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SITE OF IMPAIRMENT OF OXIDATIVE PHOSPHORYLATION IN IRRADIATED RATS

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SUMMARY

There is a significant inhibition of liver mitochondrial oxidative phosphorylation at 72 h following whole-body X-irradiation of rats with a dose of 800 rad. The three sites of phosphorylation coupled to electron transport show differential sensitivities to radiation, the terminal site being far more affected than the other two.

Studies on NAD^+ reduction by reversal of electron flow, with the energy for the process being derived from the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine, suggest that the formation of high-energy intermediates is not interfered with by irradiation. On the other hand, there is 2-fold evidence in favour of an impairment of the transphosphorylation function. Thus, by reconstitution studies, the activity of coupling factor F_1 is found to be greatly decreased in irradiated rat livers. With ATP as an energy source, phosphorylating submitochondrial particles from livers of irradiated rats show a lower rate of reduction of NAD^+.

The site-specificity of the radiation effect is discussed in relation to current concepts of oxidative phosphorylation.

INTRODUCTION

It is now generally recognised that exposure of animals to whole-body irradiation results in a significant decrease of mitochondrial oxidative phosphorylation[1–7]; even the liver, a comparatively radio-resistant organ, has been observed to show this response[2,4,5,7]. Notwithstanding the overwhelming evidence of a deleterious effect on coupled phosphorylation, considerable qualitative and quantitative inconsistencies exist between the observations of various investigators (*cf.* refs 3, 8 and 9). Such variations are traceable in many instances to apparent differences in the experimental protocol employed. Thus, studies have been carried out employing a variety of oxidisable substrates and the radiation effects have been assessed at different periods post-irradiation, from a few hours to days. Some of the more important factors, apart from individual differences between animals, that could contribute to variations in the observations have been critically discussed by Yost *et al.*[7].

The earliest report on the differential response of the three sites of phosphorylation coupled to the electron transfer chain is that of Clarke and Lang[10], who observed the first site of phosphorylation to be far more sensitive than the other two, when mitochondria were irradiated *in vitro*. On the other hand, a greater effect on the

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine.

terminal and the second sites of phosphorylation following whole-body irradiation has also been reported[7].

The present paper relates to investigations aimed at (i) quantitating the differences in the sensitivities, of the three coupled phosphorylation reactions in rat liver mitochondria, to whole-body irradiation, and (ii) defining the possible site and mechanism of impairment of oxidative phosphorylation in these animals. Contrary to general belief, the data point to irradiation acting as an inhibitor rather than as a true uncoupler. In this respect, the effect of irradiation is similar to that of oligomycin and some of the biguanides (*cf.* ref. 11). Thus, the formation of high-energy intermediates linked to oxidation of substrates in liver mitochondria proceeds normally following whole-body irradiation, whereas the catalytic activities of the coupling factor proteins, especially of F_1, are significantly lower resulting in decreased transphosphorylation.

MATERIALS AND METHODS

Chemicals

ATP (sodium salt), antimycin A, bovine serum albumin, cytochrome *c*, DEAE-cellulose, L-glutamic acid, glycine, hexokinase, NAD, oligomycin, phenazine methosulfate, protamine sulfate, sodium ascorbate, sodium succinate and Tris were obtained from Sigma Chemical Co., U.S.A. and hydroxylapatite (Bio-Gel H T) from Bio-Rad Laboratories, U.S.A. The solvents used were of 'AnalaR' grade from British Drug House Laboratory Chemicals Division of Glaxo Laboratories, India; various inorganic chemicals, of 'AnalaR' grade, as well as EDTA, glucose, sucrose and tetramethyl-*p*-phenylenediamine (TMPD) were also obtained from the same source.

Animals

All the experiments have been performed with male albino rats of Wistar strain, weighing about 125 g. The animals were reared on a nutritionally adequate laboratory stock diet.

Whole-body X-irradiation

The rats were confined, four at a time, in a rectangular wooden box and exposed to X-rays from a Siemens Stabilipan unit operated at 250 kV and 15 mA, using a 2 mm Al filter. A dose rate of 100 rad/min was obtained at a distance of 63.5 cm from the source where the animals were placed and the total dose delivered to each rat was 800 rad. Control rats were sham irradiated.

