to the latter. This was further substantiated by experiments comparing competition between Ca^{2+} and Sr^{2+} accumulation by irradiated and non-irradiated mitochondria. Exposure of isolated mitochondria to doses of up to 50 kRad of X-ray did not affect their large amplitude swelling in KCl nor the contraction induced by ATP and Mg^{2+}.

ACKNOWLEDGEMENT

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I. INTRODUCTION

Mitochondria of both animal and plant origin have been found to possess an outer and an inner membrane, the latter being folded inside forming structures called cristae (1). On these cristae lie the enzymatic assemblies for electron transport and oxidative phosphorylation (2). The outer membrane is thinner and much more permeable to small molecules than the inner membrane, which is the major permeability barrier. The inner mitochondrial membrane is selectively permeable and as such, accumulation of solute will result in water uptake causing mitochondrial swelling. Mitochondria undergo changes in their volume (swelling or contraction) in response to both internal and external factors (3,4); volume changes are in two forms: first, a "passive" form where changes occur as a result of the passive accumulation of a permeable solute from the suspending medium; and second, an "active" form where changes are caused by the energy-dependent uptake of molecules. Both forms are accompanied by the uptake of an osmotic equivalent amount of water (3).

Animal mitochondria actively accumulate a number of cations including Ca^{2+}, Sr^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+} and Zn^{2+} (5,6,7,8). Different plant mitochondria accumulate Ca^{2+} (9-14), Mg^{2+}

(15,16,17), Ba^{2+} (14) and Sr^{2+} (13,14,18,19). The accumulation of these cations in both animal and plant mitochondria utilizes the energy of oxidative phosphorylation (15,20). Three theories have been proposed to explain the mechanism by which energy is transformed from electron transport to ATP synthesis, the chemical coupling hypothesis, the conformational hypothesis and the chemiosmotic hypothesis, although the latter is by far the most widely accepted.

The chemical coupling hypothesis proposes that the passage of an electron pair along the electron transport chain is coupled in three sites to the formation of a high energy intermediate at each of the sites. This high energy intermediate discharges its energy by reacting with ADP and Pi to form ATP (2) or reacts stoichiometrically with cations as Ca^{2+} and Sr^{2+}, causing their transfer into the mitochondria (21). The chemical coupling theory suffers from the fact that no high energy intermediate has been detected yet and that a high energy compound with a $\Delta G^{o'}$ of hydrolysis of 17 kcal/mole is unlikely (13).

The conformational hypothesis according to Hackenbrock (22) proposes that energy is conserved as a conformational change in a respiratory protein and this energy is used in the formation of ATP. This theory is similar to the changes seen in muscle contraction and the action of ATP

on actomyosin. Mitochondrial conformational changes with different energy states have been reported using electron microscopy (23); however, the slowness of these changes makes them doubtful as intermediate states in energy conservation (24).

The chemiosmotic hypothesis proposed by Mitchell (25,26) states that the energy of oxidative phosphorylation is conserved by transporting H^+ ions out of the mitochondria, thereby creating an electrochemical gradient of protons across the mitochondrial membrane which is utilized to phosphorylate ADP to ATP and accumulate cations such as Ca^{2+} and Sr^{2+}.

The uptake of cations by mitochondria has been shown to compete with ATP synthesis during substrate oxidation. The processes of cation uptake and ATP synthesis presumably compete for the high energy intermediate state (2,9). This is supported by the finding that uncouplers of oxidative phosphorylation like dinitrophenol (DNP) also inhibit cation uptake. Oligomycin inhibits mitochondrial ATP synthesis (27,28), and inhibits ATP-supported cation uptake (15,29), but not metabolically dependent cation uptake (15,30). This implies that substrate-dependent cation uptake occurs at a level one step removed from the level of ATP synthesis. Plant mitochondria show an absolute necessity for phosphate as a permeant anion for

Ca^{2+} uptake in corn (9,28) and Ca^{2+} and Sr^{2+} uptake in bean mitochondria (13,18), unlike animal mitochondria which can utilize other anions during Ca^{2+} and Sr^{2+} uptake (20). Johnson (13) proposed that cation uptake in plant mitochondria involves the interaction of phosphate with the high energy intermediate causing its phosphorylation; this phosphorylated intermediate then interacts directly with the cation, causing the transport of a cation:phosphate complex into the mitochondria. However, there is little evidence to support such a mechanism of cation accumulation.

Mammalian mitochondria have a greater affinity for cations. Rat liver mitochondria take up 2.6 μmoles/mg protein Ca^{2+} and 2.5 μmoles/mg protein Sr^{2+} (6). This mitochondrial cation uptake takes preference over the process of ATP synthesis (2). However, plant (15) and yeast (31) mitochondria do not have the same capacity for cations as mammalian mitochondria. They appear to accumulate 10-20% of that taken up by animal mitochondria (14). Respiration-independent Ca^{2+} binding to mammalian mitochondria occurs at high and low affinity binding sites (32,33), while only low affinity binding sites are detected in plant (13), Blowfly flight muscle (34) and yeast mitochondria (31).

A glycoprotein has been isolated from rat liver mitochondria which posesses binding affinity for Ca^{2+} similar to

that by intact mitochondria (35,36,37). This glycoprotein is believed to be the Ca^{2+} carrier molecule in the mitochondrial membrane. When antibodies to this suspected carrier were produced in rabbits by Panfili et al. (38), the antibodies inhibited active Ca^{2+} transport by 45%, while not affecting electron transport or respiratory control in rat liver mitoplasts. Increasing the antibody concentration by 5 times inhibited Ca^{2+} transport by 84% while inhibiting electron transport and respiratory control by 37% and 19%, respectively. The authors proposed a carrier mechanism for Ca^{2+} and cation transport into the mitochondria. This carrier has only been isolated from mammalian sources.

The molar ratio of calcium to phosphate accumulated by rat liver mitochondria is 1.8 (20), while for corn mitochondria it is 1.0 for slow rates or 1.3 for massive uptake (9). While the molar ratio of Sr^{2+} to phosphate accumulated by rat liver mitochondria is generally between 1.2 and 1.4 (6), for bean mitochondria it is 1.34 (12). Massive loading of both animal and plant mitochondria with Ca^{2+} and Sr^{2+} leads to the deposition of calcium phosphate and strontium phosphate salt granules in the mitochondrial matrix. These granules are detected in electron micrographs or by X-ray analysis.

Calcium phosphate electron-dense granules were detected

inside animal mitochondria (39) and corn mitochondria (15;40). Similarly strontium phosphate electron-dense granules were reported in rat liver mitochondria (41) and in bean mitochondria (42). Massive loading of mitochondria with Ca^{2+} and Sr^{2+} also occurs *in vivo*. Carafoli (43) has shown that *in vivo*, rat liver mitochondrial fractions are capable of accumulating Ca^{2+} and Sr^{2+} far more than do other subcellular fractions.

Since the mitochondrion is a major site of ATP synthesis in the cell, the effect of ionizing radiation on this organelle is an important aspect of the radiation response of the whole cell. Structural changes in the mitochondria are often detected after exposure to ionizing radiation. Gartner (44) observed that exposure of *Drosophila melanogaster* to 99 kRad of gamma radiation induced the formation of "giant deformed" mitochondria of the mid-gut. The cristae of these mitochondria were in disarray and in many cases were lost. Similarly, swelling and destruction of cristae of mitochondria are reported for yeast irradiated with 50 kRad of X-radiation, and various mammalian tissues irradiated with dosages between 0.2 and 16 kRad (45-49). Romani *et al.* (50) did not detect any structural changes in pear mitochondria after 250-1000 kRad of gamma irradiation. The effect of radiation on oxidative phosphorylation of the mitochondria has received a great deal of attention. Early investigators could not detect any

radiation effect on mitochondrial coupling or oxidative phosphorylation. Thomson (51) and Thomson et al. (52) found no effect on oxidative phosphorylation in rat liver mitochondria exposed to 1 kRad whole body radiation. The same results were reported for isolated mitochondria exposed to 750-40,000 Rad (53). It is, however, recognized now that ionizing radiation does inhibit oxidative phosphorylation in many tissues of the irradiated animal; for example, Romantsev et al. (54) found that liver mitochondria change from "tight" to "loose" coupling after irradiation. Inhibition of oxidative phosphorylation in the mitochondria of different rat tissues after whole body irradiation with dosages of 0.5-10 kRad was reported by various authors (55-61). After massive irradiation of pear mitochondria, it was observed that a stimulation of mitochondrial respiration rate and a reversible decrease in Respiratory Control Ratios occurred at doses of 250, 500 and 750 kRad, while a dose of 1000 kRad inhibited the respiration rate and irreversibly decreased the Respiratory Control Ratios. Similar results were reported by Joshi and Gaur (65) for bean mitochondria, which showed an inhibition of respiration rate and a decrease in Respiratory Control Ratios after a dose of 500 kRad to the bean hypocotyls. These results demonstrate that plant mitochondria are much more resistant to ionizing radiation than mammalian mitochondria (50,65,66). Moreover, isolated mitochondria are more radioresistant than

mitochondria inside cells or body (67,68).

