THE EFFECT OF X-RAYS ON PHOSPHORYLATIONS *IN VIVO*

D. W. VAN BEKKUM

Medical Biological Laboratory of the National Defence Research Council T.N.O., Rijswijk, Z.-H. (The Netherlands)

INTRODUCTION

The oxidative phosphorylation of mitochondria isolated from some of the radiosensitive tissues is decreased within a few hours after total body X-irradiation[1]. This radiation effect has been demonstrated in spleen mitochondria from rats and mice as well as in rat thymus mitochondria[2]. These findings do not necessarily imply that irradiation causes a disturbance of the oxidative phosphorylations *in vivo*. It is conceivable that mitochondria isolated from irradiated animals might be more seriously affected by the rather complicated isolation technique than those from untreated controls. The experiments reported here were designed to assess the effect of total body irradiation on the rate of phosphorylation *in vivo*.

At the present time no satisfactory methods are known for determining the turnover rate of the labile phosphate groups of adenosinetriphosphate (ATP) and adenosinediphosphate (ADP) in living tissues. The main difficulty is the technical impossibility of separating the intracellular from the extracellular inorganic phosphate, which prohibits the calculation of the specific activity (s.a.) for the intracellular inorganic phosphate[3]. However to compute the turnover rate involved, the s.a. of the intracellular inorganic phosphate should be known, as this phosphate is assumed to be the pre precursor of the labile phosphate groups produced during oxidative phosphorylation. An additional obstacle to straightforward turnover studies is the presumable existence of an unknown number of individual phosphate spaces (compartments) inside the cell. However, the rate of incorporation of parenterally administered radioactive inorganic phosphate into ATP and ADP of tissues was considered as supplying a reasonable approximation of the rate of oxidative phosphorylation *in vivo*.

So far, information of this kind with regard to irradiated animals is scarce. SHERMAN AND FORSSBERG[4] reported an increased specific activity of the acid-labile phosphate groups of ATP in the liver and a decrease of this value in skeletal muscle of mice immediately after total body irradiation. The significance of these findings is not evident. The ATP content of the spleen was found to be decreased 24 hours after total body irradiation in a few animal species[5]. Probably this change reflects the altered cellular composition of the tissue, since the X-ray doses employed cause a severe depletion of the lymphoid elements of the spleen.

METHODS

Animals

Young adult male albino rats, weighing between 130 and 300 grams were paired according to body weight. One of each pair was irradiated, the other was used as a control. All procedures, excepting the irradiation, were carried out simultaneously on the experimental animal and its control.

References p. 492.

Labeling

4 hours after the irradiation the rats were anesthetized with ether and 0.5 ml of a 0.1% neutralized Na_2HPO_4 solution, which contained 100 μc $Na_2H^{32}PO_4$, was injected into the tail vein. An overdose of Nembutal (35–50 mg) was administered intraperitoneally 3 minutes before the end of the labeling period. The excised tissues were dropped into liquid air. One spleen or the combined thymus tissue of 3 rats was used for each analysis.

Irradiation

The rats were irradiated in a circular perspex cage under conditions of maximum backscatter. The physical constants of the radiation were: 200 kV, 20 mA, total filtration: 1.5 mm Cu, H.V.L.: 1.8 mm Cu, dose rate 45 r.p.m.; the total-body dose was 700 r in all cases.

Chemical analysis

In principle the method consists of a separation of the labeled nucleotides from the inorganic and other phosphates of the acid-soluble fraction, followed by a fractionation of the adenosinephosphates by ion exchange. The specific activity of adenosinemonophosphate (AMP), ADP and ATP was estimated in the corresponding eluate fractions.

