Effect of X-Irradiation on Protein Synthesis in Pig Thyroid Microsomal Suspensions[1] (35844)

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The increasing use of ^{131}I for the treatment of Graves' disease has stimulated research into the nature of its effect on thyroid tissue. Unlike the small radiation doses required to cause reproductive death in mammalian cells, large doses (5000–20,000 rads) are required to suppress the function of the thyroid gland (1–3). Consequently, the specific lesion caused by therapeutic levels of ^{131}I appears to reside in some process other than reproduction *per se*. For example, cellular processes that might be affected are oxidative phosphorylation (4, 5), enzyme integrity, including alterations in constituent amino acids (6–10), DNA–histone interactions (11), integrity of cellular membranes (12), protein synthesis (13–15), and RNA synthesis (13). Since enzymes and other proteins regulate cellular metabolism, we studied the effect of X-irradiation on thyroid protein synthesis. Because earlier studies concerned with this problem failed to separate the effects of radiation on thyroid protein synthesis from other more generalized cellular effects (13, 14), we measured protein synthesis in irradiated, subcellular fractions obtained from thyroid tissue. We found significant depression of protein synthesis in subcellular particles with radiation doses of 50,000 and 100,000 rads.

Methods. Preparation of microsomal particles. Pig thyroids obtained from a local slaughterhouse were placed on ice and within 2 hr of slaughter were minced into 2 vol of cold homogenizing medium containing 0.05 *M* Tris buffer, pH 7.6; 0.25 *M* sucrose; 0.025 *M* KCl; and 5 m*M* $MgCl_2$. In addition the homogenizing medium contained 5 m*M* β-mercaptoethanol except where otherwise indicated. The mixture was homogenized for 2 periods of 15 sec each in a Sorvall OmniMixer at full speed. The resulting homogenate was centrifuged at 10,000*g* for 15 min and the pellet was discarded. The 10,000*g* supernatant was centrifuged at 110,000*g* for 1 hr. The resulting supernatant was used throughout this experiment without dilution unless otherwise indicated. The 110,000*g* microsomal pellet was resuspended in sufficient homogenizing medium to make 0.2 ml of suspension equivalent to 1 ml of thyroid tissue. On occasion, the pellet was further freed of supernatant protein by resuspending the microsomal pellet (from 25 ml of tissue) in 10 ml of Tris buffer and centrifuging at 100,000*g* for 1 hr. This preparation is referred to as washed microsomes. All operations were performed at 0–5°.

X-irradiation. The 0.25–2-ml suspensions of microsomal particles or supernatant solutions were irradiated in small plastic cups surrounded by an ice and water mixture. The depth of the liquid in each cup was uniform within each experiment, and varied from 2 to 4 mm from one experiment to another. The cups were covered with a thin plastic film to prevent evaporation. They were irradiated with a Picker superficial X-ray unit at 90 kVp; the beam had a half-value layer of 1.0 mm of aluminum. The doses were delivered at a rate of 1920 rads/min. After each delivery of 25,000 rads the cups were shaken and repositioned under the X-ray beam. Nonirradiated control suspensions were similarly treated except for irradiation.

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TABLE I. Some Factors Affecting Protein Synthesis in Microsomal Suspensions.

Alteration	Incorporation (dpm/tube)		(%)
Full system[a]	1825	1855	100[b]
Minus microsomes	79	79	4
Boiled microsomes	97	97	5
Minus ATP	163	143	8
Minus GTP	1305	1335	72
Plus GTP, 0.4 m*M*	1871	1729	98
Minus ATP and GTP	159	141	8
Nonincubated (zero time)	60	74	4
Puromycin, 1 m*M*	233	249	13
Actinomycin D, 40 μg/ml	1741	1749	95

[a] Full system was the standard incubation mixture described in the Methods section with incubation for 1 hr.

[b] Percentage of control based on average of duplicate assays.

Incubation. Incubations were carried out at 37° for various times in a total volume of 0.25 ml. Into each milliliter of the standard incubation mixture was placed the following: Tris buffer, pH 7.6, 25 μmoles; sucrose, 125 μmoles; KCl, 50 μmoles; $MgCl_2$, 6 μmoles; ATP, 5.0 μmoles; GTP, 0.4 μmoles; ^{14}C-Leucine (U), 1 μCi; microsomal particles from 1 ml of tissue (unwashed = 4 mg of protein; and washed = 2 mg of protein) suspended in 0.20 ml of the homogenizing medium; supernatant solution from 0.03 ml of tissue (6 mg of protein) dissolved in enough homogenizing medium to make a total of 0.10 ml.