Isolation of mitochondria

Control and experimental animals were sacrificed simultaneously by stunning and decapitation, their livers quickly removed and suspended in ice-cold sucrose. Mitochondria were isolated by differential centrifugation as described by Dingle[12].

Oxidative phosphorylation studies

Phosphorylation coupled to electron transfer during the oxidation of glutamate and succinate was determined according to the method of Yost et al.[7], with minor modifications.

The terminal site of phosphorylation with ascorbate as substrate was measured using a slight modification[7] of the method of Lehninger *et al.*[13].

Phosphorylation, at the first site alone, was determined using glutamate as substrate, together with (i) antimycin A (0.20 μg/mg mitochondrial protein) and NaCN (0.25 μg/mg mitochondrial protein) to inhibit electron transport corresponding to the other two sites of coupled phosphorylation, and (ii) phenazine methosulfate (0.1 mM) to carry electrons from the flavoprotein region of the chain to molecular oxygen (*cf.* ref. 14). Details of the composition of the reaction mixture are given in the legend to Table I. In all cases, measurements of O_2 consumption and phosphorus esterification[15] were made in a Warburg respirometer during a 20-min incubation period.

Preparation of ammonia particles

Submitochondrial electron transport particles devoid of significant phosphorylating capacity, *viz.* the ammonia particles, were prepared by the method of Fessenden and Racker[16] as described for beef heart mitochondria. Ammonia particles corresponding to approx. 200 mg protein were obtained from 200 g rat liver. The capacity of these particles for substrate oxidation was assessed by measuring O_2 uptake with various substrates in a Warburg respirometer at 25 °C. Although the particles exhibited good respiratory activity (e.g. 0.48 μatom O_2 consumed per min/mg protein, with glutamate), they were incapable of coupled phosphorylation in the absence of the coupling factors; P/O ratios of 0.02–0.04 were obtained with glutamate.

Isolation and purification of coupling factors

Coupling factor I (mitochondrial ATPase; F_1) from rat liver was prepared essentially by the method detailed by Racker and co-workers[17,18] for beef heart mitochondria, excepting that disruption was achieved by sonication (20 kcycles, 2 min) instead of by use of a Nossal shaker. The temperature fractionation step (Step 4), as described by these authors was also omitted, as this resulted in a slight inactivation, the extent being different in the case of control and irradiated samples. Starting with about 400 g of rat liver, F_1 was obtained in a yield of about 75 mg protein from control and 50 mg protein from irradiated rats. In addition to its capacity to specifically restore oxidative phosphorylation in ammonia particles in the presence of an excess of the other coupling factors, F_1 was also identified by its ability to catalyse the hydrolysis of ATP.

Coupling factors F_2, F_3 and F_4 were isolated by procedures described by Fessenden and Racker[16], Racker[19] and Conover *et al.*[20]. The yields of F_2, F_3 and F_4 were approx. 45, 90 and 80 mg, on a protein basis, from 200 g of liver from both control and irradiated rats.

The ammonia particles showed a specific requirement for each of the coupling factors (with the exception that F_3 and F_4 were interchangeable) and the full complement of factors was needed for obtaining maximal P/O ratios.

Reconstitution of the phosphorylating electron transport assembly

Ammonia particles, obtained from livers of unirradiated rats, were reconstituted with the coupling factors in the usual manner[16] and P/O ratios were measured in a Warburg respirometer using succinate as substrate. Details of the composition of the assay system are described in the legend to Table III.

Preparation of phosphorylating submitochondrial particles

Liver mitochondria, obtained by differential centrifugation[12], were suspended in 0.08 M sucrose and sonicated (20 kcycles) for 2 min. The sonicate was centrifuged at 12 000 × *g* for 10 min and the pellet was discarded. The supernatant contained the phosphorylating species of submitochondrial particles and was used as such.

Reduction of NAD^+ through reversal of electron transport

The reduction of NAD^+ by reversal of electron transport[21] was energised either by high-energy intermediates formed by oxidation of substrates in a portion of the respiratory chain or by exogenous ATP. The system contained Tris–HCl buffer (pH 8.0), $MgCl_2$, sucrose, succinate, sonicated submitochondrial phosphorylating particles and antimycin A in concentrations as given in the legend to Fig. 1.

Where the energy was derived by substrate oxidation, ascorbate and TMPD were also present. The reduction was initiated by the addition of NAD^+ and was followed at 25 °C by measuring the increase in absorbance at 340 nm in a Perkin–Elmer double-beam spectrophotometer. Studies were carried out both in the absence and in the presence of oligomycin.