The frozen tissues were weighed quickly and homogenized in 20 ml of an ice-cold 0.5 *N* perchloric acid solution. The suspension was kept for at least 30 min in the cold, after which the precipitate was centrifuged off and discarded. The supernatant fluid (acid-soluble fraction) was neutralized with 3 *N* KOH and stored overnight at 1–2°C. After the potassium perchlorate precipitate had been removed by centrifugation, the solution was passed through a small charcoal column (0.6 cm × 3 cm^2). The columns were prepared from Carboraffine C (Bayer) which had been stirred in 1*N* HCl, packed into columns and washed with distilled water before use. The solution was passed through the column by slight suction at a rate of 1–2 ml/min; afterwards distilled water was washed through the column until the radioactivity of the outflow became negligible. The fluids that passed the column were collected and treated with an excess of magnesium reagent to precipitate the inorganic phosphate[6]. The mixture was kept for 12–24 hours in the cold; the ensuing precipitate was collected by centrifugation, washed with 2½% NH_4OH and dissolved in 1 *N* HCl. This solution was analysed for phosphate[7] and for radioactivity. In control experiments the inorganic phosphate fraction was subjected to the extraction procedure described by ENNOR AND STOCKEN[8]. In every case more than 97% of the radioactivity was recovered in the *iso*butanol layer.

The nucleotides were eluted from the charcoal with 20% aqueous pyridine. This resulted in the removal of 85% of the absorbed radioactivity (mean of 18 determinations). The nucleotides were separated by the method of COHN AND CARTER[9]; instead of Dowex-1 we used Amberlite I.R.A.–400–50 mesh.

Small columns (2.5 cm × 0.3 cm^2) of the HCl-treated resin were washed with distilled water until the eluate was free of chloride ions. The pyridine solution containing the nucleotides was diluted with 4 volumes of distilled water and passed through the resin. The pyridine was washed carefully from the column with distilled water until the density of the eluate remained less than 0.050 at 260 mμ (Beckman Spectrophotometer, model DU, lightpath 1 cm). Then elution was carried out with the solutions described by COHN AND CARTER[9] at a rate of 1.5 ml/minute. 20 ml fractions were collected and measured against appropriate blanks at 260 mμ. A molar extinction coefficient of $14.2 \cdot 10^3$ was used to calculate the concentration of the adenosine phosphates in the fractions[10]. Radioactivity was measured in a Veall type fluid counter. In the ADP and ATP fractions a close correlation between the optical density (260 mμ) and the radioactivity was observed (Fig. 1).

In a number of experiments the purity of the ADP and ATP fractions has been estimated The acid-soluble fraction contains a number of other nucleotides besides the adenosinephosphates, but the available data indicate that the latter are by far the most abundant in animal tissues[11]. Using the method of ENNOR AND STOCKEN[8] on representative ADP and ATP fractions before and after acid hydrolysis, more than 90% of the radioactivity was always found to be associated with the acid-labile phosphate groups. A very small amount of inorganic phosphate (less than 2½% of the total number of counts) was found, which probably resulted from some hydrolysis of ATP and ADP in the course of the fractionation and the extraction.

The identity of the nucleotides was confirmed by paper chromatography. To remove the interfering inorganic salts the fractions were passed through charcoal columns. After washing with distilled water the nucleotides were eluted with 20% aqueous pyridine. The pyridine was removed by extraction with chloroform. The resulting aqueous solution of nucleotides was concentrated at a low temperature and chromatographed on paper[12]. The spots containing phosphate groups were located with the method of HANES AND ISHERWOOD[13] and the position of the adenine groups was determined by ultraviolet contact photography[14]. Autoradiography was used to locate the radioactive phosphate; the active spots were cut out and counted with an end window G.M. tube.

References p. 492.

