Miller (1950).

Estimation of deoxyribonucleic acid. Burton's (1956) modification of Dische's (1930) diphenylamine method was used.

Chemicals. The reagents were all of A.R. grade, except triethanolamine hydrochloride, which was prepared by threefold recrystallization from aqueous ethanol of the hydrochloride obtained by neutralizing commercial-grade triethanolamine.

RESULTS

Nuclie were prepared from thymus gland, lymph node, 'intestinal mucosa', spleen, bone marrow, pancreas and kidney in medium A and from liver and brain in medium B. Preliminary experiments were carried out with each tissue to ensure not only that sufficient labile phosphate was formed but that the reaction was stopped before the rate of phosphorylation ceased to be linear. Because the quantity of material used was small it was impracticable to identify the sources of labile phosphate and the results are therefore presented as an amount of phosphate liberated in 10 min. at 100° in acid solution. The values (Table 1) sharply differentiate the radio-sensitive from the non-sensitive tissues, since only in the former group is there an increase in labile phosphate.

Table 2. *Inhibition of the formation of labile phosphate in nuclei isolated from tissues of rats exposed to whole-body X-irradiation 1 hr. previously*

	Rate of phosphorylation for various doses of X-irradiation as percentage of control values		
Dose	25 r.	50 r.	100 r.
Tissue			
Spleen	18, 30	0, 0, 0	0, 0, 0
Thymus gland	45, 77	14, 23	0, 0, 0, 0, 0
Bone marrow	0, 0	0	0
Lymph node	0, 6	0	0

The next step in the investigation was to ascertain whether this phosphorylation system was affected by ionizing radiation. We therefore irradiated rats with varying doses of total body radiation and killed them 1 hr. later. Similar rats were treated in the same way, except for the irradiation, and were used at the same time for control values. Low doses produce a very marked inhibition and complete suppression of phosphorylation results from exposure to 100 r. (Table 2).

Although 1 hr. is a comparatively short time after irradiation in which to observe any biochemical change it was important to know whether this was an immediate rather than a delayed effect. Experiments were therefore carried out with spleen and thymus to discover whether the phosphorylating ability was retained in spleen and thymus when the organs had been stored in ice-cold

medium. This was necessary since 45 min. was required to transport the animals from the X-ray machine at Harwell to the laboratory in Oxford. The rate of phosphorylation was unchanged in the nuclei from thymus kept for 1 hr., but from spleen it was reduced almost to zero. Rats were therefore exposed to 100 r. and killed within 3–5 min., and the thymus glands placed in semi-frozen medium A. One hour later nuclei were isolated from these and control glands which had been obtained at the same time and similarly treated. The nuclei from the irradiated rats were completely ineffective, whereas the control specimens had unimpaired phosphorylating ability. When the X-ray dose was reduced to 50 r. the rates of phosphorylation in two experiments were reduced to 18 and 45 % of the control values. These results clearly indicate that the response is immediate and can be compared with the block in the synthesis of DNA which has also been found at 3–5 min. post-irradiation (Ord & Stocken, 1956).

Since the inhibition of nuclear phosphorylation was complete after exposure to 100 r. of total body irradiation it was of interest to follow the rate at which the recovery occurred. Spleen and thymus were chosen as test systems and it was found (Fig. 1) that in thymus recovery began at about 60 hr. and was approaching normal values by 100 hr. In spleen (Fig. 2) there was an over-compensation which also returned to normal at about 100 hr. When a lethal dose of 1000 r. was given there was no recovery at any time before death.

Experiments were also carried out *in vitro* to eliminate the possibility that the inhibition of phosphorylation was an abscopal effect (Mole, 1953) and not due to a direct action on the nucleus. Nuclei were isolated from spleen and thymus gland in medium A and part of the preparation was exposed at 0° to γ-rays from a radium source. The irradiated and control samples were then shaken in air in the usual way and the inhibition was obtained by direct comparison. The system is rather more sensitive *in vitro* than *in vivo* (Table 3), but in view of the absence of any protection which might be afforded by the natural environment this is perhaps not surprising.

