SECTION 5

PROTEIN SYNTHESIS IN LIVER MITOCHONDRIA OF IRRADIATED RAT

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INTRODUCTION

The observations reported in the earlier sections have provided evidence for a marked impairment of liver mitochondrial oxidative phosphorylation following whole-body x-irradiation of the rat. It is conceivable that this deleterious effect may also be reflected in derangement of a multitude of cellular functions that are directly dependent on metabolic energy. Among the important anabolic processes in the cell is the synthesis of proteins and, in particular, the enzymes, since the latter are responsible for the regulation of all metabolic activities of the cell. It is not surprising, therefore, that many of the physiological and metabolic disturbances of radiation injury are traceable to specific influences on key enzymes.

While the effects of whole-body irradiation on the nuclear-ribosomal protein synthetic machinery in liver have been investigated in considerable detail to elucidate the underlying mechanisms (1 - 4), there is a comparative dearth of information on the response of the liver mitochondrial protein synthesising system to radioexposure (5 - 7). The presence of its own characteristic DNA, the various RNA species, ribosomes and other components of transcription and translation has conferred on the mitochondria a considerable degree of autonomy as evidenced by the capacity of this organelle for <u>in vitro</u> protein synthesis (cf 8, 9). The mitochondrial system for protein synthesis has been shown to possess properties distinct from those of the nuclear-ribosomal system, and the differences in their sensitivities to certain inhibitors of protein synthesis are particularly marked (10 - 13).

Investigations of the response of liver mitochondrial protein synthesis in the rat to whole body radioexposure are therefore of interest.

This section comprises studies on the following aspects of mitochondrial protein biosynthesis in irradiated rats:

(i) the effect of whole body irradiation on the protein content of hepatic mitochondria;

(ii) the *in vivo* and *in vitro* incorporation of radioactively labelled leucine into liver mitochondria of irradiated rat;

(iii) alterations in free leucine pool of liver cell sap and of liver mitochondria due to whole body irradiation; and

(iv) fractionation of liver mitochondrial proteins, labeled *in vivo* to study the incorporation of radioactivity into proteins established to be of exclusive mitochondrial origin.

In addition, the activity of liver lysosomal enzymes has been monitored, to assess their significance, if any, in the disruption of mitochondrial structure and function following radioexposure.

MATERIALS AND METHODS

Chemicals:

The various fine chemicals including the biochemicals and other inorganic chemicals and solvents were obtained from sources as indicated in the earlier sections. In addition to

these, heparin, ATP (Na salt), ribonucleic acid, haemoglobin, sodium succinate, tris (hydroxymethyl) aminomethane, various amino acids, sodium cholate and sodium deoxycholate as well as the fluors for liquid scintillation counting were purchased from the Sigma Chemical Co., St. Louis, U.S.A. Leucine-1-C^{14} was a product of the Isotope Division of this Research Centre. Uranyl acetate, urea and sodium β -glycerophosphate were obtained from British Drug House, Laboratory Chemical Division of Glaxo Laboratories, India. Ninhydrin (Indanetrione hydrate) was obtained from the British Drug House Ltd., Poole, England.

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:

Whole-body x-irradiation of rats was carried out as described in earlier sections.

Isolation of liver mitochondria:

Mitochondria from rat liver, were isolated as described in earlier sections.

Preparation of liver slices:

The livers were removed quickly after sacrificing the

rats and placed in ice-cold 0.1 M Krebs-Ringer phosphate buffer (pH 7.4). They were then cut into thin uniform slices of less than 0.5 mm thickness with the help of a Stadie-Riggs tissue slicer.

Determination of protein content:

The protein content of whole liver homogenate and of mitochondria were determined by the method of Lowry et al.(14).