Where exogenous ATP served as the source of energy, NaCN was included in the system, whereas ascorbate, TMPD and antimycin A were omitted. The reduction of NAD^+ through energy generated by substrate oxidation was found to be inhibited by 2,4-dinitrophenol whereas oligomycin showed similar inhibition when the reaction was driven by ATP.

Protein estimation

The protein content of mitochondria, the submitochondrial particles and the coupling factors was determined by the method of Lowry *et al.*[22].

RESULTS

In the first series of experiments, the ability of liver mitochondria from both control and irradiated (800 rad; 72 h post-irradiation) rats to oxidise glutamate, succinate and ascorbate, as well as their capacity for coupled phosphorylation have been compared. By the use of appropriate oxidisable substrates, either alone or together with specific inhibitors of respiration and artificial electron acceptors, it was possible in these studies to make a gross assessment of the damage sustained individually by each of the three sites. Mitochondria from irradiated rats did not show any differences in their ability to oxidise the various substrates.

From the data, summarised in Table I, it may be seen that whereas the overall inhibition of phosphorylation coupled at all three sites is 50 %, the second and third sites together show a 63 % decrease; no phosphorylation was observable at the terminal step utilizing ascorbate as substrate. With glutamate being oxidised in the presence of antimycin A and NaCN, *via* phenazine methosulfate, utilizing only the first site of phosphorylation, the lowering in P/O ratio caused by whole-body irradiation was only 20 %.

The difference between the P/O ratios with ascorbate and succinate may be taken to represent, specifically, the ratio at the second site. The values of P/O ratio at the second site so computed for control and irradiated rats are 1.07 (1.94–0.87)

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TABLE I

INHIBITION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION DUE TO WHOLE-BODY IRRADIATION

Rats were exposed to 800 rad X-rays at a dose-rate of 100 rad/min and sacrificed 72 h later. With succinate or glutamate as substrate, each flask contained sucrose (250 μmoles), phosphate buffer (pH 7.4) (40 μmoles), cytochrome *c* (0.09 μmole), ATP (6 μmoles), $MgSO_4$ (15 μmoles), KF (70 μmoles) and mitochondria corresponding to 7 mg protein in the main compartment and hexokinase (10 mg), glucose (75 μmoles) and either succinate or glutamate (20 μmoles) in the side arm. The total volume of the reaction mixture was 3.4 ml and the incubation was carried out at 25 °C for 20 min. For experiments with ascorbate, each flask contained mitochondria corresponding to 5 mg protein in sucrose (38 μmoles), phosphate buffer (pH 7.4) (20 μmoles), $MgCl_2$ (10 μmoles), EDTA (2 μmoles), ATP (5 μmoles), KF (26 μmoles) and cytochrome *c* (0.03 μmole) in the main compartment and ascorbate (100 μmoles) in the side arm. The total volume of the reaction mixture was 2.0 ml and the incubations were carried out at 25° C for 20 min. For studies on phosphorylation at Site 1, the reaction mixture was the same as used for glutamate oxidation but contained in addition, antimycin A (1.5 μg), NaCN (1.75 μg) and phenazine methosulfate (0.34 μmole). The incubations were terminated by addition of trichloroacetic acid and esterified phosphorus determined by difference between initial and final amounts of P_i present in the medium. The values represent averages based on four independent determinations ± S.E.

Substrate	*Other additions*	*Phosphorylating site(s)*	*Control* (μatoms/min per mg protein × 10^2)			*Irradiated* (μatoms/min per mg protein × 10^2)			*Inhibition* (%)
			O_2 uptake	*P_i esterified*	*P/O ratio*	*O_2 uptake*	*P_i esterified*	*P/O ratio*	
Glutamate	—	1, 2 and 3	3.10 ± 0.07	9.50 ± 0.43	3.10 ± 0.08	2.96 ± 0.18	4.49 ± 0.33	1.55 ± 0.12	50
Succinate	—	2 and 3	5.26 ± 0.24	9.99 ± 0.78	1.94 ± 0.07	5.68 ± 0.23	3.98 ± 0.35	0.71 ± 0.04	63
Ascorbate	—	3	9.00 ± 0.51	7.83 ± 0.86	0.87 ± 0.09	8.86 ± 0.33	0	0	100
Glutamate	Antimycin A + NaCN + phenazine methosulfate	1	2.95 ± 0.07	2.94 ± 0.11	0.98 ± 0.02	3.23 ± 0.19	2.58 ± 0.15	0.80 ± <0.01	20

and 0.71 (0.71–0.00), respectively. It may be roughly assessed from these data that the second site of phosphorylation is inactivated to the extent of about 30 % in the irradiated rats.