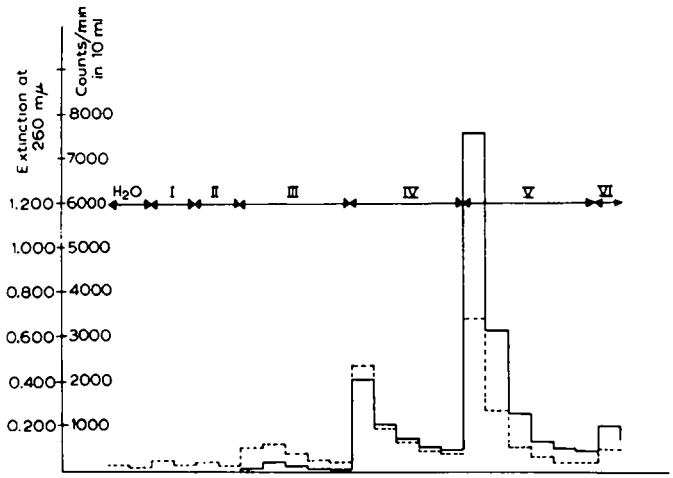

Fig. 1. Elution pattern of the nucleotides of rat spleen. Resin: Amberlite I.R.A.-400. Elution fluids: I: 0.1 *N* NH_4OH + 0.01 *M* NH_4Cl (adenosine). II: 0.01 *M* NH_4Cl (adenine). III.: 0.003 *N* HCl (AMP). IV: 0.01 *N* HCl + 0.02 *M* NaCl (ADP). V: 0.01 *N* HCl + 0.2 *M* NaCl (ATP). VI: 1 *N* HCl (unknown compounds). Drawn line: radioactivity; broken line: extinction at 260 mμ; fraction volume: 20 ml.

The results showed that the major part of the radioactivity in fractions IV and V (Fig. 1) could be accounted for by ADP and ATP respectively. 65–80% of the total number of counts on the paper chromatograms was associated with the appropriate nucleotide spots. Taking into account that some hydrolysis of ATP and ADP had probably occurred during the desalting and the chromatography, the purity of the ion exchange fractions was considered to meet the requirements of our study.

CALCULATIONS

The s.a. has been expressed as the number of counts per μmole. In the case of the nucleotides this value represents the mean of the first four 20 ml samples of the fraction, tubes showing an extinction of less than 0.085 have been omitted. The relative specific activity (r.s.a.) of the nucleotide represents the quotient of the s.a. of ADP or ATP and the s.a. of the inorganic phosphate from the same tissue sample.

RESULTS

The rate of incorporation of inorganic phosphate into ADP and ATP of thymus and spleen is shown in Table I and Figs. 2 and 3. It will be seen that labeling of the ATP and the ADP fractions occurs very rapidly, especially in the case of the thymus. Therefore, short intervals between the injection of the radioactive phosphate and the sampling of the tissues have been chosen in studying the effects of irradiation.

Table II summarizes the effect of total body irradiation on the rate of phosphorylation in rat spleen. The differences between the paired observations were used to calculate the significance of the radiation effects. In this case the *t* test of Student could be used[15]. A highly significant decrease of the s.a. of ADP and ATP as well as of the r.s.a. of ATP was found in the irradiated rats. The s.a. of the inorganic phosphate was significantly decreased in the irradiated group. However, this decrease does not account for the decreased s.a. of ADP and ATP, as may be seen from the r.s.a. values.

References p. 492.

TABLE I

LABELING OF VARIOUS PHOSPHATE GROUPS OF RAT THYMUS FOLLOWING THE INTRAVENOUS INJECTION OF RADIOACTIVE INORGANIC PHOSPHATE

Time after injection	*Blood c/min/ml* $\times 10^{-3}$	*Specific activity* $\times 10^{-3}$*			*Relative specific* activity*	
		inorganic phosphate	*ADP*	*ATP*	*ADP*	*ATP*
5 min	133	111	65	138	0.59	1.24
10 min	77	101	96	170	0.95	1.68
15 min	70	98	107	192	1.09	1.98
20 min	85	124	126	261	1.02	2.11

Tissue values were obtained by the analysis of the pooled thymus of 3 or 4 rats. The 20 min values represent the means of 2 experiments. The blood data are means of measurements on 3 rats, except for the 20 min value (8 rats).

* Defined in the text.