Label incorporation studies in vivo:

The rats were intraperitoneally administered 20/uCi/100 g body wt. of DL-leucine-1-C^{14} (sp.act. 47.9 mCi/mmole) and sacrificed 1 h later. The liver was homogenised in cold (4°C) 0.25 M sucrose containing 0.05 M Tris (pH 7.4), 0.025 M KCl and 0.01 M $MgCl_2$ to a ten per cent suspension. Mitochondria were isolated as described earlier, washed with 0.25 M sucrose containing 50 mM L-leucine and suspended in 0.25 M sucrose. Aliquots of the homogenate and mitochondrial suspension (0.5 ml each) were taken in centrifuge tubes and the protein precipitated by the addition of 10% TCA. The precipitate was washed first with 5% TCA at 90°C, then twice with cold 95% ethanol and finally twice with absolute ether. The precipitate was dissolved in formic acid and aliquots were spotted on filter paper strips (Whatmann no 3, 7 cm x 1.8 cm). The strips were dried with the help of a hair-dryer, rolled and transferred to counting vials. The vials were filled with 10 ml toluene containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis(4-methyl-5-

phenyloxazole-2-yl) benzene as fluors and the radioactivity was measured in a Beckman LS-100 liquid scintillation spectrometer.

The uptake of ^{14}C-leucine *in vivo* by the free amino acid pool has been studied 20 min after administration of the label. The free amino acid extracts (TCA soluble fraction) of liver homogenate or of mitochondrial sonicate were washed repeatedly with diethyl ether and the radioactivity in 0.5 ml aliquots determined after spotting on filter paper strips as above.

Label incorporation studies with isolated mitochondria:

The incorporation of 1-^{14}C-leucine by isolated mitochondria was studied according to the method of Loeb and Hubly (15) with slight modifications; cycloheximide was not included in the incubation medium, as it has been observed recently that this inhibitor also affects mitochondrial protein synthesis (16). The incubation medium contained the following components in a total volume of 10 ml: 10 mM potassium phosphate; (pH 7.4); 70 mM Tris HCl (pH 7.4); 50 mM KCl; 11 mM $MgCl_2$; 11 mM potassium succinate; 110 mM sucrose; 2 µM leucine; and the other 19 amino acids at a concentration of 20 µM each; leucine-1-C^{14} (0.3 µc/ml) and 1 mM ATP. The incubation flasks were equilibrated at 37°C in air for 5 min in a shaker water bath. Mitochondria corresponding to 40 mg of protein and 20 µg RNAse were added to each flask and incubation carried out for 45 min. After incubation, aliquots were transferred to chilled centrifuge tubes containing 2 ml of 10% trichloroacetic acid.

The labeling of the free amino acid pool of mitochondria, following in vitro incubation with 1-^{14}C-leucine, was also determined. The incubation was carried out for only 10 min after which the mitochondria were separated and washed as before. The mitochondrial pellet was suspended in 2.0 ml 0.08 M sucrose, sonicated and treated with 5 ml of 10% trichloroacetic acid. After removal of the precipitated protein, the supernatant was washed 8 times with diethyl ether and the radioactivity was determined in 0.5 ml aliquot.

Leucine content in the free amino acid pool:

Livers from control and experimental (24 and 48 h post-irradiation) rats were made into 10% homogenate in isotonic sucrose, the mitochondria isolated and sonicated as described earlier. To 2 ml of either 10% homogenate or the sonicated mitochondria (1 ml sonicate ≡ 1 g liver), 5 ml of 10% trichloroacetic acid was added. The precipitate was centrifuged, the supernatant was washed 8 times with diethyl ether and the leucine was separated by thin layer chromatography on silica gel G, using n-propanol and water in the ratio of 7:3 (17). The amino acids were visualised by spraying with the reagent (18) containing 0.3 g ninhydrin, 97 ml of n-butanol and 3 ml of glacial acetic acid and heating to 60^{o}C for about 30 minutes. The spot corresponding to leucine was identified by comparison with standard and after elution with 0.01 N HCl, the amino acid was estimated colorimetrically (19). One ml of the eluent was mixed with 0.5 ml of cyanide-acetate buffer (pH 5.4)

and 0.5 ml of 3% ninhydrin in ethylene glycol monomethyl ether and placed in a water bath at 100°C for 15 minutes. Immediately after removing from the water bath, 5 ml of isopropyl alcohol-H_2O (1:1) diluent was added. The mixture was shaken vigorously, allowed to cool at room temperature and the colour was read at 540 nm.