In a study of substrate oxidation and coupled phosphorylation pertaining to the third site at different periods, it was observed that while the oxidation of ascorbate was not significantly influenced, the extent of inhibition of the P/O ratio increased progressively with time (Table II). A significant lowering of about 30 % in the P/O is observable as early as 4 h following irradiation, whereas the ability for coupled phosphorylation appears to be totally lost by 72 h.

TABLE II

INHIBITION OF OXIDATIVE PHOSPHORYLATION AT TERMINAL SITE: TIME STUDY

Rats were exposed to 800 rad X-rays at a dose-rate of 100 rad/min and sacrificed at different periods as indicated. The composition of the reaction mixture for studying phosphorylation coupled to ascorbate oxidation is detailed in the legend to Table I. The values represent averages based on four independent determinations ± S.E.

Post-irradiation period (*h*)	*μatoms/min per mg protein* × 10^2		*P/O ratio*	*Inhibition* (%)
	O_2 *uptake*	P_i *esterified*		
0	9.00 ± 0.51	7.83 ± 0.86	0.87 ± 0.09	0
4	9.35 ± 0.71	5.49 ± 0.30	0.59 ± 0.02	32
24	9.25 ± 1.03	2.63 ± 0.44	0.28 ± 0.02	67
48	7.05 ± 0.21	1.90 ± 0.07	0.27 ± 0.01	69
72	8.86 ± 0.33	0	0	100

To elucidate further the nature of radiation-induced impairment of oxidative phosphorylation, especially of the third site, the reduction of NAD^+ by reversed electron flow, with succinate as electron donor and with the oxidation of ascorbate furnishing the needed energy has been investigated.

In the absence of oligomycin (Fig. 1a), the kinetics of NAD^+ reduction, in both control and irradiated, showed three distinct phases—a rapid reduction during the first minute, a considerably slower rate thereafter and no further reduction after some time. During the first minute, the control phosphorylating submitochondrial particles form NADH at almost twice the rate as the irradiated ones, whereas the two show identical rates thereafter. There is no further reduction in control beyond 10 min, whereas it is still observable up to 20 min in the irradiated; the total amounts of NADH formed, however, are similar.

The inclusion of oligomycin in the system almost totally abolishes the initial rapid rate of NAD^+ reduction in both control and irradiated particles (Fig. 1b). The subsequent steady-state rate is, however, not influenced by the presence of oligomycin. There are no significant differences in the rates of NAD^+ reduction by the phosphorylating particles from control or irradiated rats, in the presence of oligomycin. In both cases, a linear rate of reduction is sustained for up to 25 min.

Studies have also been carried out on the kinetics of NAD^+ reduction energised by exogenous ATP. As compared to the earlier experiments, in which the oxidation of ascorbate provided the energy, the reduction of NAD^+ in presence of added ATP is rapid; the control and the irradiated particles, however, exhibit significant differ-

ences (Fig. 2). During an incubation period of 4 min, the phosphorylating particles show greater NAD^+ reduction in the ATP system, than during (i) 10 min in the absence of oligomycin (Fig. 1a) or (ii) 25 min in the presence of oligomycin (Fig. 1b), when energy to drive the reaction was derived from oxidation of ascorbate.

The inhibition of mitochondrial oxidative phosphorylation, due to whole-body irradiation, has also been investigated in terms of effects on the catalytic