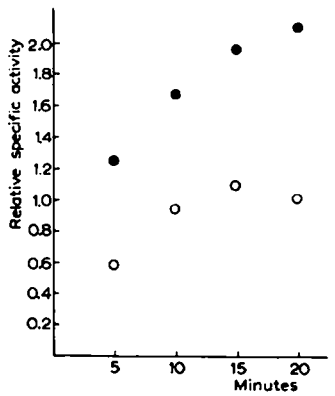

Fig. 2. Rate of incorporation of inorganic phosphate into ADP and ATP of rat thymus. ● Relative specific activity of ATP; ○ relative specific activity of ADP. Each value is the result of an analysis of the pooled thymus tissue of 3 rats. The 20 min values represent means of 2 determinations.

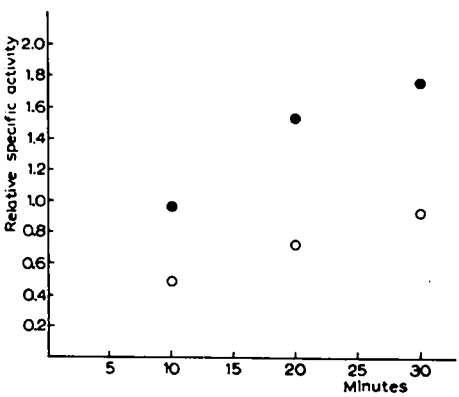

Fig. 3. Rate of incorporation of inorganic phosphate into ADP and ATP of rat spleen. ● Relative specific activity of ATP; ○ relative specific activity of ADP. Number of experiments: 10 min: 1; 20 min: 3; 30 min: 2.

TABLE II

THE EFFECT OF TOTAL BODY IRRADIATION ON THE RATE OF LABELING OF VARIOUS PHOSPHATE GROUPS IN RAT SPLEEN

	Specific activity $\times 10^{-3}$*			*Relative specific activity**	
	inorganic phosphate	*ADP*	*ATP*	*ADP*	*ATP*
Control	114 ± 13	82 ± 7	154 ± 21	0.72 ± 0.06	1.35 ± 0.12
Irradiated	99 ± 18	65 ± 7	112 ± 18	0.66 ± 0.06	1.13 ± 0.08
P of difference	< 0.05	< 0.001	< 0.001	< 0.05	< 0.001

Values in the table represent means of 10 experiments ± the standard deviation.

* Defined in the text.

References p. 492.

The amount of ATP and of ADP per gram of spleen tissue has been roughly assessed from the optical density at 260 mμ of the ion exchange eluates. The mean values obtained in the irradiated and the control tissues did not differ.

Because of the unequivocal results with spleen, only 4 experiments were performed with thymus. The results (Table III) show a similar trend as in the case of the spleen, except for the s.a. of the inorganic phosphate. A statistical analysis of the r.s.a. data using WILCOXON's test[15], showed that the r.s.a. of ATP was significantly lower in the irradiated group ($P > 0.03$).

TABLE III

THE EFFECT OF TOTAL BODY IRRADIATION ON THE RATE OF LABELING OF VARIOUS PHOSPHATE GROUPS IN RAT THYMUS

	Specific activity × 10 [3]*			*Relative specific activity**	
	inorganic phosphate	*ADP*	*ATP*	*ADP*	*ATP*
Control	129	73	135	0.61 ± 0.04	1.13 ± 0.06
Irradiated	137	71	125	0.52 ± 0.03	0.92 ± 0.08

Values in the table represent means of 4 experiments ± the standard deviation.

* Defined in the text.