The reaction was stopped by addition of a solution containing 5% TCA and 0.1% nonradioactive leucine. The resulting mixture was heated at 90° for 15 min. The precipitates were then poured onto glass filter discs, vacuum filtered, and each disc was further washed 6 times with 10-ml portions of cold 5% TCA containing 0.1% leucine. The discs were then placed into a glass counting vial and the precipitate was dissolved in 1% NaOH. After taking aliquots for determination of protein content by Lowry's method, the remainder was counted in a Packard liquid scintillation spectrometer. The counting efficiency was about 70%.

Results. Optimum conditions for protein synthesis in nonirradiated microsomal suspensions. Basic requirements for amino acid incorporation into the proteins of pig thyroid homogenates were similar to those reported for other species and organs (16, 17). Microsomal particles, an electrolyte solution, an energy source and β-mercaptoethanol were required (Tables I and II). The optimum amount of $MgCl_2$ added to the incubation mixture was 5–7 μmoles/ml while that of ATP was 5 μmoles/ml. An energy generating system consisting of ATP, phosphoenolpyruvate, and pyruvate kinase employed in preliminary experiments was not superior to 5 m*M* ATP and was discarded. Doubling of

TABLE II. Effect of β-Mercaptoethanol on Incorporation of ^{14}C-Leucine into Pig Thyroid Microsomal Suspensions.[a]

Alteration	Incorporation (dpm/tube)		(%)
0.005 *M* β-mercaptoethanol in homogenizing media only	1701	1849	100[b]
0.005 *M* β-mercaptoethanol in incubation media only	1303	1121	68
0.05 *M* β-mercaptoethanol in incubation media only	1730[c]		98
0.005 *M* β-mercaptoethanol placed into microsomal and supernatant fractions only immediately after final centrifugation	1606	1650	92
0.005 *M* β-mercaptoethanol in incubation and homogenizing media	1782	1740	99
No β-mercaptoethanol	380	392	22

[a] Incubation conditions are those described in Methods section with incubation for 1 hr.

[b] Percentage of control based on average of duplicate assays.

[c] One sample only.

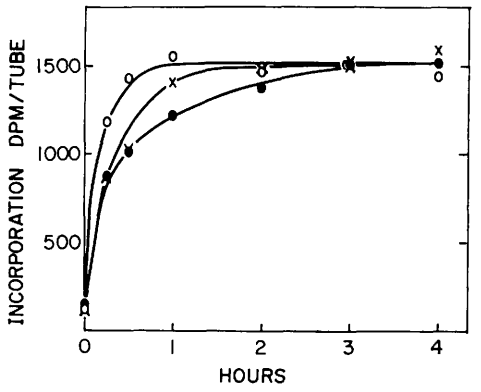

FIG. 1. Time course of radioactive leucine incorporation into pig thyroid microsomal suspensions. Experimental conditions are listed in the Methods section. All points from 15 to 60 min are averages of 2–4 assays: (- O) nonirradiated control; (-X) microsomal fraction irradiated with 50,000 rads; (- ●) microsomal fraction irradiated with 100,000 rads.

GTP concentration did not enhance amino acid incorporation, while omitting GTP had a minor effect (Table I). Puromycin treated, boiled microsome, and nonincubated (zero time) controls failed to incorporate appreciable amino acids. Actinomycin D failed to inhibit protein synthesis even at a concentration of 40 μg/ml (Table I). In nonirradiated control samples, incorporation of amino acids ceased after about 30–40 min (Fig. 1) and could not be increased when standard amounts of more ^{14}C-leucine, ATP, or supernatant fraction were added 1 hr after incubation under standard conditions. However, ^{14}C-leucine incorporation could be increased as much as 60% above the 1 hr control when additional microsomes alone were added 1 hr after incubation under standard conditions.

Amino acid incorporating ability was optimum in nonirradiated controls in the presence of about 2 mg of total protein/0.25 ml of incubation mixture (Fig. 2). Presumably some factor was deficient at lower concentrations while higher concentrations of endogenous leucine diluted the radioactive leucine. On the other hand, washed microsomal particles alone (0.5 mg of total protein) were able to synthesize protein quite well, presumably due to the presence of residual supernatant elements in that fraction (Fig. 2).