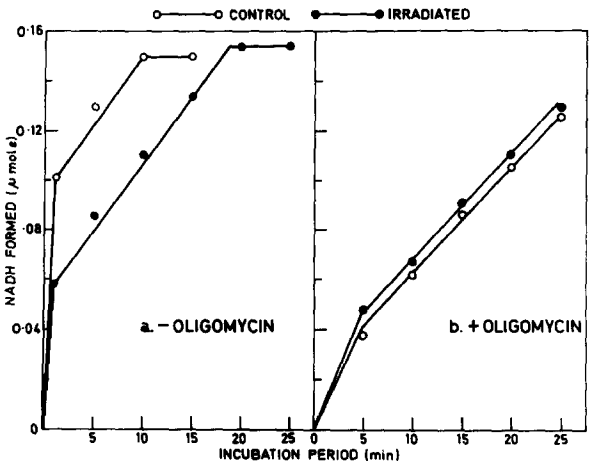

Fig. 1. Effect of irradiation on NAD^+ reduction energised by oxidation of ascorbate, in (a) absence of oligomycin, and (b) presence of oligomycin. Sonicated submitochondrial phosphorylating particles (4.5 mg) were incubated with Tris–HCl buffer (pH 8.0) (165 μmoles), $MgCl_2$ (18 μmoles), sucrose (750 μmoles), succinate (21 μmoles), antimycin A (1.8 μg), ascorbate (120 μmoles) and TMPD (0.96 μmole) at 25 °C. Reaction was initiated by addition of NAD^+ (3 μmoles) and increase in absorbance at 340 nm was measured in a Perkin–Elmer double-beam spectrophotometer. In experiments using oligomycin, it was added at 0.2 μg/mg particle protein. The total volume was 3 ml. Reduction of NAD^+ is shown in terms of μmoles of reduced NAD^+ formed per 5 mg of particle protein.

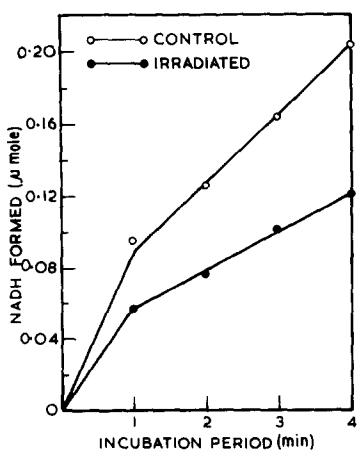

Fig. 2. Effect of irradiation on NAD^+ reduction energised by exogenous ATP. Sonicated submitochondrial phosphorylating particles were incubated in a system as described in Fig. 1 with the exception that ascorbate, TMPD and antimycin A were omitted, whereas ATP (18 μmoles) and NaCN (1.62 μg) were included. Reduction of NAD^+ is shown in terms of μmoles of reduced NAD^+ formed per 5 mg of particle protein.

activities of the different coupling factors in a reconstituted phosphorylating system (Table III). The "ammonia particles" from control rat livers, on supplementation with coupling factors F_1, F_2 and F_3, also derived from control animals, yield an average P/O ratio of 0.63, with succinate as substrate. When coupling factor F_1 from control rats is replaced by F_1 from irradiated rats, the P/O ratio falls markedly by over 75 %; when F_1 from irradiated rats was added in addition to, and not as a replacement for, F_1 from control animals, the P/O ratio remains unaltered. Similar experiments, in which one coupling factor at a time was derived from the irradiated animals, show that whole-body irradiation causes a far less loss of activity in any of these as compared to the effect on F_1. It is also seen that similar P/O ratios are obtained when the "ammonia particles" are supplemented with F_1, F_2 and either one of F_3 or F_4.

TABLE III

EFFECT OF WHOLE-BODY IRRADIATION ON MITOCHONDRIAL COUPLING FACTORS

Rats were exposed to 800 rad X-rays at a dose-rate of 100 rad/min and sacrificed 72 h later. Ammonia particles from livers of control rats were reconstituted with the coupling factors derived from control and irradiated animals, in various combinations as indicated, and P/O ratios determined with succinate as substrate. Each flask contained in a total volume of 1.2 ml: (a) Main compartment: (i) 0.4 ml of a solution made up of glucose (56 μmoles), sucrose (72 μmoles), $MgSO_4$ (20 μmoles), ATP (3 μmoles), ADP (2 μmoles), hexokinase (54 units; 3 mg) and bovine serum albumin (2 mg); (ii) 0.2 ml of a solution of F_1 (3 mg); (iii) 0.1 ml of F_2 (0.5 mg); (iv) 0.2 ml of F_3 (3 mg) or 0.2 ml of F_4 (4 mg) and (v) 0.2 ml ammonia particles (4 mg). Since F_2 was in 0.3 M potassium phosphate buffer (pH 7.5), no additional buffer was added to the incubation medium. (b) Side arm: 0.1 ml of sodium succinate (50 μmoles). (c) Central well: 0.2 ml of KOH (10 %). O_2 uptake and phosphorus esterification were measured during an incubation period of 30 min at 25 °C. The values represent averages based on four determinations using pooled preparations of the ammonia particles and the various coupling factors ± S.E.