The effect of ionizing radiation on mitochondrial protein synthesis has been investigated in both animal and plant sources. Various authors report the enhancement of mitochondrial protein synthesis in rat tumour and mouse liver after in vivo irradiation with 400-2000 Rad of X-rays (69,70,71). However, Romani and Fisher (72) report the suppression of mitochondrial protein synthesis in pears irradiated in vivo with 250 and 1000 kRad of gamma-radiation. This difference in result is undoubtedly more a function of dose range than a demonstration of differential radiation response between plant and animal mitochondria.

There are several reports concerning radiation effects on mitochondrial enzymes. For example, succinic dehydrogenase activity was decreased in rat and guinea pig liver mitochondria after in vivo irradiation with 900 Rad of X-rays (73) and in mouse liver mitochondria after in vivo irradiation with 600 Rad (74); this latter exposure also reduced NADH-cytochrome c-reductase and L-amino acid oxidase activities. However, the activities of cytochrome oxidase, catalase and acid phosphatase in pear mitochondria increased when exposed to 250 kRad of gamma-radiation in vivo, while after 1000 kRad exposure, mitochondrial catalase and acid phosphatase activities were decreased

(62). The disparities in apparent radiosensitivities of mitochondria are probably due to the variation in mitochondrial sources, dose range and experimental procedures, including isolation procedure and metabolic state of the mitochondria during irradiation. These disparities were fully discussed by Yost et al. (57).

The radiation effect on the ionic permeability of mitochondria has been dealt with only in a few reports. Wills (75) reported that rat liver mitochondria irradiated with 5-100 kRad from an electron beam accumulate less K^+ and Ca^{2+} and lose these ions more readily than non-irradiated mitochondria. This radiation effect appears to be on mitochondrial membranes and not on mitochondrial ATPase or oxidative phosphorylation. Similar results are reported for Mn^{2+} uptake by irradiated liver mitochondria (76). It has been reported that Ca^{2+} uptake in pea mitochondria is inhibited by irradiation in vivo and in vitro with 5-10 kRad of X-radiation (77). In addition, these authors reported the increase in Ca^{2+} leakage from the mitochondria after irradiation, and concluded that the radiation effect was on the passive and not on the energy-dependent uptake of Ca^{2+}.

Investigation into the effect of radiations other than ionizing have been carried out mainly on UV, although a few dealt with microwaves. Roy and Abboud (78) reported

the inhibition of respiration, loss of coupling and inhibition of Ca^{2+} and Sr^{2+} uptake in bean mitochondria irradiated in vitro with far UV radiation. No effect on rat liver mitochondrial respiration rates, respiratory coupling or Ca^{2+} uptake was detected after microwave irradiation (79,80). Exposure of liver mitochondria to visible light causes the inactivation of succinate and NADH-dehydrogenase and production of lipid peroxides. This damage is maximal between 370 and 720 nm, and is dependent on the presence of oxygen (81).

The ultimate goal of studying isolated organelles and their responses to different treatments is to gain more insight into what role they play in the cell and its responses to those treatments. The large amounts of Ca^{2+} and Sr^{2+} accumulated by animal and plant mitochondria attest to the important role of mitochondria in some mechanism of regulation of ion movement inside the cell. In plants, mitochondrial uptake of Ca^{2+} is too slow to play a controlling role in rapid response reactions (13). The precipitation of insoluble Ca^{2+} and Sr^{2+} salts inside the mitochondria has been reported for animals (20) and plants (13,15). This could serve as a salt regulation mechanism inside the whole cell.

The importance of the mitochondria in cellular radiosensitivity is illustrated by the difference in their numbers

from a few radiosensitive cells such as spermatogonia or lymphocytes, to over 1000 per cell in liver cells (82). And according to Goldfeder (83) and Goldfeder et al. (84), there exists a correlation between the number of mitochondria in a cell and its radiosensitivity, and these play a role not only in initial radiation damage but in subsequent repair processes.

This study will investigate the effect of X-irradiation on some metabolic functions of isolated bean mitochondria. The effect of radiation on respiration rate as well as on active and passive calcium and strontium uptake will be studied. If a radiation effect on active uptake exists, the relation of this to any radiation effects on electron transport and energy coupling will be investigated. Finally, the effect of radiation on the relative uptake of calcium and strontium in the presence of the other ion will be studied. This is important, in view of the 90-strontium contamination of the environment from fallout and nuclear plant effluents.

II. MATERIALS AND METHODS

Plants and Growth Conditions

Yellow Garden Bush beans (Phaseolus vulgaris, var. Earliwax) were grown on Vermiculite and tap water in the dark at 27±1°C and 60% relative humidity for 7 days.

Experimental Procedure

Isolation of the Mitochondria (modified from Johnson (13))

Etiolated bean hypocotyls were cut from the plants under green light, then homogenized at 10°C in a Waring blender for 20 seconds with twice their weight of chilled Isolation Medium containint 0.40 M D-Mannitol, 4 mM $MgCl_2$, 1 mg/ml Bovine Serum Albumin Fraction V (B.S.A.), and Tris-Tricine buffer, 0.050 M, pH 7.5. The homogenate was strained through a 100 µm, then a 10 µm nylon mesh. It was then centrifuged in cold tubes at 2000 x g for 10 minutes at 2°C in an I.E.C. B-20 refrigerated centrifuge. The supernatant was then centrifuged at 12,000 x g for 10 minutes as above and the resulting pellet resuspended in 2-3 ml of the Isolation Medium and kept on ice for the duration of the experiment.

Irradiation

One ml of mitochondrial stock suspension (approximately 30 mg

protein) was placed in a small polyethylene vial covered with Parafilm and irradiated on ice with 260 kVp, 7 mA, 2 mm Al. filter X-rays at a dose of 1,000 R/minute, delivered from a Muller MG300 X-ray machine. X-ray dose rate was measured with a Victoreen Condenser R-meter, model 570; absorbed dose was determined by Fricke chemical dosimetry (85).

Analysis

Protein Determination

Mitochondrial protein concentration was measured using the Biuret method. A 0.1 ml aliquot of mitochondrial suspension was diluted to 1.0 ml with double distilled water and added to 4.0 ml of Biuret reagent. The mixture was incubated for 30 minutes at room temperature with a blank made up of 1 ml double distilled water and 4 ml Biuret reagent. The absorbance of the mitochondrial sample was determined at 540 nm in a Bausch & Lomb Spectronic 700, single beam, non-recording spectrophotometer. Net protein was determined from a standard concentration curve using B.S.A. as a standard, and expressed as mg protein/ml of mitochondrial suspension.

Respiration

Oxygen consumption measurements were followed with a Y.S.I. Clark O_2 electrode, model 4004, operated at 0.8 V output. Voltage was recorded on a B & L VOM-5 chart recorder at 100 mV, full scale. A 0.2 ml sample mitochondria (approxi-

mately 6 mg protein) was added to 3 ml of Isolation Medium. When the respiration rate was stable, 0.1 ml each of succinic acid, 3 mM, and phosphoric acid, 3 mM, were added and state IV respiration rate was measured. Then 0.1 ml of ADP, 300 µM, was added to measure the state III respiration rate. Respiratory Control Ratio (R.C.R.) was calculated as the ratio of respiration rates of states III/IV. All respiration measurements were carried out at room temperature with constant stirring.