Because of the interest in protection against exposure to ionizing radiation some preliminary experiments have been carried out with cysteamine (Bacq *et al.* 1951). Groups of rats were given doses of 150 mg. of neutralized cysteamine hydrochloride/kg. by intramuscular injection 5 min. before exposure to 100 r. of total body irradiation. At 1 and 48 hr. after irradiation, when the control values were still zero, the thymus nuclei isolated in medium C from the cysteamine-treated rats gave 10 and 90 % respectively of normal values.

We have also attempted to use cysteamine as a protective agent *in vitro*, but unfortunately the phosphorylation system is inhibited by the concentrations which give satisfactory results *in vivo*. Low concentrations of sulphydryl groups (e.g. 5 μg./ml.) are difficult to maintain in aqueous solution at physiological pH and we have not yet been able to demonstrate any protection either by glutathione or cysteamine. Some protection, however, is afforded by anaerobiosis. If a suspension of thymus nuclei is exposed to 48 r. in the absence of oxygen and then shaken aerobically, the

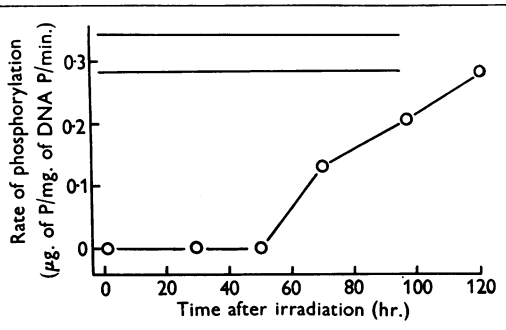

Fig. 1. Recovery of nuclear phosphorylation in the thymus gland of the rat after 100 r. of total-body radiation. The region within the parallel lines represents the mean of the control values with its standard error.

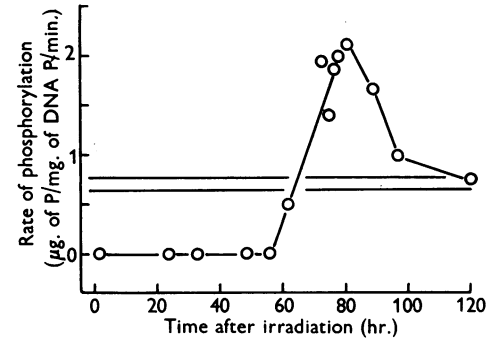

Fig. 2. Recovery of nuclear phosphorylation in rat spleen after 100 r. of total-body radiation. The region within the parallel lines represents the mean of the control rates with its standard error.

Table 3. *Effect of γ-irradiation* in vitro *on the formation of labile phosphate in nuclear suspensions*

Tissue	Radiation dose (r.)	Rate of phosphorylation as percentage of control values
Thymus gland	22	70, 68, 64
	30	40
	44	0, 0, 0, 0
Spleen	22	0, 7, 26
	44	0, 0

rate of phosphorylation is about 20 % of the corresponding non-irradiated controls. This dose of γ-radiation given aerobically is sufficient to suppress completely the conversion of the mononucleotides into the triphosphates.

DISCUSSION

The characteristic features of phosphorylation of the nucleotides bound to nuclei prepared from calf-thymus gland have been thoroughly investigated and discussed by Osawa *et al.* (1957). We have repeated their experiments with rat instead of calf thymus and, except that we have been able to prepare active nuclei in a medium which does not contain sucrose, our results agree with theirs. We have shown that nuclear phosphorylation is not restricted to thymus but is demonstrable in spleen, intestinal mucosa, bone marrow and lymph node. No phosphorylation could be detected in brain, kidney, liver or pancreas and since we failed to obtain a satisfactory preparation from testis and heart muscle we were unable to investigate these two sources. Neither have we been able to demonstrate nuclear phosphorylation in regenerating liver, but the explanation for this may reside in the differences in enzyme complement, e.g. regenerating liver has ten times the inorganic pyrophosphatase activity of thymus nuclei.

The results provide a clear separation of the radio-sensitive from the non-sensitive tissues. They also show that nuclear phosphorylation is abolished by low doses of radiation given either *in vivo* or *in vitro*. Some of the difficulties in the mode of expression of the results have been avoided by reference to the DNA content of the preparation; but because the heterogeneity of the cell population varies with different tissues and because the preparations may be contaminated with metabolically inert nuclei, it cannot be claimed that the results either indicate an order of radio-sensitivity or allow a direct comparison between the various organs. One further problem of interpretation could arise if any cell migration had occurred after irradiation and before the tissues were taken for testing, but in the experiments described here we have no reason to suppose that this is a complicating factor.