DISCUSSION

The results leave no doubt that the rate of incorporation of parenterally administered inorganic phosphate into the acid-labile phosphate groups of spleen and thymus adenosine phosphates is significantly decreased shortly after total body irradiation. Obviously the interpretation of these findings should be made with great caution. The decrease of the s.a. of the acid-labile phosphate groups following irradiation may be the result of a decrease of the s.a. of the intracellular inorganic phosphate, supposing that the latter is the immediate precursor. The finding that the r.s.a. of ATP and ADP is also decreased does not rule out this possibility, because changes of the s.a. of the intracellular inorganic phosphate will probably not be proportionally reflected in the s.a. of the tissue inorganic phosphate. This masking effect can be expected to be more pronounced in the experiments with thymus; the amount of radioactive phosphate in the blood being relatively large at the time of analysis (5 min after the injection of the label, see Table I).

If the exchange of inorganic phosphate between the intra- and the extracellular compartments occurs by way of passive diffusion our results might indicate an inhibition of this process after irradiation. However, the available radiobiological data are not consistent with such an explanation, on the contrary ionizing radiation has been found to increase the permeability of the cell membrane for various substances[16, 17].

In case of the alternative mechanism of phosphate transfer, namely of a phosphorylation at the cell membrane, a decreased s.a. of the intracellular inorganic phosphate would in itself be the result of an inhibition of phosphorylation. In fact the extremely rapid equilibration between tissue inorganic phosphate (which includes some highly labeled extracellular phosphate) and the acid-labile phosphate groups of ATP, as observed in thymus, seems to be reconcilable with a mechanism of active phosphate transfer.

References p. 492.

In view of these considerations our findings may best be interpreted as reflecting a decreased rate of phosphorylation in the spleen and the thymus of irradiated animals. It is realised that this phosphorylation may include oxidative as well as anaerobic phosphorylation. Since the latter process is known to be much less efficient, its contribution to the total phosphorylation is supposed to be relatively small.

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SUMMARY

The incorporation of ^{32}P into ATP and ADP of spleen and thymus was measured shortly after the intravenous administration of radioactive inorganic phosphate to rats.

4 hours after total body X-irradiation with a dose of 700 r, both the specific activity and the relative specific activity of the ADP and ATP of the spleen were significantly decreased. Similar results were obtained with the thymus. These changes may result from an inhibition of the phosphorylation in the irradiated tissues.

REFERENCES

1 D. W. VAN BEKKUM AND O. VOS, *Brit. J. Exptl. Pathol.*, 36 (1955) 432.
2 D. W. VAN BEKKUM, *Radiobiology Symposium, 1954*, Butterworths, London, 1955, p. 201.
3 A. H. ENNOR AND H. ROSENBERG, *Biochem. J.*, 56 (1954) 302.
4 F. G. SHERMAN AND A. FORSSBERG, *Arch. Biochem. Biophys.*, 48 (1954) 293.
5 E. M. UYEKI, *Federation Proc.*, 13 (1954) 413.
6 B. L. GRISWOLD, F. L. HUMOLLER AND A. R. MCINTYRE, *Anal. Chem.*, 23 (1951) 192.
7 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
8 A. H. ENNOR AND L. A. STOCKEN, *Australian J. Exptl. Biol. Med. Sci.*, 28 (1950) 647.
9 W. E. COHN AND C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 4273.
10 M. L. EIDINOFF, H. C. REILLY, J. E. KNOLL AND D. H. MARRIAN, *J. Biol. Chem.*, 199 (1952) 511.
11 R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
12 B. MAGASANIK, E. VISCHER, R. DONIGER, D. ELSON AND E. CHARGAFF, *J. Biol. Chem.*, 186 (1950) 37.
13 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
14 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 49 (1951) 407.
15 G. W. SNEDECOR AND W. G. COCHRAN, *Statistical Methods*, 5th ed., The Iowa State College Press, Ames, Iowa, 1956.
16 D. BILLEN, G. E. STAPLETON AND A. HOLLAENDER, *J. Bacteriol.*, 65 (1953) 131.
17 S. P. STRUGGER, A. T. KREBS AND Z. S. GURLACH, *Am. J. Roentgenol., Radium Therapy Nuclear Med.*, 70 (1953) 365.

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