Swelling and Contraction

A 0.2 ml aliquot mitochondria (approximately 6 mg protein) was added to 3 ml of Swelling Medium, containing 0.10 M KCl, 1 mg/ml B.S.A. and 0.02 M Tris, pH 7.5, in a 1 cm path length plastic cuvette. Swelling was measured as a decrease in absorbance of the above mixture at 520 nm in a Bausch & Lomb Spectronic 700. At the end of the swelling period, 0.1 ml of ATP, 0.1 M and $MgCl_2$, 0.2 M, were added and contraction was measured as an increase in absorbance until completion. Both processes were carried out at room temperature with occasional stirring.

Cation Accumulation

Mitochondria (0.6-1.0 mg protein) were added with 0.1 µCi of $^{45}CaCl_2$ or $^{85}SrCl_2$ to 5 ml of Accumulation Medium containing 0.40 M D-Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 0.002 M $SrCl_2$ and/or $CaCl_2$, 50 µM Rotenone, 0.01 M succinic acid and 0.002 M phosphoric acid. In some

experiments, a respiratory inhibitor, 2 mM KCN, or an uncoupling agent, 100 μM DNP, were added. All were incubated in a water bath at 27°C for 10 minutes. At the end of the incubation period, the mitochondria were collected by filtering the medium through a 0.45 μm Metricell membrane filter in a Delrin filter assembly, then washed with 10 ml of 0.6 M Mannitol. The filters were collected and transferred to glass scintillation vials (for ^{45}Ca activity measurements) or to plastic test tubes with a cap (for ^{85}Sr activity measurements).

Isotope Activity Measurements

Filters with ^{45}Ca-loaded mitochondria were transferred to glass liquid scintillation vials and 10 ml of Aquasol were added to them. Their activity was then measured in an Ansitron liquid scintillation spectrometer. In some instances, 15 ml of a liquid scintillation mixture of toluene, POPOP and PPO were added instead of Aquasol, and the activity measured in a Unilex II Nuclear Chicago liquid scintillation counter.

Filters with ^{85}Sr-loaded mitochondria were transferred to capped plastic tubes and their activities measured in a Picker deep well NaI solid crystal scintillation counter.

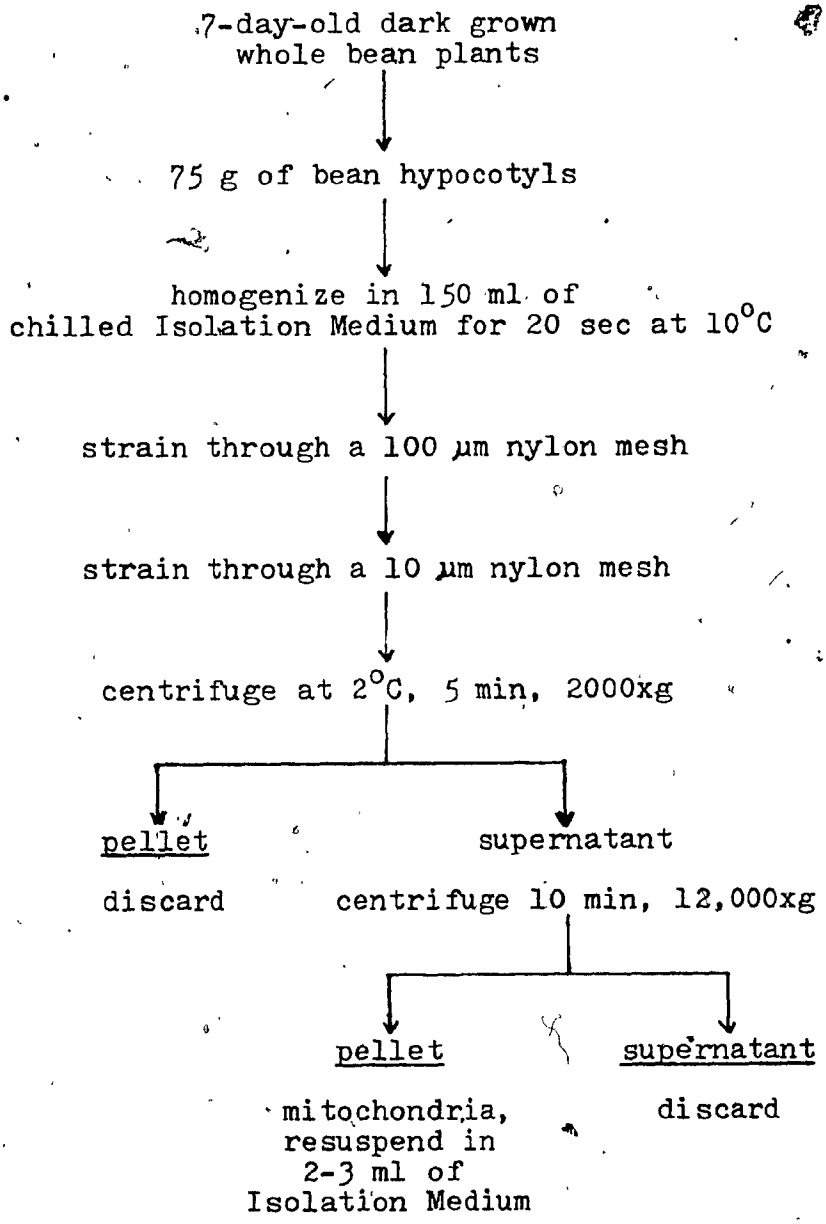
Isotope counts were corrected for residual activity on filters without the mitochondria. The total activity

administered to the mitochondria (0.1 μCi) was counted, and the absolute amount of cation uptake was calculated from the ratio of mitochondrial activity to total activity.

FIGURE 1

MITOCHONDRIAL ISOLATION PROCEDURE

ISOLATION OF MITOCHONDRIA



III. RESULTS

Mitochondria were isolated from bean hypocotyls as outlined in Figure 1. State III and state IV respiration rates were measured polarographically with succinate as substrate, at 20°C. The state III (ADP and phosphate present) respiration rate of isolated mitochondria measured within the first two hours post-isolation was 42 ± 5 nmoles oxygen per minute per mg protein while Respiratory Control Ratios (RCR) of 2.0-4.4 were generally observed. The quality of the mitochondria was judged by these criteria as adequate for active transport studies.

Conditions for strontium and calcium uptake were optimized (Figures 2-5). Greater than 90% uptake was shown by mitochondria incubated for 10 minutes at 2 mM salt concentration. Maximum uptake was 0.13 µmoles/mg protein strontium and 0.11 µmoles/mg protein calcium.

It is frequently observed that there are few instantaneous effects of ionizing radiation on physiological processes; however, radiation damage increases progressively with time after initial exposure (86). In this study, the effects of radiation on respiration and cation accumulation by isolated bean mitochondria were studied immediately and

FIGURE 2

TIME COURSE OF CALCIUM UPTAKE IN PLANT MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml. B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $CaCl_2$, 0.1 μCi 45-Calcium and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 0-25 minutes.