Label incorporation with liver slices:

Liver slices (750 mg) were incubated in a total volume of 7.5 ml of 0.1 M phosphate buffer (pH 7.4) containing DL-leucine-1-C^{14}, 4 μCi, (sp.act. 47.9 mCi/mmole) at 37°C in conical flasks open to air in a shaker water bath for 30 minutes. Following incubation, the supernatant was decanted off and the liver slices were homogenized in 7.5 ml of 0.25 M sucrose containing 50 mM L-leucine. The homogenate was centrifuged at 500xg and the supernatant was centrifuged again at 10000 x g. The pellet was washed 3 times in the same sucrose solution and was finally suspended and homogenised in 1.5 ml of 0.25 M sucrose. Aliquots of the mitochondrial suspension were transferred to centrifuge tubes, the protein precipitated and washed as above, dissolved in formic acid and the radioactivity incorporated was measured by liquid scintillation counting.

Fractionation of ^{14}C-leucine labeled mitochondrial proteins:

Liver mitochondria obtained from rats administered ^{14}C-leucine (page No.110- *in vivo* labeling studies) were washed and suspended

in 0.4 N KCl, frozen and thawed, and centrifuged at 12000 x g for 10 min. The resulting pellet was suspended in 0.4 N KCl, frozen and thawed and recentrifuged at 17000 x g for 10 min. The supernatants from the two centrifugations were combined, the protein precipitated from aliquots, washed, dissolved in formic acid and radioactivity measured.

The KCl-insoluble material was suspended in 0.25 M sucrose to a final concentration of 20 mg per ml and used for the isolation of structural protein, according to the original method of Criddle et al. (20), with modifications and washings procedures of Lenaz et al. (21) and Yang and Criddle (22). Deoxycholate and cholate were then added to the 0.4 N KCl-insoluble pellet at a concentration of 2 mg and 1 mg, respectively, per mg of mitochondrial protein. The clarified protein suspension thus obtained was kept in an ice bath for 10 min and then centrifuged at 35000 x g for 10 min. The soluble protein was treated with $(NH_4)_2SO_4$ to a final concentration of 15% and allowed to remain overnight in an ice bath. The structural protein was collected by centrifugation at 27000 x g for 10 min and then extracted three times with 90% acetone. After removal of the lipids with acetone, the protein was washed three times with 0.4% trichloroacetic acid in methanol. The extracted protein was washed further with methanol, dialyzed against distilled water and lyophilized. The lyophilized protein was extracted with 8 M urea (pH 5.5), and the insoluble protein, believed to be of exclusively mitochondrial origin (23), was separated by centrifugation. It was

washed, dissolved in formic acid and radioactivity incorporated into the fraction counted.

Activity of lysosomal enzymes:

The free and total activities of three typical lysosomal enzymes, viz., acid phosphatase, cathepsin and ribonuclease have been determined by standard procedures (24).

For assaying acid phosphatase activity, the incubation mixture contained in a total volume of 2.0 ml, 0.05 M sodium β-glycerophosphate, 0.05 M acetate buffer (pH 5.0), 0.005 M $MgSO_4$ and 0.4 ml of 10% homogenate (for total activity determination, 2 ml of 10% homogenate was treated with 0.2 ml of 2.5% triton X 100, and 0.4 ml of this mixture was used). After the incubation at 37°C for 15 min, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid and the inorganic phosphorous liberated was estimated according to the method of Fiske and Subba Row (25).

The assay mixture for measuring cathepsin activity contained in a total volume of 3 ml, 0.00026 M haemoglobin and 0.17 M acetate buffer pH 5.0, in addition to the enzyme (0.5 ml of 10% homogenate). After 15 min incubation, the reaction was stopped by adding 5 ml of ice cold 0.3 M trichloroacetic acid, cooled in ice immediately and filtered in the ice box. Aromatic degradation products in the filtrates were measured by the Folin-Ciocalteu reagent, with tyrosine as standard. The sample (0.5 to

1.0 ml) was made up to 2 ml with water and treated with 4 ml of 0.5 N NaOH and 1.2 ml of Folin phenol reagent (1:3 dilution). The colour was read at 660 nm.