With these reservations, the effects of ionizing radiation on mitochondrial and nuclear phosphorylation may be compared. Osawa *et al.* (1957) showed that the effects of metabolic inhibitors on the two systems were not identical, and it is clear that a similar situation exists for the radiation response. It was first observed by Potter & Bethel (1952) that mitochondria isolated from spleens of rats which had been exposed to 800 r. 1 hr. previously had a reduced capacity to couple oxidation and phosphorylation. This finding has been confirmed and extended in mice by Ashwell & Hickman (1952) and in rats by van Bekkum, Jongepier, Nieuwerkerk & Cohen (1953). van Bekkum (1956) has also reported that phosphorylation is uncoupled in mitochondria from thymus but not from liver or regenerating liver, even when the dose is several thousand roentgens. Although in spleen and thymus a rather high dose is required to elicit an immediate mitochondrial response, it might be worth while extending these observations to a more complete range of tissues to see whether there is parallelism in susceptibility with the nuclear lesion. In addition to mitochondria requiring much greater doses than the nuclei to produce damage it appears that they also require time for development of the maximum effect. Experiments carried out *in vitro* differentiate even more sharply the mitochondrial and nuclear systems. Potter & Bethel (1952) exposed spleen mitochondria to 2000 r. at 2°, Ord & Stocken (1955*a*) gave 1000 r. to spleen mitochondria respiring at 38°, and van Bekkum (1956) gave as much as 20 000 r. without any effect on oxidative phosphorylation.

The evidence suggests that the mitochondrial defect becomes important only when near-lethal doses of whole-body radiation have been received, and supports the earlier ideas which have stressed the importance of the cytoplasm in radiation damage (Trowell, 1955, 1958; Ord & Stocken, 1955*b*, 1958; Schjeide, Mead & Myers, 1956).

The part played by nuclear phosphorylation in the economy of the cell cannot yet be completely defined, but it is known from the work of Osawa *et al.* (1957) and Allfrey *et al.* (1957) that most of the chemical reagents which uncouple phosphorylation inhibit the incorporation of alanine into nuclear protein and that loss of the nucleotides bound to the nucleus results in a failure of protein synthesis. More recently it has been suggested that nuclear phosphorylation may also be involved in the processes leading to the formation of DNA (Ord & Stocken, 1958). It is now well established that the synthesis of DNA is restricted to a limited period of the life cycle of the dividing cell (Howard & Pelc, 1953; Lajtha, Oliver, Kumatori & Ellis, 1958) and that the formation of DNA as measured by isotope incorporation is more readily inhibited in the pre-synthetic (G_1) period than in the synthetic (S) period itself (Howard & Pelc, 1953; Kelly, 1954; Holmes & Mee, 1954; Lajtha *et al.* 1958). Low doses of radiation given in the G_1 period delay the onset of DNA synthesis and mitosis, and it may be that the bound phosphorylated nucleotides are essential for the formation or alignment of an activated complex within the nucleus. When the preliminaries are completed,

only high doses of radiation, which probably interfere with the template itself, can prevent the final condensation of the deoxynucleotides on the template. The biochemistry of the premitotic (G_2) phase is unknown and its radio-sensitivity is still being explored; it is therefore not possible to implicate nuclear phosphorylation in this stage of the mitotic cycle.

Nuclear phosphorylation appears to be a sensitive index of radiation damage, but as we have detected a loss of catalase from thymus nuclei at the same dose level it is possible that other biochemical lesions will be found.

SUMMARY

1. When nuclei prepared from spleen, thymus gland, lymph node, bone marrow and intestinal mucosa of the rat are shaken aerobically at 0° in a suitable medium, acid-labile phosphate is formed. This is not so for the radio-insensitive tissues—brain, liver, kidney and pancreas.

2. Low doses of whole body X-irradiation abolish the nuclear phosphorylation.

3. Low doses of γ-irradiation given to suspensions of nuclei also prevent nuclear phosphorylation.

4. In spleen and thymus there is a recovery of the ability to effect nuclear phosphorylation at about 100 hr. after 100 r. of total-body radiation.

5. The possible significance of the observations in relation to the radio-biological lesion is discussed.

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