SECTION 3

INFLUENCE OF SOME EXPERIMENTAL CONDITIONS ON RADIATION INDUCED IMPAIRMENT OF OXIDATIVE PHOSPHORYLATION

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INTRODUCTION

Studies reported in the earlier section have demonstrated a significant deleterious effect of whole-body radioexposure on hepatic mitochondrial oxidative phosphorylation. It is now well recognised that among the many metabolic disturbances arising from whole-body irradiation of animals, some may be categorised as being primary, whereas others arise secondarily as a consequence of such primary damage. The mediation of various endocrine glands in the development of some radiation responses has also been postulated from time to time (cf 1).

The evidence in literature, on the factors that may contribute to the radiation induced alterations in oxidative metabolism, is rather fragmentary. The observation that isolated mitochondria require very high doses of radiation (2) as compared to the dose *in vivo* for obtaining a similar degree of derangement of oxidative metabolism suggests that the effect may be indirect. That the impaired oxidative phosphorylation is possibly abscopal in nature is also indicated by studies using hormonal inhibitors or those in which specific organs are shielded from exposure to radiation (3 - 5). It is difficult to draw any generalised inference from the findings of different investigations as they have not been carried out under comparable conditions. On the other hand, it would appear that no single study has made an in-depth assessment of all the factors that are of likely relevance to the induction of radiation damage to mitochondrial function.

In the studies reported in this section, the following aspects of damage to mitochondrial oxidative metabolism following whole-body irradiation of rats have been investigated:

(i) the relevance, if any, of the decreased food intake of the irradiated animals to the observed effects on coupled phosphorylation;

(ii) the possibility of damage to oxidative metabolism resulting from whole-body irradiation acting as a general stress and consequent hyperactivity of the adrenals;

(iii) the influence of shielding a part of the body, so as to prevent the exposure of the liver to ionising radiation, on coupled phosphorylation in hepatic mitochondria;

(iv) comparison of the effects of whole-body irradiation with those following exposure of isolated liver mitochondria; and

(v) the possibility of modifying radiation effects on mitochondrial oxidative phosphorylation by prior administration of some radioprotective chemicals or agents that are known to affect mitochondrial metabolism in vivo.

MATERIALS AND METHODS

Chemicals:

In addition to the chemicals whose sources are indicated in the earlier section, serotonin (5-hydroxy-tryptamine), and 2-aminoethyl isothiouronium bromide hydrobromide (AET) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ethyl-α-p-

chlorophenoxy isobutyrate (CPIB) was obtained as a gift from Imperial Chemical Industries Limited, Pharmaceuticals Division, Macclesfield, Great Britain.

Animals:

Male Albino rats of Wistar strain, weighing about 125 g and reared on a nutritionally adequate laboratory stock diet were used.

Bilateral adrenalectomy:

Access to the adrenals was gained via the dorsal route with the rats under light ether anesthesia and the endocrine glands were excised out carefully to ensure their total removal. The operated animals were provided with drinking water containing 0.9% saline. They were irradiated 7 days following adrenalectomy and were sacrificed 72 h after irradiation. Control rats were sham operated.

Liver shielding:

Each rat was placed on a wooden board with its dorsal side up and with its legs firmly tied to the board to render it immobile. Five lead strips, 6.5 cm wide and of a total thickness of 3.0 cm, and bent to a dimension slightly larger than the abdominal contour were placed over the rat so that the portion of the body 3.5 cm from the head of the rat to 4.5 cm from the base of the tail was shielded when the animal was exposed to radiation. Control animals were sham irradiated in the same manner.

Pair feeding studies:

In one set of experiments the daily food intake of the irradiated rats was measured and the controls were pair fed the same amount of diet, with a 24 h phase difference between the two groups.