Ribonuclease activity was measured at 37°C in a total volume of 2 ml containing 3 mg of purified ribonucleic acid and 0.1 M sodium acetate buffer (pH 5.0). The reaction was stopped by adding 2 ml of ice cold 10% (w/v) perchloric acid containing 0.25% (w/v) uranyl acetate. The mixture was centrifuged after standing for 1 hour in the cold and the extinction of the supernatant read at 260 nm in Beckman Model DU spectrophotometer.

RESULTS

Studies on mitochondrial protein content of liver reveal significant alterations following whole body exposure of rats to a lethal dose of 800 R of x-rays. From the data presented in Table 1, it is observed that there occurs an increase in the amount of hepatic mitochondrial protein per unit mass of the tissue, being maximum between 24 and 48 h following irradiation. The mitochondrial protein content shows a decline beyond this period although it still remains elevated above the unirradiated values even at 72 h post radio-exposure. However, at 168 h the mitochondrial protein content is considerably less than in the unirradiated animals.

Attempts have also been made to assess the effect of whole body irradiation on the rate of synthesis of liver mitochondrial proteins, in the whole animal, in liver slices and in

Table 1

EFFECT OF WHOLE-BODY IRRADIATION ON LIVER MITOCHONDRIAL PROTEIN CONTENT

Post-irradiation period (h)	Mitochondrial protein (mg/g liver)	Per cent of control
0	26.0 ± 1.8	100
16	30.1 ± 3.1	115
24	41.2 ± 3.3	158
48	41.1 ± 3.6	158
72	33.5 ± 1.6	127
168	16.2 ± 1.5	62

The rats were subjected to 800 R of whole-body x-irradiation and killed at various intervals thereafter.

The values represent averages of six independent determinations ± S.E.M.

isolated mitochondria. The experimental approach has involved studies on the incorporation of radioactivity into proteins from ^{14}C labeled leucine, with due consideration to the alteration if any, in the pool size and labeling of endogenous free leucine of liver cell sap and mitochondria.

Observations on the in vivo labeling of total liver protein and mitochondrial protein by intraperitoneally administered ^{14}C-leucine in control and irradiated rats are summarised in Table 2. The incorporation of ^{14}C-leucine into both total liver protein and mitochondrial protein shows a more than two-fold enhancement at 24 h following irradiation of the animal. The labeling of total liver protein continues to show the same degree of increase over the unirradiated animals even at 48 h and beyond this period it shows a decrease and is significantly less than normal at 144 h post-irradiation. The liver mitochondrial protein shows maximum labeling at 24 h and falls off progressively after this to less than 50 per cent of control values in rats killed at 144 h after radioexposure. The extent of increase in the labeling due to irradiation up to 72 h following the exposure of the rats, is greater in the case of total liver protein as compared to the mitochondrial protein. The maximum increase observed in the labeling of total liver protein is 236% of control at 48 h as compared to 183% of control in case of mitochondrial protein at 24 h post irradiation. The fall in the incorporation of ^{14}C-leucine into protein, at a late period, following irradiation, viz., 144 h, is less marked in the total liver protein than in the mitochondrial protein.

Table 2

IN VIVO INCORPORATION OF DL-LEUCINE-1-C^{14} INTO TOTAL PROTEIN AND MITOCHONDRIAL PROTEIN OF LIVER

Post-irradiation period (h)	Radioactivity incorporated into Total liver protein		Liver mitochondrial protein	
	dpm/mg protein	Per cent of control	dpm/mg protein	Per cent of control
0	1755 ± 111	100	2316 ± 165	100
24	3950 ± 179	225	4233 ± 372	183
48	4144 ± 53	236	3063 ± 31	132
72	2394 ± 83	136	2743 ± 257	118
144	1235 ± 57	70	1073 ± 98	46

The rats were exposed to 800 R of whole body x-irradiation. DL-leucine-1-C^{14} was administered intraperitoneally (20 μCi/100 g body wt.) 1 h, prior to killing the animals at different time intervals following irradiation. The values represent averages of four independent determinations ± S.E.M.