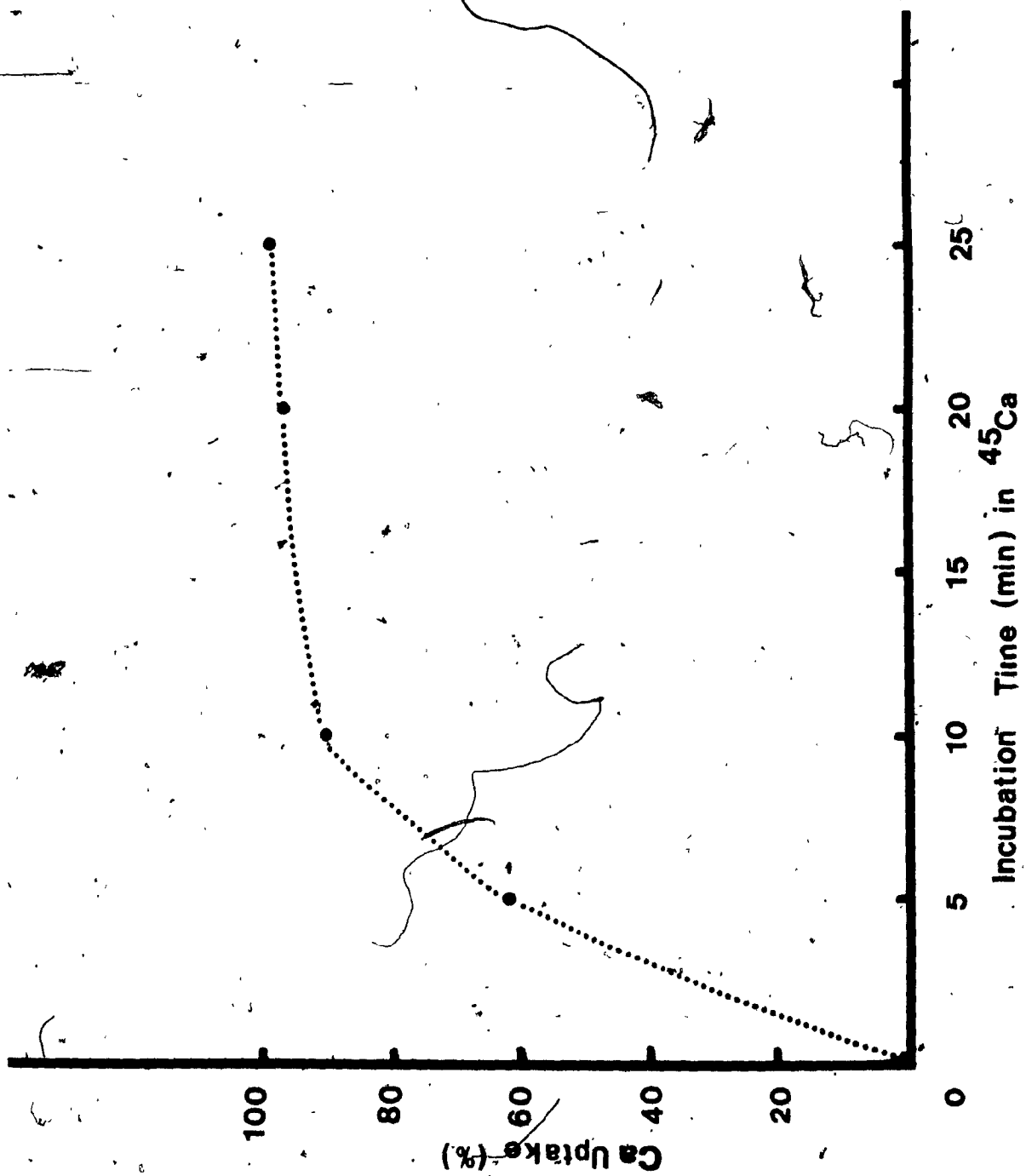

Ca Uptake (%)
100
80
60
40
20
0
5
10
15
20
25
Incubation Time (min) in ^{45}Ca

FIGURE 3

TIME COURSE OF STRONTIUM UPTAKE IN PLANT MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $SrCl_2$, 0.1 μCi 85-Strontium and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 0-25 minutes.

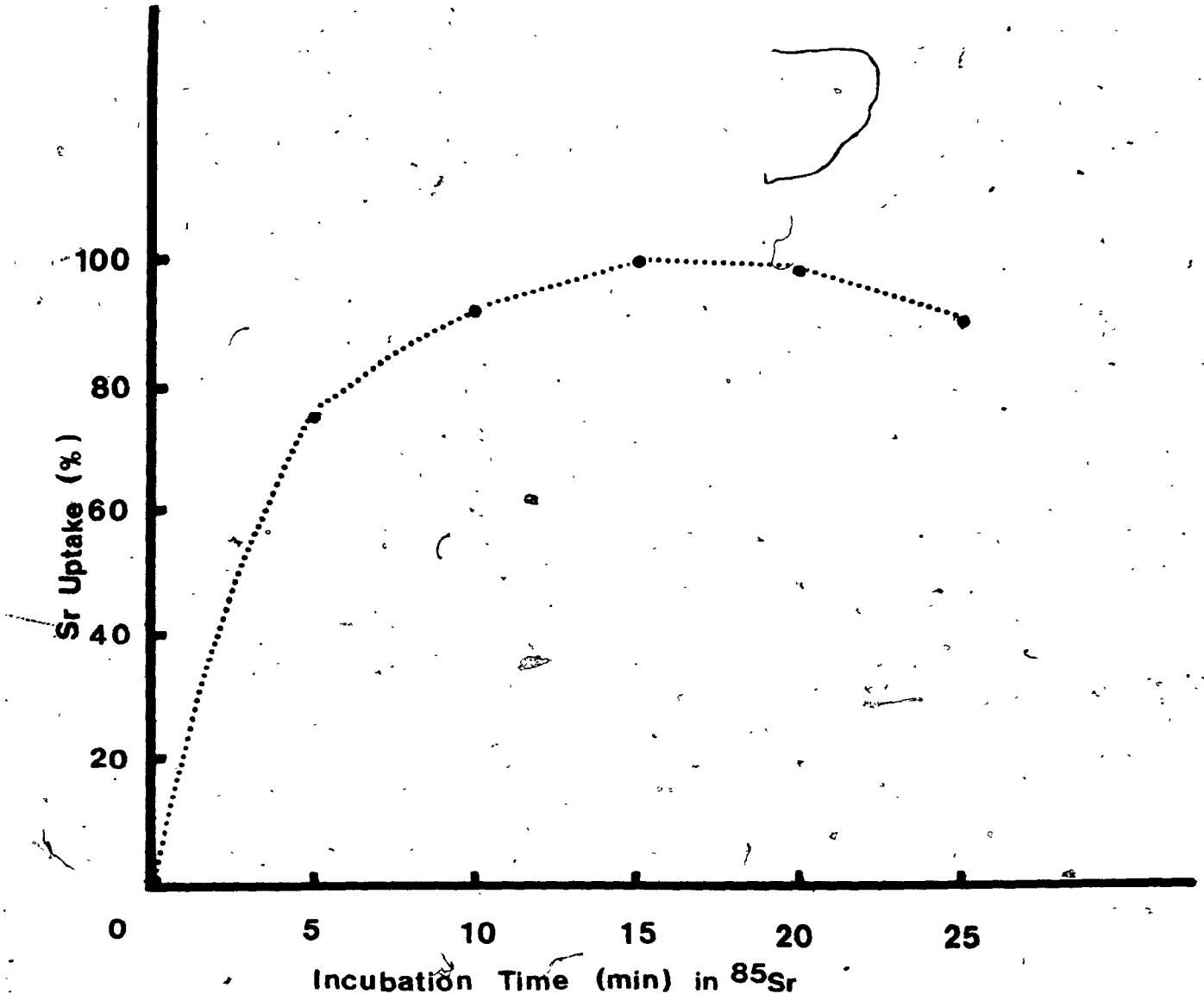

100
80
60
40
20
Sr Uptake (%)
0
5
10
15
20
25
Incubation Time (min) in 85Sr

FIGURE 4

EFFECT OF $CaCl_2$ CONCENTRATION ON CALCIUM UPTAKE BY PLANT MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 0-3 mM $CaCl_2$, 0.1 μCi 45-Calcium and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