Protein synthesis in X-irradiated samples. Time course. After 50,000–100,000 rads of irradiation microsomal particles showed a reduced ability to incorporate radioactive leucine into protein (Fig. 1). Although the amount of reduction from the nonirradiated control values was significant ($p<0.01$) for both radiation doses when samples were incubated for 15–60 min, apparently there was less effect at 60 min; and no reduction was observed in samples incubated for 2–4 hr. Thus, radiation altered the microsomal fraction in some manner to reduce the initial rate of protein synthesis, but apparently the maximum synthesis was predetermined by some other factor(s) found both in the irradiated and nonirradiated microsomes. This phenomenon indicates the need to look for radiation effects on tissue homogenates after short incu-

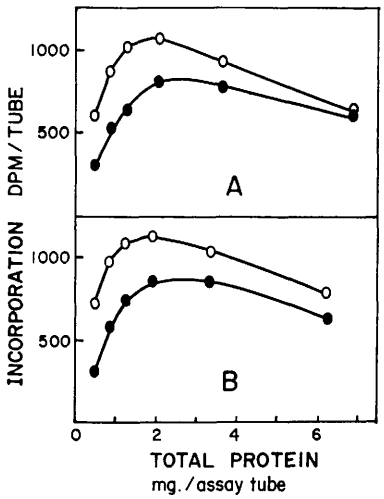

FIG. 2. Effect of supernatant concentration on X-ray inhibition of protein synthesis in pig thyroid microsomal suspensions. Experimental conditions are the same as listed in the Methods section, except for the varying protein concentration (the first point in each curve represents the presence of the washed microsomal fraction only); β-mercaptoethanol (10 m*M*) was placed in the incubation mixture only; and the incubation time was 30 min. Each point represents duplicate assays, from which a zero time sample was subtracted: (- O) nonirradiated samples; (- ●) irradiated with 100,000 rads; (A) increasing amounts of non-irradiated supernatant were incubated with irradiated microsomes; (B) mixtures containing a constant amount of microsomal fraction and various concentrations of supernatant solution were irradiated before incubation.

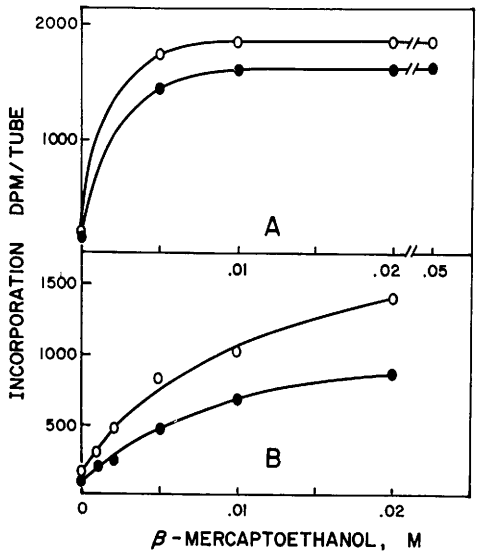

FIG. 3. Effect of β-mercaptoethanol concentration on X-ray inhibition of protein synthesis in pig thyroid microsomal suspensions. Experimental conditions were the same as in the Methods section except for a supernatant protein concentration of 0.75 mg/incubation tube, and incubation time of 30 min, and absence of β-mercaptoethanol in the homogenizing media. Each point represents two or more assays from which zero time incorporation has been subtracted. Microsomal and supernatant fractions were combined before irradiation in all samples. (- O) Non-irradiated; (- ●) irradiated with 100,000 rads. (A) β-Mercaptoethanol was added in the concentrations shown to the combined supernatant and microsomal fractions immediately after the final centrifugation. Subsequently, the samples were irradiated and incubated without additional β-mercaptoethanol. (B) β-Mercaptoethanol was added in the concentrations shown only during incubation.

bation times.

Effect of β-mercaptoethanol. Sulfhydryl containing compounds are required for protein synthesis in microsomal systems and are known to reduce radiation damage (10). Therefore, β-mercaptoethanol would be expected to decrease radiation damage while promoting an increase in protein synthesis. In nonirradiated samples, β-mercaptoethanol had a maximum effect when added to the homogenizing medium at a concentration of 5 m*M* before homogenization or when added to the incubation mixture at 10 times that amount (Table II). Further addition of β-mercaptoethanol slightly decreased protein synthesis. When β-mercaptoethanol was added to the supernatant and microsomal fractions at 5 m*M* immediately after centrifugation at 110,000*g*, the effect was only slightly less than maximum (Table II). Addition of β-mercaptoethanol (5 m*M*) both during homogenization and during incubation produced no better incorporation than when it was used alone at optimum levels at either time (Table II).

β-Mercaptoethanol added, even in small amounts, before irradiation with 100,000 rads apparently prevented most radiation damage, but did not completely overcome it even at higher concentrations (Fig. 3A). Both the irradiated sample and the nonirradiated control had near maximal incorporation with only 5 m*M* β-mercaptoethanol, which was added after the final centrifugation, in agreement with the data in Table II. On the other hand, β-mercaptoethanol added in increased amounts to the incubation mixture after irradiation (Fig. 3B) had little effect on radiation damage, which was more pronounced than in the previous experiment, when compared to the nonirradiated control.