Data on the incorporation of ^{14}C-leucine into protein by isolated liver mitochondria are presented in Table 3. No significant change in the labeling is observable with liver mitochondria isolated from rats 24 h following whole body irradiation, whereas at 48 h and 72 h, it is considerably decreased.

The labeling of mitochondrial protein by ^{14}C-leucine has also been assessed in liver slices. From the results of these studies, summarised in Table 4, it would appear that whole body irradiation does not significantly influence such label incorporation, although it showed a very slight stimulatory trend at all the periods studied up to 72 h following irradiation of the rats.

The leucine content of the free amino acid pool of the liver cell sap and of mitochondria have been determined in unirradiated rats as well as in animals that had been 24 h and 48 h previously exposed to radiation. The labeling of the free leucine pool by the exogenous ^{14}C-leucine both in the whole animal and in isolated mitochondria has also been investigated and the data are contained in Table 5. There is a significant decrease in the size of the endogenous free leucine pool of both the liver cell sap and of mitochondria of the irradiated rats. On the other hand, there is a greater labeling of the free leucine pool by the exogenous ^{14}C-leucine in the rats analysed at 24 h post-irradiation; in the 48 h sample, there is a decreased labeling. The specific activities of the free leucine pool, calculated from the foregoing

Table 3

INCORPORATION OF DL-LEUCINE-1-C^{14} BY ISOLATED LIVER MITOCHONDRIA

Post-irradiation period (h)	Radioactivity incorporated dpm/mg protein	Per cent of control
0	616 ± 46	100
24	690 ± 93	112
48	385 ± 11	62
72	514 ± 15	83

The rats were subjected to 800 R of whole body x-irradiation and killed at various post-irradiation periods. Liver mitochondria (± 40 mg protein) were incubated in a medium containing DL-leucine-1-C^{14}, (3 µCi) in a total volume of 10 ml for 45 min at 37°C.

The values represent averages of four independent determinations ± S.E.M.

Table 4

INCORPORATION OF LEUCINE-1-C^{14} INTO MITOCHONDRIAL PROTEIN BY RAT LIVER SLICES

Post-irradiation period (h)	Radioactivity incorporated dpm/mg protein	Per cent of control
0	1700 ± 100	100
24	2015 ± 50	119
48	2150 ± 150	126
72	1870 ± 121	110

Rats were subjected to 800 R of whole body x-irradiation and sacrificed at various post-irradiation periods. Liver slices (750 mg) were incubated in a medium containing DL-leucine-1-C^{14}, 4 µCi (sp.act. 47.9 mCi/m.mole) in a total volume of 7.5 ml at 37°C for 30 min and the radioactivity incorporated in the mitochondria determined.

The values represent averages of four independent determinations ± S.E.M.

Table 5

FREE LEUCINE CONTENT OF WHOLE LIVER AND LIVER MITOCHONDRIA AND ITS LABELING

		Leucine in free amino acid pool		Radioactivity in amino acid pool		Sp. activity of the free leucine pool	
		Whole liver	Liver mitochondria	Whole liver	Liver mitochondria	Whole liver	Liver mitochondria
		(µg/mg protein N)		(dpm/mg protein N)		(dpm/µg leucine)	
Control		65 ± 5	123 ± 9	2445 ± 25	293 ± 23	37.6	2.4
Irradiated	24 h	46 ± 3	74 ± 5	3365 ± 122	356 ± 19	73.0	4.8
	48 h		69 ± 6	-	204 ± 11	-	2.9
Per cent control	24 h	69	60	137	122	194	200
	48 h	-	56	-	70	-	121

Rats were subjected to 800 R of whole body x-irradiation and killed at 24 and 48 h post-irradiation. Free leucine was estimated in the total liver cell sap as well as in the liver mitochondria. Radioactivities in free leucine pool of control and irradiated (24 h) rats were determined 20 min following administration of leucine (20 µCi/100 g body wt.). For the labeling of free leucine pool *in vitro*, mitochondria isolated from liver of control and irradiated rat (48 h) were incubated for 10 min as described under Table 3.