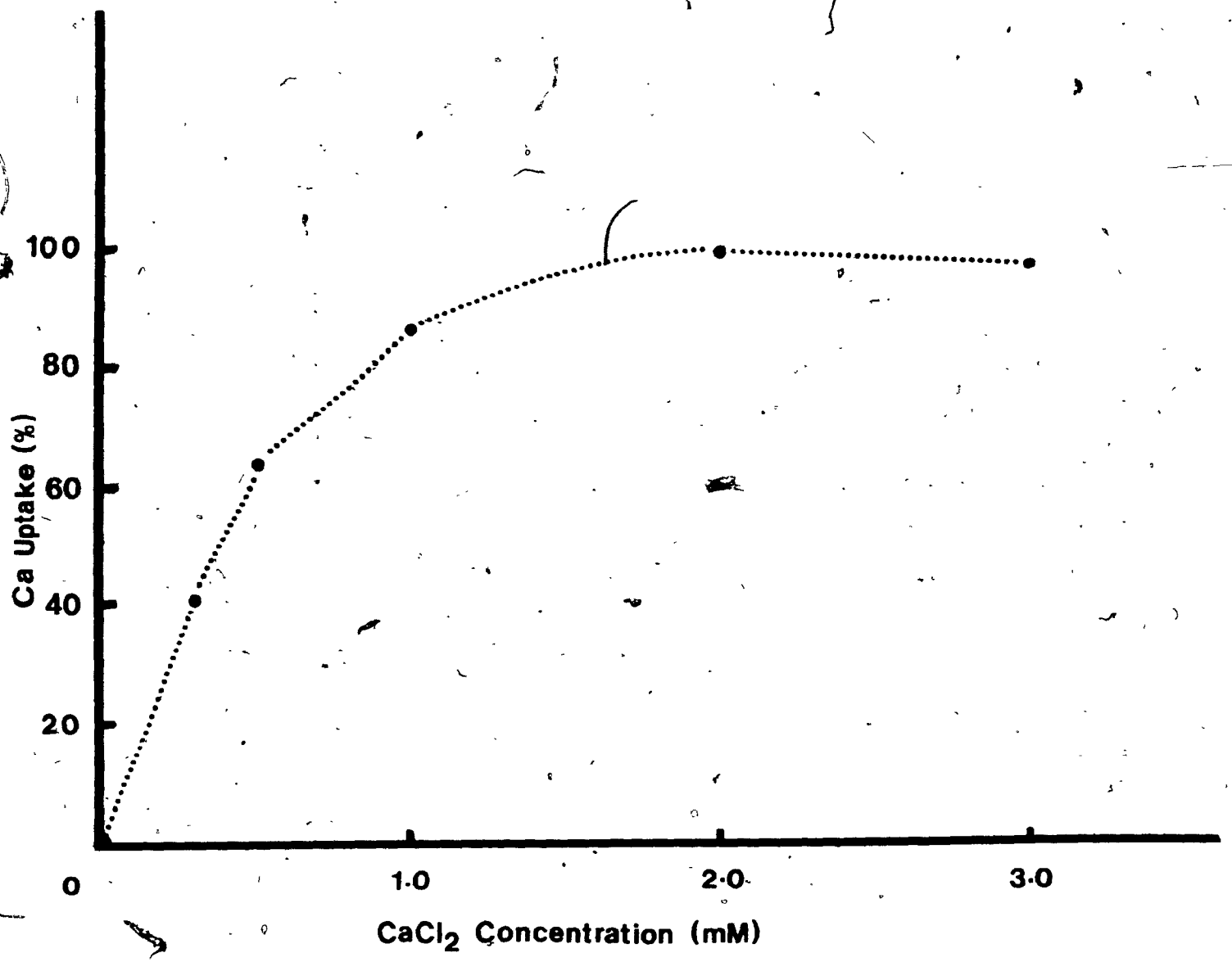
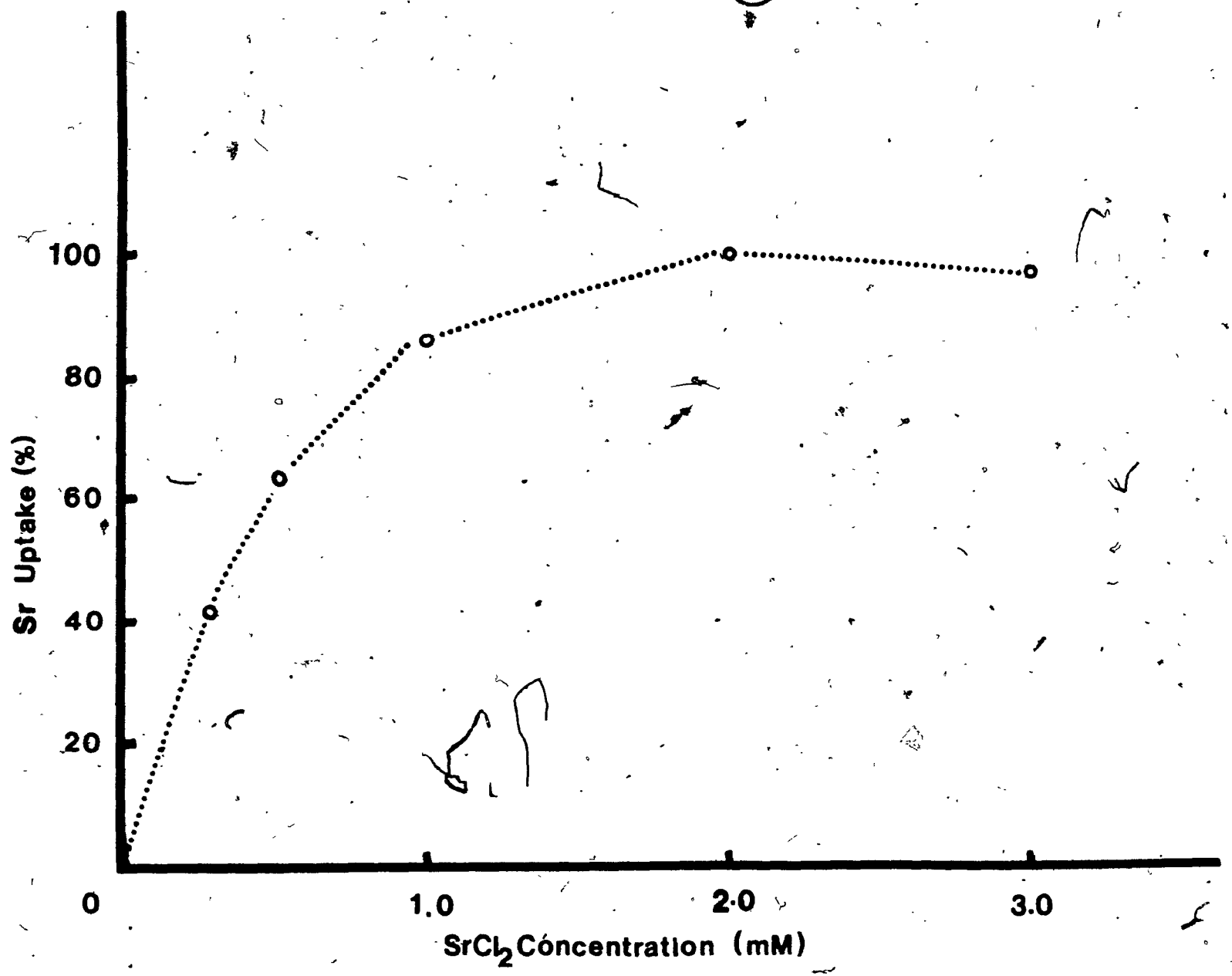

100
80
60
40
20
Ca Uptake (%)
0
1·0
2·0
3·0
CaCl2 Concentration (mM)

FIGURE 5

EFFECT OF $SrCl_2$ CONCENTRATION ON STRONTIUM UPTAKE BY PLANT MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 0-3 mM $SrCl_2$, 0.1 μCi 85-Strontium and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.


100
80
60
40
20
0
Sr Uptake (%)
1.0
2.0
3.0
SrCl2 Concentration (mM)

one hour after irradiation. The results are presented in Table I, and show that no difference between irradiated and non-irradiated mitochondria in uptake of Ca^{2+} and Sr^{2+} was observed immediately after irradiation. By contrast, one hour after irradiation, calcium and strontium uptake by irradiated (50 kRad) mitochondria was decreased by 12% and 26% respectively; however, cation uptake by non-irradiated mitochondria did not change. For this reason, all subsequent studies on radiation effects in mitochondria were carried out at one hour post-irradiation.

Irradiating isolated mitochondria with 10, 25 and 50 kRad of X-rays did not produce detectable effects on their oxygen consumption during state IV (presence of succinate) or state III (presence of succinate and ADP) respiration and thus no apparent effect on the Respiratory Control Ratios up to one hour post-irradiation. This does not preclude small changes (less than 10%) in respiratory functions.