Administration of serotonin and AET:

Serotonin creatine sulphate was dissolved in water (7.5 mg/ml) and injected intraperitoneally at a dose of 30 mg /kg body weight of rat. 5, 2 amino ethyl thiourium bromide-hydrobromide (AET) was taken in 0.9% NaCl (45 mg/ml), neutralised with 0.1 N NaOH and administered intraperitoneally at dose of 180 mg/kg body weight. The solutions of both these radioprotective agents were prepared just prior to use and were given to the animals 10 min before radioexposure.

Administration of CPIB (∝-p-chlorophenoxy isobutyrate):

After being maintained for 10 days on the normal stock diet, experimental animals were given a dietary supplement of 0.5% (W/W) of CPIB for another 10 days, after which some of the animals were irradiated.

<u>In vitro</u> irradiation of mitochondria:

A gamma cell 200 (Atomic Energy of Canada, Ltd.) containing cobalt-60 was used as the source of radiation for these studies. Liver mitochondria were irradiated at a dose rate of 3000 r/min

either as a pellet or as a suspension in 0.154 M/ KPO_4 buffer (pH 7.4) (20 mg of protein/ml), in glass test tubes kept immersed in a beaker containing ice. Dosimetry was carried out using ferrous sulfate and ceric sulfate (6).

RESULTS

Data on the effect of restricting the dietary intake of control rats to that of the irradiated ones, on oxidative phosphorylation in liver mitochondria are presented in Table 1. It would appear that the decreased food intake *per se* results in a slightly enhanced oxygen uptake without any alteration in the P/O ratio. The oxygen consumption as well as P/O ratio of mitochondria from livers of irradiated rats are decreased as compared to those of both the *ad-libitum* and pair-fed control rats.

The possible role of the adrenals in contributing to the radiation effects has been assessed by studying mitochondrial oxidative metabolism in adrenalectomised rats subjected to whole body irradiation. From the results summarised in Table 2, it may be observed that adrenalectomy in itself results in a significant decrease of both oxygen uptake as well as P/O ratio. It is even more interesting that it enhances the inhibitory effect of irradiation on P/O ratio, so that in the adrenalectomised rats, no coupled phosphorylation is evident at 72 h post-irradiation.

Shielding a portion of the abdomen, inclusive of the liver, during exposure of the rats to x-rays considerably minimises

Table 1

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM IRRADIATED RATS - COMPARISON WITH PAIR FED CONTROL

Group	O_2 uptake (μ atom/min/mg protein) x 10^2	Pi esterified (μ atom/min/mg protein) x 10^2	P/O ratio	Inhibition (%)
Ad-libitum fed control	5.12 ± 0.43	10.24 ± 0.72	2.00 ± 0.01	-
Pair-fed control	5.84 ± 0.42	11.40 ± 0.82	1.98 ± 0.02	-
Irradiated	3.82 ± 0.18	1.22 ± 0.07	0.32 ± 0.04	84

The rats were subjected to 800 R of whole body x-irradiation and oxygen uptake and coupled phosphorylation measured 72 h later with succinate as substrate. The values represent averages of four independent determinations ± S.E.M.

Table 2

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM IRRADIATED RATS – EFFECT OF BILATERAL ADRENALECTOMY

Group	O_2 uptake (µ atom/min/mg protein)	Pi esterified x 10^2	P/O ratio	Inhibition (%)
Control	5.61 ± 0.82	10.66 ± 0.43	1.91 ± 0.11	-
Adrenalectomised	4.92 ± 0.39	7.72 ± 0.52	1.57 ± 0.08	17
Irradiated	5.21 ± 0.41	5.00 ± 0.31	0.96 ± 0.04	50
Adrenalectomised and irradiated	5.72 ± 0.63	Not measurable	0	100

The adrenalectomised rats were killed 10 d after removal of the endocrines. Those of them which were irradiated, were exposed to 800 R, 7 d following adrenalectomy and were killed 72 h post-irradiation. Oxygen uptake and coupled phosphorylation were measured with succinate as substrate. The values represent averages of four independent determinations ± S.E.M.

the radiation effect on hepatic mitochondrial oxidative metabolism, although it does not totally abolish it (Table 3). Thus, the lead-shielded animals do not show any significant radiation effect up to 24 h post-irradiation and even by 72 h the degree of derangement is only about half of that suffered by the rats which are whole-body irradiated without any shielding (Section 2, Table 2). By far the most significant difference between these studies and the earlier one without shielding is with respect to the reversibility of the damage. The shielded rats exhibit total recovery by 96 h as against the progressively increasing damage shown by those that are not.