Coupling factors derived from		*P/O ratio*	*Inhibition* (%)
Control	*Irradiated*		
F_1, F_2, F_3	—	0.63 ± 0.02	0
F_2, F_3	F_1	0.15 ± 0.01	76
F_1, F_2, F_3	F_1	0.63 ± <0.01	0
F_1, F_3	F_2	0.48 ± <0.01	24
F_1, F_2	F_3	0.45 ± 0.01	29
F_1, F_2, F_4	—	0.65 ± 0.02	0
F_1, F_2	F_4	0.55 ± 0.01	15

DISCUSSION

In the present investigations, a significant impairment of oxidative phosphorylation has been observed in rat liver mitochondria 72 h following whole-body exposure of the animals to 800 rad of X-rays. Decreased phosphorylation due to irradiation has been reported by other investigators as well and a comparison of the data reveals similarities as well as differences. While Thomson *et al.*[9] and Scaife and Hill[3] have not been able to obtain any effect on P/O ratio of liver mitochondria 4 h after exposure to 1000 rad, significant uncoupling using a variety of substrates that utilise either 2 or 3 sites of phosphorylation has been reported by Hall *et al.*[4] to occur as early as 3 h after irradiation. Pronounced decrease in phosphorylating efficiency at the terminal site has also been noted in our investigations at such an early period. However,

whereas Hall *et al.*[4] have observed a return to normality between 24 and 72 h, our data show the deleterious effect to be progressive. A similar trend, as observed by us, has been reported in regenerating liver of irradiated rats[5].

An important finding in the present studies relates to the extreme sensitivity of the terminal phosphorylating site of the electron transfer chain as compared to the other two. By the use of suitable oxidisable substrates that transfer electrons either through the entire chain or from the flavoprotein stage, Hall *et al.*[4] have concluded that there are no differences in the extent of inactivation of the first two sites in liver mitochondria due to irradiation of the rat; similar observations have also been made with thymus mitochondria[3]. Yost *et al.*[7] have reported a greater sensitivity of the third site of phosphorylation, as denoted by experiments using ascorbate. However, an analysis of their data reveals that the difference in the extent of inhibition between the second and third sites is not as pronounced as seen in our studies. In this context, it is interesting to note that the inhibition of oxidative phosphorylation observed following *in vitro* irradiation of liver mitochondria[10,23] is reported to be more in the first site[10]. The several-fold difference in the doses required to elicit the response, *in vivo* and *in vitro*, also serves to emphasise the possible differences in the modes of action of ionising radiation in the two cases.

In an attempt to locate the site of radiation interaction, in terms of the partial reactions involved, recourse has been taken to studies on NAD^+ reduction by reversal of electron flow mediated by an energy source. It is now well established that this reaction can be driven not only by ATP but also by the high-energy intermediates, which have been implicated in its synthesis (*cf.* ref. 24). With energy originating from ascorbate–TMPD oxidation, it was generally observed that the kinetics of NAD^+ reduction was rather slow. Such a low rate has also been reported by other investigators[25–27] in washed submitochondrial particles and is believed to be due to the lack of a soluble stimulatory factor of either mitochondrial origin[25] or from the supernatant[26,27].

In the absence of oligomycin, the initial rate of formation of NADH is very high and is followed by a slower reaction persisting for several minutes. A likely explanation for the early spurt in both control and irradiated particles is that this represents the reduction that is energised by the endogenous ATP present in the preparations; the irradiated particles presumably having a lower amount of ATP show a somewhat lesser initial spurt as compared to the control particles. While the presence of endogenous ATP or of Mg^{2+} in these submitochondrial particles has not been established in the present studies, it has been ascertained that the initial rapid increase in absorption does not represent an artifact arising from turbidity changes. The subsequent slower rate of NAD^+ reduction very likely reflects the rate at which high-energy intermediates are being generated by the oxidation of ascorbate. It may further be inferred from the equal rates of NAD^+ reduction obtaining beyond the first minute in the submitochondrial particles from livers of control and irradiated rats that the formation of high-energy intermediate(s) proceeds normally in the latter.