The effect of X-radiation on calcium and strontium uptake by mitochondria was studied at 10, 25 and 50 kRad, and is presented in Table II. No significant differences in calcium and strontium accumulation between irradiated and non-irradiated mitochondria were found at doses of 10 and 25 kRad, while at a dose of 50 kRad, calcium and strontium accumulation by irradiated mitochondria decreased by

TABLE I

EFFECT OF POST-IRRADIATION TIME ON STRONTIUM AND CALCIUM UPTAKE BY NON-IRRADIATED AND IRRADIATED (50 kRAD) MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $SrCl_2$ or $CaCl_2$, 0.1 μCi 85-$SrCl_2$ or 45-$CaCl_2$ and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

	Uptake by non-irradiated mitochondria (μmoles/mg p)		Uptake by irradiated mitochondria (μmoles/mg p)	
	Calcium	Strontium	Calcium	Strontium
0 hr post-irradiation	0.082 [a]	0.092 [a]	0.081 [a]	0.097 [a]
1 hr post-irradiation	0.085 [a]	0.090 [a]	0.071 [b]	0.072 [b]

[a] These values are not significantly different from one another at the 95% confidence level, using a paired data t-test.

[b] These values are significantly different from the other values at the 95% confidence level, using a paired data t-test.

STATISTICS (T-TEST),

	t-values		d.f.	
	Calcium	Strontium	Calcium	Strontium
0 hr vs. 1 hr non-Irr.	0.8384	1.7801	8	10
0 hr non-Irr. vs. Irr.	0.1552	0.9460	8	10
0 hr vs. 1 hr Irr.	2.431	4.202	8	10
1 hr non-Irr. vs. Irr.	5.101	4.655	8	10

TABLE II

UPTAKE OF CALCIUM AND STRONTIUM BY IRRADIATED BEAN MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 µM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $SrCl_2$ or $CaCl_2$, 0.01 µCi 85-$SrCl_2$ or 45-$CaCl_2$ and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

X-ray dose (kRad)	Calcium Uptake (µmoles/mg p)		Strontium Uptake (µmoles/mg p)	
	non-irradiated	irradiated	non-irradiated	irradiated
10	0.076	0.077 [a]	0.082	0.084 [a]
25	0.069	0.073 [a]	0.086	0.075 [a]
50	0.099	0.069 [b]	0.111	0.073 [b]

[a] These values are not significantly different from their non-irradiated counterparts at the 95% confidence level, using a one-way Analysis of Variance.

[b] These values are significantly different from their non-irradiated counterparts at the 95% confidence level, using a one-way Analysis of Variance.

STATISTICS (ONE-WAY ANALYSIS OF VARIANCE)

Dose (kRad)	Strontium F values	Calcium F values
10	0.0023	1.493
25	3.124	2.579
50	28.01	24.81

N.B. Each radiation dose was done at a different time than the others. Absolute uptakes with a radiation dose are comparable but uptakes between doses are not.

approximately 30%, in comparison to non-irradiated mitochondria. All subsequent studies on the radiation effects on calcium and strontium accumulation by mitochondria were carried out at a dose of 50 kRad.

In order to evaluate the relative effect of radiation on active (energy dependent) and passive (energy independent) uptake of cations, studies were carried out on irradiated and non-irradiated mitochondria incubated as follows:

(a) with electron transport medium, succinate as a substrate, phosphate as an accompanying anion, and at a temperature of 27°C.

(b) with electron transport medium, but at a temperature of 0°C where active transport processes are largely inhibited and passive diffusion is unaffected.

(c) in the absence of substrate (succinate) and an accompanying anion (phosphate), in which active transport is inhibited by the absence of respiratory substrate; however, passive transport is unaffected.

(d) with electron transport medium plus the addition of a mitochondrial metabolic poison (KCN) in which electron transport and hence ATP production is inhibited, leading to the elimination of active transport, leaving passive transport undisturbed.

(e) with electron transport medium plus the addition of a mitochondrial uncoupler (DNP), in which all high

energy states are inhibited by virtue of uncoupling from electron transport chains; this causes the inhibition of active transport only.

(f) with electron transport medium and the addition of an inhibitor of cation accumulation (Ruthenium Red), which binds to the mitochondrial membrane preventing all transport processes.

Accumulation of calcium and strontium ions in each of the above conditions are presented in Tables III and IV respectively. The tables show that anything that diminishes the electron transport and ATP production in the mitochondria, inhibits significantly both irradiated and non-irradiated mitochondrial uptake of calcium and strontium, and that there is still significant inhibition of cation uptake in irradiated compared to non-irradiated mitochondria. These inhibitions are statistically significant at the 95% level, using a two-way ANOVA test. The same statistical test showed no interaction between radiation and any of the inhibiting conditions, which means that the magnitude of differences between irradiated and non-irradiated were not significantly altered in any of these situations; in other words, the radiation effects have not been drastically modified by inhibitors of energy-dependent transport. The membrane dye Ruthenium Red inhibited completely the uptake of both ions in irradiated and non-irradiated mitochondria. Table V shows

TABLE III

EFFECT OF TEMPERATURE, CYANIDE, DINITROPHENOL AND THE ABSENCE OF PHOSPHATE AND SUCCINATE ON CALCIUM UPTAKE BY IRRADIATED (50 kRAD) AND NON-IRRADIATED BEAN MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $CaCl_2$, 0.1 μCi ^{45}-$CaCl_2$ and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

Medium	Uptake by non-irradiated mitochondria (μmoles/mg p)	Uptake by irradiated mitochondria (μmoles/mg p)
Complete	0.105	0.074
0°C	0.057	0.036
-Pi, -Succ.	0.059	0.040
+2 mM KCN	0.056	0.038
+100 uM DNP	0.051	0.033

STATISTICS (2 x 2 ANALYSIS OF VARIANCE PERFORMED FOR COMPLETE WITH EACH TREATMENT)

	F (Treatment)	F (Irradiated)	F (Interaction)
0°C	21.03	56.08	0.7582
-Pi, -Succ.	18.23	46.43	1.094
+KCN	16.05	53.79	3.194
+DNP	29.53	105.8	1.895

The above statistics show a significant ($p < 0.01$) difference between treatments, and between irradiated and non-irradiated samples, but no significant interaction ($p > 0.05$) between treatments and irradiation.

TABLE IV

EFFECT OF TEMPERATURE, CYANIDE, DINITROPHENOL AND THE ABSENCE OF PHOSPHATE AND SUCCINATE ON STRONTIUM UPTAKE BY IRRADIATED (50 kRAD) AND NON-IRRADIATED BEAN MITOCHONDRIA

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 μM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $SrCl_2$, 0.1 μCi 85-$SrCl_2$ and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

Medium	Uptake by non-irradiated mitochondria (μmoles/mg p)	Uptake by irradiated mitochondria (μmoles/mg p)
Complete	0.126	0.089
0°C	0.068	0.043
-Pi, -Succ.	0.071	0.050
+2 mM KCN	0.067	0.055
+100 uM DNP	0.064	0.046

STATISTICS (2 x 2 ANALYSIS OF VARIANCE PERFORMED FOR COMPLETE WITH EACH TREATMENT

	F (Treatment)	F (Irradiated)	F (Interaction)
0°C	67.38	197.1	2.191
-Pi, -Succ.	77.44	205.4	5.068
+KCN	22.98	80.37	3.724
+DNP	38.96	150.1	4.773

The above statistics show a significant difference ($p < 0.01$) between treatments, and between irradiated and non-irradiated samples, but no significant interaction ($p > 0.05$) between treatments and irradiation.

TABLE V

PASSIVE (ENERGY-INDEPENDENT), ACTIVE (ENERGY-DEPENDENT) AND TOTAL UPTAKE OF CALCIUM AND STRONTIUM BY IRRADIATED (50 kRAD) AND NON-IRRADIATED BEAN MITOCHONDRIA

Uptake	Calcium Uptake (μmoles/mg p)			Strontium Uptake (μmoles/mg p)		
	non-irradiated		irradiated	non-irradiated		irradiated
Total [a]	0.105	*	0.074	0.126	*	0.089
Passive [b]	0.059	*	0.040	0.071	*	0.050
Active [c]	0.046	**	0.034	0.054	**	0.039

[a, b] These uptake values were reported in Tables III and IV as normal and no succinate, no phosphate treatments, respectively.