Observations on the effect of irradiation of isolated liver mitochondria on oxidative phosphorylation are presented in Table 4. The radiation dose required to elicit a response of similar magnitude during in vitro irradiation to that observed in the whole animal is many times greater. Qualitatively, however, the effects seem to be similar since the decreased phosphorylation is not accompanied by any increase in the rate of oxygen uptake. When the mitochondria are irradiated as a pellet (as obtained by centrifugation at 10000 xg for 10 min and subsequent decanting off the supernatant), a dose as high as 600 KR is required to decrease the efficiency of coupled phosphorylation by about 25 per cent. A somewhat smaller dose of radiation (300 KR) is seen to be sufficient to obtain a significant damage to the phosphorylating function when the mitochondria are irradiated as a suspension in an aqueous medium.

Table 3

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM IRRADIATED RATS -

EFFECT OF SHIELDING LIVER (PART OF ABDOMEN)

Post-irradiation period (h)	O_2 uptake (μ atom/min/mg protein)	Pi esterified $\times 10^2$	P/O ratio	Inhibition (%)
0	4.81 ± 0.41	8.64 ± 0.32	1.80 ± 0.09	0
24	4.21 ± 0.36	7.14 ± 0.52	1.70 ± 0.09	6
72	4.63 ± 0.11	5.66 ± 0.22	1.22 ± 0.12	32
96	5.40 ± 0.21	9.72 ± 0.43	1.80 ± 0.11	0

The liver shielded animals were exposed to x-rays (800 R) with a part of their upper abdomen (as described in text) covered by 3 cm thickness of lead. The determinations were made at various time intervals following irradiation, using succinate as substrate. The values represent averages of four independent determinations ± S.E.M.

Table 4

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA IRRADIATED IN VITRO

State of mitochondria	Radiation dose (KR)	Control			Irradiated			Inhibition (%)
		O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified	P/O ratio	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified	P/O ratio	
Dry pellet	600	3.61 ± 0.21	10.11 ± 0.41	2.80 ± 0.09	3.42 ± 0.23	7.18 ± 0.25	2.10 ± 0.15	25
Suspension (in 0.154 M KPO_4 buffer)	300	4.12 ± 0.38	11.54 ± 0.25	2.80 ± 0.12	3.82 ± 0.20	6.30 ± 0.14	1.65 ± 0.09	41

Liver mitochondria were isolated by differential centrifugation and irradiated at 1 - 4°C either as a pellet or as an aqueous suspension. Oxidative phosphorylation was measured immediately after irradiation with glutamate as substrate. The values represent averages of four independent determinations ± S.E.M.

Studies on the modifying influence, if any, of serotonin, a known radioprotective agent, on liver mitochondrial function of irradiated rats reveal almost total protection and the data are tabulated in Table 5. The findings from similar studies with AET, which has been documented to be one of the most effective chemicals for protection against radiation injury, are presented in Table 6. The administration of serotonin per se brings about a significant reduction in oxygen uptake by the liver mitochondria in the rats killed immediately after whole body irradiation. However, no such alteration in oxygen uptake is noticeable at 72 h and 96 h post-irradiation in the serotin administered rats. Protection with AET does not show any influence on the rate of oxidation of substrate at any of the time periods studied. While both these radiation protection chemicals are quite effective in preventing the decrease in P/O ratio of liver mitochondria, serotonin is slightly more efficient than AET. A mixture of the two was not any more effective than the individual components, but was found to be far more toxic as seen by increased mortality in even control rats.