The absence of the initial rapid rate of reduction, when oligomycin is included in the system, affords additional support to the inference of ATP involvement in this effect. The control and irradiated samples show almost identical activities in the presence of oligomycin; however, the amounts of NAD^+ reduced during 20–25 min are significantly lower in both control and irradiated particles as compared to the

amounts formed in the absence of oligomycin. In the presence of oligomycin, the high-energy intermediate(s) alone can serve as the energy source and the finding that the rate of reduction of NAD^+ in the mitochondrial particles from irradiated rats is unaffected provides conclusive evidence of radiation not interfering with this intermediary step in the utilisation of oxidative energy for ATP synthesis.

When the reduction of NAD^+ was carried out in the absence of oxidative energy, but with exogenous ATP, a significant difference is observed between the control and irradiated particles. With ATP as energy source, the formation of NADH is rapid, the rate being similar to that observed during the first minute in the experiments with ascorbate as oxidisable substrate, in the absence of oligomycin. The significantly decreased rate of reduction of NAD^+ by the irradiated particles in the presence of ATP can be taken as an indication of an interference with the transphosphorylation reaction.

Studies on the reconstitutability of a phosphorylating system, by the addition of coupling factors to ammonia particles, also point to a loss of function involved in the final step of ATP synthesis in liver mitochondria of irradiated animals. Thus, coupling factor F_1, which has been visualised to play a role in the transphosphorylation reaction resulting in ATP formation[18], shows a marked fall in activity due to irradiation. It may be pointed out that in addition to this decreased catalytic activity of F_1, the livers from irradiated rats also yielded a smaller amount of this coupling factor. The possibility of an inhibitory effect of F_1 from irradiated animals on the reconstituted system is ruled out by the experiment in which no deleterious effect was observed when F_1 from both control and irradiated rats was added together with the other complements to the ammonia particles.

The major point of interest arising from these studies relates to the nature of radiation-induced impairment of oxidative phosphorylation. On the basis of our current concept of the partial reactions involved in oxidative phosphorylation coupled to electron transport[28,29], it is difficult to reconcile the two salient findings of the present studies. These relate to: (i) whole-body irradiation exerts a site-specific inhibition, where the terminal phosphorylating assembly associated with the cytochrome *c* region is far more sensitive than the other two; (ii) irradiation does not interfere with the generation of high-energy intermediates as a consequence of substrate oxidation but has its locus of action at the transphosphorylation reaction involving coupling factor F_1.

Among the chemicals that have been shown to exert site-specific uncoupling and/or inhibition of oxidative phosphorylation are 2,4-dinitrophenol[30] and the guanidine derivatives[31]. These agents have been shown to act at a step prior to the formation of a common pathway for the synthesis of ATP from the three separate energy conservation sites of the electron transfer chain. The specificity of these agents has, thus, been related to their capacity for specific interaction with primary or secondary high-energy compounds formed at the different sites (*cf.* ref. 11). On the other hand, oligomycin which acts on the transphosphorylase system, inhibits equally well phosphorylation coupled to all the three sites.

The present findings may be considered in the light of one of the following possibilities. Contrary to the generally accepted concept, the pathways of energy transfer from the three coupling sites to the final formation of ATP need to be represented as being independent of each other. This may arise either due to the

topography of the various components of respiratory chain in the mitochondrial membrane or due to differences in the specificity of interaction of transphosphorylase with the different high-energy phosphates (at the present time, designated as a single entity) arising from the three energy-generating sites. These suggestions, although hypothetical, are not altogether without supporting evidence from the work of other investigators. The spatial distribution of the different carriers of the electron transport chain in definite positions on the mitochondrial membrane has been suggested by Racker[32], and from their studies on the ease of accessibility of arsenate to the three phosphorylating sites, Azzone and Ernster[33] have indicated that the first phosphorylating site may be more superficially situated in the mitochondria than the other two. It has been shown by Beyer[34] that ATP synthetase II, which restores phosphorylation coupled to the oxidation of both NADH and succinate in submitochondrial particles with low phosphorylating ability, has little or no effect on phosphorylation during oxidation of cytochrome *c* reduced by ascorbate. It is not unlikely that these findings may have a bearing on the site-specific inhibition of mitochondrial oxidative phosphorylation following whole-body irradiation.

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