[c] Active uptake was calculated as the difference between total and passive uptakes.

* $p < 0.01$ comparing non-irradiated and irradiated using a paired data t-test.

** $p < 0.05$ comparing non-irradiated and irradiated using a paired data t-test.

total, energy dependent (active) and energy independent (passive) calcium and strontium uptake in irradiated and non-irradiated mitochondria. The total and passive uptakes are given in Tables III and IV as uptake in complete and absence of phosphate and succinate media, respectively. Energy dependent (active) uptake was calculated as the difference between total and passive uptakes. Table V also shows that active cation uptake in irradiated mitochondria is significantly less than in non-irradiated mitochondria.

It has been proposed that the accumulation of calcium and strontium by mitochondria is carrier-mediated, and that these cations share that carrier (13). Competition experiments were carried out in which the uptake of calcium was measured with and without 2 mM $SrCl_2$ and likewise, strontium uptake was measured in the presence or absence of 2 mM $CaCl_2$. These results are presented in Table VI and indicate that there is a significant difference in uptake of calcium and strontium in the presence or absence of the other competing ion. However, no significant difference is found between irradiated and non-irradiated in the presence of the competing ion.

Changes in the volume of the mitochondria (swelling and contraction) are important parameters in the study of mitochondrial permeability. Swelling or contraction is

TABLE VI

INHIBITION OF CALCIUM AND STRONTIUM UPTAKE IN NON-IRRADIATED AND IRRADIATED (50 kRAD) BEAN MITOCHONDRIA BY $SrCl_2$ AND $CaCl_2$, RESPECTIVELY

Incubation medium contained 0.4 M Mannitol, 0.05 M Tris-Tricine buffer, pH 7.5, 1 mg/ml B.S.A., 5 µM Rotenone, 0.01 M Succinate, 2 mM Phosphate, 2 mM $SrCl_2$ or $CaCl_2$, 0.1 µCi 85-$SrCl_2$ or 45-$CaCl_2$ and 0.6-1.0 mg mitochondrial protein. Temperature was 27°C, incubation time 10 minutes.

$CaCl_2$	$SrCl_2$	Calcium Uptake (µmoles/mg p)		Strontium Uptake (µmoles/mg p)	
		non-irradiated	irradiated	non-irradiated	irradiated
2 mM	-	0.131	0.113	-	-
2 mM	2 mM	0.087	0.075	-	-
-	2 mM	-	-	0.161	0.128
2 mM	2 mM	-	-	0.097	0.087

STATISTICS (2 x 2 ANALYSIS OF VARIANCE)

	F (Treatment)	F (Irradiated)	F (Interaction)
Calcium	26.44	3.440	0.1137
Strontium	28.01	4.758	1.283

From the above statistics, a significant difference at the 99% level was found between treatments with and without the competing ion, while no significant difference at the 95% level was found between irradiated and non-irradiated, and interaction between irradiation and treatment.

TABLE VII

SWELLING AND CONTRACTION BY NON-IRRADIATED AND IRRADIATED (50 kRAD) BEAN MITOCHONDRIA (6 MG MITOCHONDRIAL PROTEIN)

Swelling was initiated in 3 ml of 0.1 M KCl, 1 mg/ml B.S.A. and 0.02 M Tris, pH 7.5. Contraction was initiated by addition of 0.1 ml of 0.1 M ATP and 0.2 M $MgCl_2$.

	Non-irradiated Mitochondria	Irradiated Mitochondria
Swelling (%) [1]	10.9	12.1 *
Contraction (%) [2]	61.3	61.6 *

[1] % swelling was calculated as the percentage change in absorbance at 520 nm from time zero to minimum absorbance.

[2] % contraction was calculated as the percentage change in absorbance from minimum to maximum absorbance following addition of ATP and $MgCl_2$.

* Not statistically significant ($p > 0.05$) from non-irradiated, using one-way Analysis of Variance.

measured as the per cent change in light scattering at a wavelength of 520 nm of the mitochondrial suspension. The swelling in a 0.1 M KCl solution and subsequent contraction upon addition of ATP and Mg^{2+} of irradiated and non-irradiated mitochondria were measured and the results presented in Table VII. These observations indicate that there is no significant difference in the per cent swelling (calculated as the % change in light scattering) or per cent contraction of irradiated and non-irradiated mitochondria.

IV. DISCUSSION

Instant radiation damage seldom occurs at the physiological level; maximum damage always occurs some time after irradiation (68,87). Table I shows that there is no change in uptake of Sr^{2+} and Ca^{2+} right after irradiation, while significant inhibition of their uptake occurs one hour post-irradiation. This is probably due to the radiation-induced production of short-lived free radicals from radiolysis in the mitochondria and their surroundings, which interact to form lipid peroxides in a chain reaction leading to the accumulation of biological damage caused by peroxide action on mitochondrial constituents (87).

This study has shown that bean hypocotyl mitochondria accumulate about 0.13 μmoles per mg protein of Sr^{2+} in ten minutes at 27°C in the presence of phosphate and succinate. This mitochondrial uptake of Sr^{2+} is somewhat less than that reported by other authors (6,12,13,14,18,19). This study also showed that bean hypocotyl mitochondria accumulated 0.11 μmoles per mg protein Ca^{2+} in ten minutes at 27°C in the presence of phosphate and succinate. This Ca^{2+} accumulation by bean mitochondria is less than the values reported by others (9-14,20). This difference in rates of uptake is probably due to the degree of purity

of the mitochondria, variations in mitochondrial sources, and the difference in incubation media. The results are consistent with other reports (14) that plant mitochondria accumulate more strontium than calcium.

Respiration rates for isolated bean mitochondria were found to be 30-40 nmoles O_2 per minute per mg protein at 20°C with succinate as a substrate in the presence of ADP. This is less than that reported for bean mitochondria (13,18), but quite similar to the 90 natoms O per minute per mg protein for bean mitochondria reported by Venugopal and Patwardhan (11) at the same temperature. The above differences are probably due again to the different degrees of purity of the mitochondria used, methods of preparation and incubation conditions.

The Respiratory Control Ratios for bean mitochondria of 2.0-4.4 with succinate as a substrate are the same as those reported for bean and corn mitochondria by other authors (9,11,13,14,18). Near maximum uptake of Sr^{2+} and Ca^{2+} by bean mitochondria in the presence of succinate and phosphate occurs after 10 minutes of incubation at 27°C (Figures 2 and 3), and 2 mM concentration of either ion (Figures 4 and 5). This is similar to results reported by other investigators (13,14,18).

It has been observed that Sr^{2+} and Ca^{2+} uptake by plant

mitochondria in the presence of a substrate is dependent on the presence of phosphate, which cannot be replaced by other anions (9,12,15,19); by way of explanation, it has been suggested that Sr^{2+} and Ca^{2+} form strontium or calcium phosphate complexes which are then actively transported to the mitochondria.

Respiration rates and respiratory uncoupling of bean mitochondria were not altered by exposure to 10, 25 and 50 kRad X-radiation up to 1 hour post-irradiation. This is not unusual, considering the high resistance of some plants to radiation (50,65,66). Also, the mitochondrion is known to be one of the most resistant cell organelles (82). This resistance is not surprising in view of the large amounts of the enzymes catalase and superoxide dismutase associated with the mitochondria (88). These enzymes break down H_2O_2 and $O_2^{\cdot}$ respectively, which are major radiolysis products of water. Furthermore, isolated mitochondria are more radioresistant than those irradiated in situ, inside the cell (67,68). Inhibition of respiration rates and loss of respiratory coupling of the mitochondria after exposure to ionizing radiation have been reported by several authors (54,61,62,64); Romani et al. (63) reported damage to respiration in pear mitochondria irradiated with 1000 kRad of gamma-radiation, and Joshi and Gaur (65) found similar results for bean mitochondria irradiated with 500 kRad of gamma-radiation.