The influence of the prior feeding of CPIB, on mitochondrial oxidative phosphorylation, can be assessed from the data presented in Table 7. Administration of the drug for ten days prior to whole-body irradiation (800 rads) shows only a marginal effect on the radiation induced decrease in oxidative phosphorylation. Rats fed this drug also show a decrease in P/O ratio which increases with time, and these animals do not show any difference in mortality from the irradiated rats not administered the drug. Liver mitochondria

Table 5

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM IRRADIATED RATS - EFFECT OF PRE-TREATMENT WITH SEROTONIN

Post-irradiation period (h)	Without serotonin				Serotonin-treated			
	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified	P/O ratio	Inhibition (%)	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified	P/O ratio	Inhibition (%)
0	5.12 ± 0.71	10.24 ± 0.96	2.00 ± 0.10	-	2.92 ± 0.21	5.69 ± 0.64	1.95 ± 0.05	-
72	3.62 ± 0.69	2.82 ± 0.23	0.78 ± 0.09	61	3.43 ± 0.39	6.86 ± 0.83	2.00 ± 0.07	-
96	4.25 ± 0.43	3.19 ± 0.47	0.75 ± 0.04	62	3.89 ± 0.46	7.78 ± 0.34	2.00 ± 0.09	-

The rats were exposed to 800 R of x-rays and sacrificed at different time intervals following irradiation. The serotonin treated rats were administered 30 mg/kg body wt of the drug 10 min prior to irradiation. Succinate was used as substrate for determining the efficiency of oxidative phosphorylation. The values represent averages of four independent determinations ± S.E.M.

Table 6

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM X-IRRADIATED RATS - EFFECT OF PRE-TREATMENT WITH AET

Post-irradiation period (h)	Without AET				AET-treated			
	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified x10^2	P/O ratio	Inhibition (%)	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified x10^2	P/O ratio	Inhibition (%)
0	4.62 ± 0.91	8.55 ± 1.2	1.85 ± 0.09	-	4.12 ± 0.38	7.83 ± 0.75	1.90 ± 0.10	-
72	3.92 ± 0.62	2.67 ± 0.83	0.68 ± 0.04	64	3.11 ± 0.42	4.98 ± 0.63	1.60 ± 0.15	16
96	4.10 ± 0.23	2.50 ± 0.51	0.61 ± 0.09	66	3.40 ± 0.25	6.26 ± 0.89	1.84 ± 0.03	3

The rats were exposed to 800 R of x-rays and sacrificed at different time intervals following irradiation. The AET treated rats were administered 180 mg/kg body wt of the drug 10 min prior to irradiation. Succinate was used as substrate for measuring P/O ratio. The values represent averages of four independent determinations ± S.E.M.

Table 7

OXIDATIVE PHOSPHORYLATION IN LIVER MITOCHONDRIA FROM IRRADIATED RATS - EFFECT OF PRIOR FEEDING WITH CPIB

Post-irradiation period (h)	Control				CPIB-fed			
	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified x10^2	P/O ratio	Inhibition (%)	O_2 uptake (μ atom/min/mg prot.)x10^2	Pi esterified x10^2	P/O ratio	Inhibition (%)
0	4.68 ± 0.12	9.12 ± 0.26	1.95 ± 0.01	-	3.22 ± 0.25	6.25 ± 0.34	1.94 ± 0.02	-
72	4.23 ± 0.23	3.38 ± 0.31	0.80 ± 0.08	59	3.13 ± 0.14	3.76 ± 0.18	1.20 ± 0.15	38
96	3.82 ± 0.33	2.48 ± 0.23	0.65 ± 0.02	67	4.11 ± 0.21	3.69 ± 0.11	0.90 ± 0.09	54
168	5.14 ± 0.31	0.36 ± 0.06	0.07 ± 0.00	96	3.82 ± 0.16	1.53 ± 0.16	0.40 ± 0.08	79

The rats were fed 0.5% of CPIB in the diet for 7 days prior to whole body radio exposure at 800 R. Mitochondrial oxidative phosphorylation has been measured using succinate as substrate. The values represent averages of four independent determinations ± S.E.M.

from the drug-treated rats show, in general, a significant lowered ability to oxid ise succinate.