The values represent averages of four independent determinations ± S.E.M.

data, show an almost two-fold increase in the values, both for whole liver and liver mitochondria at 24 h following whole body irradiation. The specific activity of the free leucine pool of liver mitochondria from the rats examined 48 h after radioexposure also showsan increase over control, although it is considerably less than that observed at 24 h post-irradiation.

In another study, the liver mitochondrial proteins have been grossly fractionated on the basis of their solubilities in 0.4 M KCl and 8 M urea, into fractions believed to be (a) exclusively synthesised by mitochondria and (b) those that are predominantly of cytoplasmic origin. The findings of these investigations are summarised in Table 6. The labeling of total mitochondrial proteins is elevated about 40% in rats 48 h following whole body irradiation. The fractionated mitochondrial proteins, however, show divergent trends; the structural proteins insoluble in 8 M urea and which have been shown to be exclusively synthesised by the mitochondria show decreased labeling, whereas the proteins soluble in 0.4 M KCl and which are mostly of cytoplasmic origin incorporated almost 50% more radioactivity in the irradiated rats.

In view of the considerable structural damage suffered by the mitochondria, the influence of irradiation on the activity of three typical lysosomal enzymes has also been monitored at various periods subsequent to exposure of the animals to radiation. It may be observed from the results presented in Table 7 that there are no significant alterations in the rate of free:bound

Table 6

FRACTIONATION OF LIVER MITOCHONDRIAL PROTEINS FOLLOWING THEIR IN VIVO LABELING

Mitochondrial protein fraction	Radioactivity incorporated (dpm/mg protein) Control	Irradiated	Per cent of Control
Total mitochondrial protein (cytoplasmic and mitochondrial origin)	2636 ± 25	3662 ± 190	138
Structural protein insoluble in 8 M urea (exclusive mitochondrial product)	340 ± 9	276 ± 22	81
0.4 M KCl soluble protein (mostly synthesised in cytoplasm)	3368 ± 119	4990 ± 147	148

Rats were subjected to 800 R of whole body x- irradiation. Control and irradiated (48 h) rats were intraperitoneally administered leucine-1-C^{14} (20 μCi/100 g body weight) and sacrificed 1 h later. The mitochondrial proteins were grossly fractionated as detailed in the text and the radioactivity incorporated into each fraction determined.

The values represent averages based on four independent determinations ± S.E.M.

Table 7

EFFECT OF WHOLE-BODY X-IRRADIATION ON LIVER LYSOSOMAL ENZYMES

Post-irradiation period (h)	Acid phosphatase		Cathepsin		RNAse	
	Ratio of free to bound activity	Percent of control	Ratio of free to bound activity	Per cent of control	Ratio of free to bound activity	Per cent of control
0	0.36	-	0.70	-	0.51	-
24	0.37	103	0.75	107	0.79	155
72	0.40	111	1.25	178	2.20	431
120	0.39	108	3.00	429	1.50	294

The rats were subjected to 800 R of whole body x-irradiation and killed at various post-irradiation periods. Ratios of specific activities of free to bound forms of lysosomal enzymes were determined in 10% liver homogenate.

The values represent averages of four independent determinations.

activities of acid phosphatase at any time following irradiation. Both cathepsin and RNAse show an increase in the proportion of the free activities but these become marked only at 72 h post-irradiation. At such late intervals, both these enzymes show a 3- to 4-fold increase in the ratio of the activities of their free and bound forms.

DISCUSSION

The increase in the incorporation of radioactivity into liver proteins in the irradiated rats is in conformity with the observations reported in literature (1 - 4). The present studies indicate that the labeling in vivo of mitochondrial proteins of liver is also enhanced following whole body irradiation of the animal, up to 48 h post-irradiation. Experiments with liver slices from irradiated rats also show an increased labeling of mitochondrial proteins, although to a considerably lesser extent. On the other hand, the incorporation of label by isolated liver mitochondria is not increased as a result of radioexposure; it does not show any alteration up to 24 h whereas at later periods it falls markedly.