Decreases in oxidative phosphorylation of the mitochondria after exposure to ionizing radiation have been reported. For example, Goldfeder and Selig (59), using rat tumor mitochondria, demonstrated the inhibition of oxidative phosphorylation (measured as P:O ratio) by 10 kRad of X-radiation; similar results were reported by others for a variety of mammalian tissue mitochondria (55-58, 60). However, Altenbrum and Kobbert (53), using isolated rat liver mitochondria irradiated with 750-40,000 Rad of X-radiation, found no inhibition of oxidative phosphorylation.

The mitochondrial uptake of Ca^{2+} and Sr^{2+} was inhibited by approximately 30% only at a dose of 50 kRad, while no inhibition occurred at doses of 10 and 25 kRad (Table II). This implies a threshold for this effect between 25 and 50 kRad. This is consistent with the observation that most physiological processes have a threshold for radiation damage, unlike mutation induction, which is believed to have no threshold. One would expect some dose proportional increase in radiation damage above the threshold. Wills (75) reports the inhibition of Ca^{2+} uptake by rat liver mitochondria irradiated *in vitro* from an electron beam at doses of 10, 20 and 50 kRad. Kalachova *et al.* (77) report the inhibition of Ca^{2+} uptake by pea mitochondria irradiated *in vivo* and *in vitro* by X-rays at doses of 5 and 10 kRad.

The apparent difference in radiosensitivity between my results and those reported elsewhere stems from the different mitochondrial sources used, incubation media and conditions, and most important, the fact that in my experiments the mitochondria were anaerobic during irradiation, which makes them more radioresistant. Upon irradiation, some free radicals produced by hydrolysis of water may remove a hydrogen atom from an organic molecule, leaving behind an organic radical. In the absence of O_2, the organic radical may combine with a hydrogen radical and the organic molecule is restored. But when O_2 is present, it competes with the hydrogen radical by combining with the organic radical to prevent restoration of the organic molecule. Thus the presence of O_2 causes more biological damage by creating more free radicals and by preventing the restoration of damaged biological molecules.

The nature of the inhibition of Ca^{2+} and Sr^{2+} uptake by bean mitochondria caused by 50 kRad of X-rays was investigated by measuring the uptake of the above ions at low temperatures, in the absence of substrate and the accompanying anion, in the presence of an inhibitor of oxidative phosphorylation (KCN) and an uncoupler of oxidative phosphorylation (DNP) (Tables III and IV). The above treatments cause the inhibition of either electron transport or the production of ATP and other high energy states,

thereby inhibiting the active, but not the passive or bound uptake of Ca^{2+} and Sr^{2+}. Therefore, if the active uptake is completely inhibited by radiation, and there is no radiation effect on passive transport, i.e., exclusively an active transport effect, no inhibition of the uptake of Ca^{2+} and Sr^{2+} by irradiated mitochondria should occur in the presence of metabolic poisons, uncouplers and other treatments in Tables III and IV. If active uptake is only partially inhibited by radiation, then one might expect some reduction in the magnitude of the radiation effect in the presence of inhibitors. Finally, if the effect is completely on passive diffusion, there will be no change in radiation effect with inhibitors. What we see is that the uptake is still significantly less in the irradiated mitochondria exposed to the inhibiting treatments, thereby suggesting that the radiation-induced inhibition of Ca^{2+} and Sr^{2+} uptake by bean mitochondria is on the passive component of that uptake. But Table V shows that there is an effect on active transport (measured as the difference between total and passive uptakes) also, which is slightly less than that on passive transport; it is, however, statistically significant. The inability of 50 kRad X-rays to inhibit respiration or uncouple the irradiated mitochondria suggests that the inhibition of the active component is related to changes in the carrier mechanism or binding sites and not due to alterations of bioenergetic processes.

Ruthenium Red is a membrane specific stain, and it is believed that it binds to the mucopolysaccharide components of these membranes (13,89). Ruthenium Red is also an inhibitor of both energy-dependent and passive and bound Ca^{2+} and Sr^{2+} uptake by mitochondria (13,89-92). It completely inhibited the uptake of the above ions in both irradiated and non-irradiated bean mitochondria. Therefore, we see that the ionic binding (adsorption) of Ca^{2+} and Sr^{2+} to the mitochondrial membrane is negligible because treatment with Ruthenium Red, which inhibits both energy-dependent and passive cation uptake, results in no detectable uptake (charge binding, for example).

The uptake of Ca^{2+} and Sr^{2+} by non-irradiated bean mitochondria is inhibited in the presence of each other; however, the magnitude of the competition effect was unaltered in irradiated mitochondria (Table VI). It has been proposed, by Johnson (13) for bean mitochondria and by Carafoli (93) for rat liver mitochondria, that Ca^{2+} and Sr^{2+} compete for the carrier and hence inhibit each other's energy-dependent uptake. Passive uptake of each ion is unaffected by the presence of other competing ions. If X-irradiation of mitochondria had inhibited the energy-dependent mechanism of Ca^{2+} and Sr^{2+} uptake, we might have observed a difference in Ca^{2+} or Sr^{2+} uptake between the irradiated and non-irradiated mitochondria

in the presence of the other ion. Likewise, effects on energy-linked processes and non-specific effects on the active transport of ions will not be expected to alter the competition between Sr^{2+} and Ca^{2+}. Table VI shows that there is no statistically significant difference in uptake of Ca^{2+} and Sr^{2+} in the presence of the other ion between the irradiated and non-irradiated mitochondria. Thus, it seems that the X-radiation effect is on both the active and passive components of cation uptake by bean mitochondria, which is in agreement with data from Table V, and that the effect on active transport is not linked to respiratory uncoupling or inhibition of electron transport. In addition, the X-radiation effect does not seem to alter the relative affinity of the carrier for the cation.

One of the important membrane-related functions in the mitochondria is that of swelling and contraction in both animal (2) and plant (4,13) mitochondria. Mitochondrial swelling in KCl is not dependent on endogenous energy-linked processes and is a passive, spontaneous process (15). Contraction of the mitochondria is dependent upon the addition of ATP and Mg^{2+} (4,94) or the addition of an oxidizable substrate (4,95).

The data in Table VII shows that 50 kRad X-irradiation has no significant effect on the swelling or on the

contraction of bean mitochondria. However, at doses of 250 kRad and higher, Joshi and Gaur (96) reported an increase in swelling and a decrease in contraction in irradiated bean mitochondria. The lack of a radiation effect in this study on mitochondrial swelling and contraction is probably due to the relatively low dosage applied. For example, Joshi and Gaur (96) had to use five times our maximum dose to get a measurable effect.

According to Bacq and Alexander (87), the major biological effect of ionizing radiation is the disruption of cellular and organelle membranes. Alteration of cellular permeability has been reported for different tissues by various authors (97-101). Cellular organelles also experience alteration of their permeability after exposure to ionizing radiation; these effects were reported by Wills (75) for mitochondria, Tarshis et al. (102) for nuclei, Wills and Wilkinson (103) for lysosomes and Brandes et al. (104) for tumor microsomes. In my experiments, the inhibition of mitochondrial uptake of Ca^{2+} and Sr^{2+} following 50 kRad of X-rays was found to be on both the "passive" component of that uptake, i.e. that component which is not dependent on electron transport or oxidative phosphorylation, and the "active" component, i.e. that component which is energy-dependent. This effect is unrelated to impairment of electron transport or energy coupling. It could be due to the lipid peroxides formed

upon irradiation, leading to the destruction of some membrane components (103,105,106,107) and the oxidation of sulfhydryl groups and formation of disulfide bridges (108), for restoration of permeability after radiation was accompanied by a decrease in the disulfide bridges under active metabolism (101,109,110). My conclusion is in agreement with several reports: Wills (75) reported inhibition of Ca^{2+} uptake in rat liver mitochondria by electron beams; Gorizontova and Trebenok (76) demonstrated a decrease in Mn^{2+} uptake by liver mitochondria after whole body irradiation; and Kalachova et al. (77) observed an inhibition in uptake of Ca^{2+} by X-irradiated pea mitochondria. All of these reports attribute the radiation effect on mitochondrial uptake to factors other than alterations in electron transport mechanisms or energy coupling.

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