DISCUSSION

Although the studies described in the earlier section had clearly demonstrated a decreased capacity of liver mitochondria from irradiated rats to carry out phosphorylation coupled to the oxidation of substrates, the possibility remained that this decrease reflected differences in the nutritional status of the control and irradiated rats. Whole body irradiation is known to result in a severe degree of inanition (cf 7) and starvation has been reported to have an adverse influence on mitochondrial oxidative phosphorylation (2). The present studies permit a comparison of the irradiated rats with pair fed controls and it would appear that the impairment of oxidative phosphorylation following whole-body radioexposure is not related to the decreased food intake of these rats. It should be mentioned, that whereas the ad libitum fed control and the irradiated rats were sacrificed at the same time, the pair-fed animals could be killed only after the 24 h phase difference necessitated by the methodology. However, every precaution was taken to have the experiments conducted under identical conditions, in so far at least as all the known experimental variables are concerned.

Some of the biochemical abnormalities in the irradiated rat are believed to be secondary to endocrinal disturbances. In

particular, it has been suggested that the radiation effect on oxidative phosphorylation in different tissues is indirect and mediated through a hormonal response involving the pituitary-thyroid or the pituitary-adrenal axis (5). Adrenal hyper-activity has been specifically implicated in the derangement of hepatic oxidative metabolism (5) in irradiated rats. The present findings do not, however, favour an involvement of the adrenal hormones in the radiation induced lowering of P/O ratio in liver mitochondria. While these studies do not rule out the contribution of other endocrine glands to the radiation effect, the failure of adrenal-ectomy to abolish the radiation damage to oxidative metabolism is indicative of such an effect not representing a generalised response to a stress condition.

Notwithstanding the fact that a specific involvement of the adrenals has been shown to be unlikely, three lines of evidence point to the abscopal nature of the derangement of liver mitochondrial oxidative phosphorylation resulting from whole body irradiation.

While the radiation effect is considerably minimised, it is still quite significant when a portion of the abdomen inclusive of the liver is shielded during exposure of the animals to x-rays. This would strongly suggest neurohormonal involvement as a major factor in the impairment of hepatic mitochondrial function. The manifestation of a radiation effect in a tissue which has itself not been irradiated could also arise from the diffusion of radio-lytic products from other irradiated tissues. It is of interest

to note in this context that gamma globulin from irradiated rabbits when administered into mice bearing ascites tumour considerably lowered the mitotic index in the tumour cells (8). Other studies in this laboratory have shown that *in situ* perfusion of liver with irradiated isotonic sucrose solution for a brief period can also lead to a significant deterioration of coupled phosphorylation (9). These observations serve to emphasise the possibility of tissues or organs manifesting deleterious effects without their being directly exposed to radiation. The decreased response as well as the better recovery of the shielded rats observed in the present study may also be due, at least in part, to the non-exposure of other vital organs such as spleen which are highly radiosensitive.

Yet another indication of the indirect nature of radiation damage to mitochondria is the great disparity in the radiation dose required to elicit the response in the whole animal and in isolated mitochondria. It would appear that mitochondria *per se* are relatively radioresistant and that the observed effect following whole body irradiation is not a direct consequence of radiation but a secondary manifestation of radiation injury. This view is further supported by the fact that the decrease in P/O ratio in the whole body irradiated rats is gradual with time and is not seen immediately following irradiation.

Although the irradiation of isolated rat liver mitochondria and its effect on oxidative phosphorylation have been studied earlier (10 - 14), some of these investigations (2, 14) are complicated by

the fact that the irradiation has been carried out in an aqueous medium containing sucrose. Irradiated sucrose solution itself is known to exert a pronounced toxic effect on mitochondria suspended in it, resulting in decreased oxidative phosphorylation (9). Thus, the impairment of oxidative phosphorylation observed in mitochondria irradiated in sucrose solution could represent the superimposition of a direct effect, if any, on the toxicity resulting from radiolytic products of sucrose. The present findings show a decreased oxidative phosphorylation even when the mitochondria are irradiated as a pellet or in an aqueous medium devoid of sucrose. On the other hand, Scaife and Hill (13) have not been able to observe any decrease in P/O ratio of liver mitochondria following their irradiation; this could, however, be due to the very low doses of radiation employed by these investigators. The greater sensitivity of the mitochondria when irradiated as a suspension could presumably result from the increased availability of free radicals following radiolysis of water.