Previous reports on the labeling of mitochondrial proteins have assessed the very early (1 - 2 h) effects of whole-body irradiation at supra-lethal doses (1000 - 2000 R) in rats (5) and mice (7). An increase in the labeling of mitochondrial protein to the extent of 15 - 70% has been reported in these

earlier studies. The present investigations have shown an increased ability of the mitochondria to incorporate label into its proteins even at a time when the mitochondria is significantly damaged in terms of its energy generating function.

The variations in the labeling data between the whole animal experiments and liver slices on the one hand and the isolated mitochondria on the other, may arise from the intrinsic differences between the two systems. Although the mitochondria possess a complete protein synthetic machinery (cf 8, 9), it has been established that the mitochondrial DNA can code for only a small fraction of the total proteins (cf 10 - 13), the others being derived from the cytoplasm. Thus, the in vivo experiments (as also those with liver slices) would permit the labeling of mitochondrial proteins of both cytoplasmic and mitochondrial origin. On the other hand, the isolated mitochondria would be capable of incorporating label only into those proteins which are of exclusive mitochondrial origin. It is, therefore, conceivable that the differences between the in vivo and in vitro labeling of mitochondrial proteins merely reflect qualitative and/or quantitative differences in the responses of the mitochondrial and the nuclear-ribosomal protein synthetic systems. The increase in ^{14}C-lysine incorporation into the microsomal fraction of liver, following whole body irradiation of rats, has been shown to be much greater than that in mitochondria (5). Differences between the two systems are also seen in the effects of a variety of inhibitors of protein synthesis (11 - 13, 26 - 29) or mutagenic agents (30). The

mitochondrial DNA has been shown to be more susceptible to radiation-induced damage than the chloroplast DNA in cells of A.mediterranea (31).

Studies on the fractionation of mitochondrial proteins following their in vivo labeling, provide definite evidence of the differential response of the mitochondrial and cytoplasmic-ribosomal systems. Thus, although the incorporation of radio-activity into total mitochondrial protein shows an increase of about 40% as a result of exposing the animals to radiation, the labeling of a structural protein fraction that has been established to be synthesised by the mitochondria shows a decline of about 20%.

Although whole-body irradiation results in enhanced labeling of liver mitochondrial protein, as has also been shown by others (1 - 4), it would appear from the data on the endogenous free leucine pool that this does not reflect a stimulation of protein synthesis. There is a marked reduction of the free leucine pool of liver cell sap as well as of the liver mitochondria at 24 and 48 h after whole body irradiation. In addition to this significant decrease in the pool size, there is an increase in the labeling of the pool (by entry of the ^{14}C-leucine), which could presumably arise from altered permeabilities of cellular and sub-cellular membranes due to radioexposure; there is evidence in literature of leakage of even macromolecules including enzymes from liver mitochondria of irradiated animals (32). The specific activity of the free leucine pool (the precursor) is thus very

much higher in the liver of the irradiated animals and if due allowance is made for this, the increased labeling of mitochondrial protein cannot be taken as representing a parallel change in the rate of its synthesis. The increase in the specific activity of the precursor, viz., the free leucine pool of either the liver cell sap or mitochondria, at a point of time midway in the label incorporation studies, is two-fold in the rats studied at 24 h post-irradiation. In these animals, the increase in the specific activity of the mitochondrial protein, following the completion of the labeling study, ranges between 10 and 80%. Since the true rate of synthesis is proportional to the ratio of the specific activity of the product to that of the precursor, whole-body irradiation actually inhibits the rate of synthesis of mitochondrial proteins. It would, however, appear from the differences in the labeling pattern that the synthesis of the mitochondrial proteins of cytoplasmic origin may be affected to a lesser extent than those endogenously synthesised by the organelle itself.

It is of relevance in this context to discuss the work of Mukherjee and Goldfeder (7), in which they claim a stimulation of liver mitochondrial protein synthesis in mouse liver at 1 h following exposure to 2000 R of x-rays. An analysis of their observations, both <u>in vivo</u> and <u>in vitro</u>, reveals that the increase in the specific activity of precursor pool is of the same order as the increase in the total incorporation into mitochondrial protein. Additionally, the study suffers from the limitation that

only the total free amino acid pool and not that of free leucine itself, has been estimated. The data presented by these investigators do not warrant any inference of stimulated protein synthesis.