Localization of radiation damage. X-Irradiation of only supernatant fraction with 100,000 rads produced no detectable effect on subsequent protein synthesis; in contrast, a definite effect was seen when only washed microsomes were irradiated (Table III). When both supernatant and microsomal fractions were irradiated separately, there was an enhanced inhibition of protein synthesis. This effect was even more pronounced when supernatant and microsomal fractions were combined before irradiation.

Effect of supernatant fraction on radiation damage. Addition of the proper amount of supernatant fraction not only determined the optimum amount of protein synthesis, but also affected the amount of radiation damage. When no supernatant fraction was added to the washed microsomal particles following irradiation, the incorporation of amino acids was depressed to 51% of that in the nonirradiated control; but when sufficient supernatant fraction (represented by increased protein concentration) was present in the incu-

TABLE III. Effect of X-Irradiation at Various Stages of Preparation on Protein Synthesis.[a]

Fraction irradiated	Incorporation (dpm/tube)		Incorporation (%)
Nonirradiated control	497	517	100[b]
Supernatant only	504	506	100
Microsomes only	370	410	77
Supernatant and microsomes irradiated separately	291	279	56
Supernatant and microsomes irradiated together	231	225	45

[a] Incubation conditions were the same as described in the Methods section except for an incubation time of 30 min, a supernatant protein concentration of 0.75 mg/incubation tube, the use of washed microsomes and 5 m*M* β-mercaptoethanol in the incubation mixture only. Fractions were irradiated with 100,000 rads.

[b] Percentage of control based on average of duplicate assays.

bation mixture, radiation damage was greatly reduced (Fig. 2).

In order to ascertain whether this sparing element was sensitive to radiation, a series of microsomal suspensions containing increasing concentrations of supernatant fraction were irradiated and then incubated with radioactive leucine in the usual manner. In the absence of the supernatant fraction the inhibition of protein synthesis was 60%, and again higher concentrations of supernatant solution reduced radiation damage (Fig. 2). The apparent inability of the irradiated supernatant to completely overcome radiation damage suggested that the sparing element was somewhat sensitive to radiation.

Discussion. Doses of 50,000–100,000 rads depressed thyroid protein synthesis by a cell-free system, primarily due to damage to the microsomal fraction. This effect was altered considerably by the experimental conditions. Postirradiation incubation time, when prolonged to several hours, obscured radiation effects (Fig. 1). The presence of β-mercaptoethanol during the isolation of tissue fractions virtually eliminated the effect of radiation, a result which was consistent with its known properties (6). On the other hand, addition of β-mercaptoethanol following irradiation, did not reverse the effect of radiation damage (Fig. 3B). The radiation-induced oxidation of sulfhydryl groups to easily reducible disulfide bonds does not entirely explain this effect of radiation. At these high doses, some of the damage might be due to the reduction of disulfide bridges (10) or to some irreparable damage to amino acids or other organic molecules (7–9).

In contrast to β-mercaptoethanol, some component of the supernatant fraction was able to reverse radiation damage to the microsomal fraction (Fig. 2A) and was still partially effective when the supernatant fraction was also irradiated (Fig. 2B). On the other hand, irradiation of the supernatant fraction alone did not decrease protein synthesis even when the supernatant was present in suboptimal amounts (Table III). These findings suggest the presence of a repair mechanism in the supernatant fraction which might be enzymatic in nature. Perhaps this repair mechanism accounts for the relative insensitivity of protein synthesis to radiation damage at doses in the therapeutic range of 5000–20,000 rads.

Earlier work of Kucan (18, 19) and more recently that of Ekert and Pattein (15) demonstrated that γ-irradiation reduced cell-free protein synthesis by ribosomal suspensions prepared from bacterial sources and utilizing synthetic messenger RNA. Both groups further increased the sensitivity to radiation by separating the ribosomes into their 50 S and 30 S subunits by means of reduced magnesium concentrations either before or after irradiation. They attribute the increased sensitivity to activation and release of an endonuclease from the 30 S subunit. Since our report deals with less refined fractions isolated from a mammalian source, using conventional magnesium concentrations (0.01 *M*), it would be difficult to compare our results with theirs; but it is interesting to note that in both cases radiation sensitivity

was markedly increased by further exposing the ribosomal system to a seemingly unnatural environment—either by dividing the ribosomes into subunits or by using supernatant concentrations well below the physiological range.

Summary. X-Irradiation of microsomal particles prepared from pig thyroid homogenates by conventional methods with 100,000 rads before incubation with radioactive leucine reduced protein synthesis as much as 60% of that of a nonirradiated control. The effect of the radiation was most pronounced at early incubation times (15–30 min) and at low concentrations of the supernatant fraction. β-Mercaptoethanol, which was necessary for protein synthesis, reduced the amount of X-ray damage to protein synthesis considerably when added before X-irradiation, but had minimal effect when added after X-irradiation.

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