Serotonin and AET are effective radioprotective chemicals and the present studies point to their ability also to prevent the radiation induced damage to liver mitochondrial oxidative metabolism. These observations lend additional support to the concept that damage to mitochondrial function could be of major significance in the development of radiation injury.

The quality and quantity of mitochondria has been suggested as an important factor determining the radiation sensitivity of cell types. In view of the reported finding that administration of the

hypocholesterolemic drug, CPIB, to rats increases the mitochondrial content of liver (15), the influence of this drug on the radiation induced impairment of mitochondrial metabolism has been investigated. Although rats administered this drug do not show the same degree of protection as serotonin and AET at earlier time periods, liver mitochondria from these animals have normal P/O ratios of 96 h post-irradiation.

SUMMARY

The decrease in liver mitochondrial oxidative phosphorylation resulting from whole body irradiation of rats is not related to the lowered food consumption of these animals. The effect does not also appear to be mediated through adrenal hyperactivity, as in a general stress response, since it is not abolished by adrenalectomy.

The great disparity in the *in vivo* and *in vitro* radiation doses required for the manifestation of radiation damage and the failure of liver shielding in totally preventing the decreased efficiency of hepatic mitochondrial phosphorylation point to the indirect nature of the deleterious effect of radioexposure on mitochondrial oxidative phosphorylation.

Prior administration of serotonin and AET offer complete protection against radiation induced decrease in mitochondrial oxidative phosphorylation. Treatment with the hypocholesterolemic drug, CPIB, also enables total recovery of the deranged mitochondrial function.

REFERENCES

1. Bacq, Z.M. and Alexander, P., 'Fundamentals of Radiobiology' p. 394, 453, Pergamon, London, 1961.
2. Clarke, I.D. and Lang, J., Rad. Res. 24, 142, 1965.
3. Bacq, Z.M. and Alexander, P., 'Fundamentals of Radiobiology' p. 395, Pergamon, London, 1961.
4. Ord, M.G. and Stocken, L.A., Mechanisms in Radiobiology, Vol. 1, p. 259 (Errera, M. and Forssberg, A. eds.) Academic Press Inc., New York, 1961.
5. Benjamin, T.L. and Yost, H.T., Rad. Res. 12, 613, 1960.
6. Robert, D.S. (Sr.), Proc. Annual Meeting of Am. Chem. Soc., Atlantic City, New Jersey, 1965.
7. Bacq, Z.M. and Alexander, P., 'Fundamentals of Radiobiology' Pergamon, London, p. 407, 1961.
8. Dalos, B., Doklen, A. and Horvath, M., Int. J. Rad. Biol. 16, 50, 1969.
9. De, A.K., Aiyar, A.S. and Sreenivasan, A., Rad. Res. 37,202,1969.
10. Potter, R.L., Bethel, F.N., Fed... Proc. 11, 170, 1952.
11. Ord, M.G. and Stocken, L.A., Brit. J. Radiol. 28, 279, 1955.
12. Van Bekkum, D.W., in Ciba Found. Symp. 'Ionizing Radiation and Cell Metabolism', J. & A. Churchill, p. 77, London 1956.
13. Scaife, J.F. and Hill, B., Can. J. Biochem. Physiol. 40, 1025, 1962.
14. Yost, H.T. and Robson, H.H., Biol. Bull. 113, 198, 1957.
15. Ramakrishna Kurup, C.K., Aithal, H.N. and Ramasarma, T., Biochem. J. 116, 773, 1970.