It does not appear likely that restricted availability of energy may be responsible for the decreased rate of protein synthesis. Unlike the uncouplers such as dicoumarol, warfarin, dinitrophenol and salicylate, which have also been reported to inhibit protein synthesis in liver (26, 28, 29), irradiation does not interfere with the formation of the high energy intermediates. Although it has been claimed that ATP is an obligatory energy source for protein synthesis (33), there is considerable evidence in literature (26, 34) that the high energy intermediates can sustain protein synthesis.

It is a paradoxical observation that while the biosynthesis of mitochondrial protein is impaired following whole-body irradiation, there is a significant rise in mitochondrial protein content between 16 and 48 h post irradiation, although at later periods it decreases sharply. Although the present studies do not provide any explanation for this, it is possible that this phenomenon represents an effort by the cell to cope up with the radiation damage. The impairment of oxidative phosphorylation may trigger some compensatory mechanism in the mitochondria, by which the turnover of the mitochondrial protein is considerably slowed down to enable conservation of the existing proteins for maintenance of an essential cellular function. Experimental evidence has been presented in recent years that a

mitochondrial protease may regulate the turnover of mitochondrial protein (35). It is, therefore, possible to visualise a significant increase in the mitochondrial protein occurring in spite of a decreased rate of biosynthesis. An increase in the mass of the individual mitochondrion of liver following whole body irradiation has been reported as early as in 1959 (36) and has since been confirmed by more recent observations (37).

The change in the morphology of hepatic mitochondria leading to larger sizes (37), following whole body radioexposure, may also be dictated by the differential responses of the nuclear-cytoplasmic and the mitochondrial protein synthesising apparatus. The failure of the mitochondrial protein synthetic rate to keep up with the nuclear-ribosomal translation in the livers of irradiated animals may result in an increase in the proportion of the proteins transferred from the cytoplasm to those of the mitochondrial origin. A larger mitochondrion with fewer cristae may make it possible for the organelle to possess a lower comparative content of structural proteins of mitochondrial origin. A quantitative study of the synthesis of defined proteins of mitochondrial and extra-mitochondrial origin and of those such as cytochrome oxidase and ATPase, which are known to consist of subunits that are biosynthesised by both the systems (38), may provide interesting data.

As already stated, the mitochondrial protein content falls off rapidly at 72 h and later, following irradiation, This time period also coincides with the time when there is marked

increase in the ratio of the free to the bound activities of some of the lysosomal enzymes and it is tempting to speculate that the two events may be related. The 'enzyme release' hypothesis of Bacq and Alexander (39) envisages damage to subcellular membranes and the consequent loss of compartmentation of enzymes as an important factor in the development of cellular radiation injury. Electron microscopic evidence has indeed been presented to indicate considerable damage to the structure of mitochondria including their disintegration a few days following exposure of the animals to radiation (40).

SUMMARY

The *in vivo* incorporation of ^{14}C-leucine into liver mitochondrial proteins is enhanced up to 48 h following whole-body irradiation. A similar increase in labeling is also observed with liver slices from irradiated rats incubated *in vitro*, but not by isolated mitochondria. That these differences in the labeling pattern may arise from differential effects of whole-body irradiation on protein synthesis in mitochondria and the nuclear-ribosomal system are indicated by studies on fractionation of the mitochondrial proteins labeled *in vivo*.

The increased labeling, however, does not denote a stimulation of the rate of synthesis of mitochondrial proteins, but reflects a contraction in the size of the endogenous free leucine pool and its increased labeling by the radioactive precursor.

Although mitochondrial protein content shows an increase up to 48 h post-irradiation, there occurs a significant fall at later periods. This decrease coincides with the time when lysosomes reveal a high degree of damage as evidenced by a several-fold increase in the ratio of the activities of the free to the bound forms of some hydrolytic enzymes.

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