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I am submitting herewith a thesis written by Mudamala Rajasekhar Reddy entitled "Effects of X-Irradiation on Protein Synthesis in L5178Y and Mouse Fetal Liver Erythroid Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

Joseph Fuhr, Major Professor

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and recommend its acceptance:

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Graduate Studies and Research

EFFECTS OF X-IRRADIATION ON PROTEIN SYNTHESIS IN L5178Y AND MOUSE FETAL LIVER ERYTHROID CELLS

A Thesis

Presented for the

Master of Science

Degree

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Mudamala Rajesekhar Reddy

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DEDICATION

This Thesis is affectionately dedicated to my mother and the loving memory of father.

ACKNOWLEDGEMENTS

The author is deeply grateful for his mother, sisters, brothers-in-law, and uncle, who have loved and supported him wholeheartedly in this work. His sincere gratitude is extended to Dr. Joseph Fuhr, his Major Professor, for his invaluable advice and moral support during the past one-and-one-half years. Appreciation is also extended to Dr. David Fox and Dr. Charley C. Congdon for their interest and critical review of this manuscript. The efforts and advice of the personnel at the University of Tennessee Memorial Research Center, especially that of Mr. William R. Conover, are greatly appreciated.

ABSTRACT

The *in vitro* effects of X-irradiation on protein synthesis have been studied in murine luekemic lymphoblasts (L5178Y) and fetal mouse liver erythroid cells. Responses resulting from different total doses of irradiation, as well as from various dose rates, were noted. RNA and protein synthesis in L5178Y cells were unaffected. Protein synthesis was inhibited by irradiation of fetal mouse liver erythroid cells of 13- to 14-day gestation. Hemoglobin synthesis was apparently inhibited irreversibly as the result of an earlier inhibitory effect upon RNA synthesis. As the result of time studies and effects of actinomycin D, the hypothesis has been advanced that inhibition of hemoglobin synthesis, at least in fetal mouse liver erythroid cells, can be attributed to a primary effect of irradiation upon transcription of messenger RNA. The radiosensitivity in the two cell types was compared by measuring the responsiveness to actinomycin D.

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I. INTRODUCTION

Exposure of biological systems to ionizing radiations has been known for some time to produce biological changes. The accelerated use of radioactive sources in industry, medicine, and research during the past decade has promoted a special interest in this subject. Although cell death has been correlated with the ability of ionizing radiations to produce chromosomal aberrations (Dewey *et al.*, 1971a, b; Carrano, 1973a, b), little is known about the biochemical mechanisms associated with radiation injury. Because the DNA of a cell contains the genetic information stored in the chromosome, extensive work has been done on the radiation effects on DNA metabolism (Kelly, 1957; Lajatha, 1960; Hahn and Hevesy, 1940). Relatively few results are available, however, on the radiosensitivity of RNA metabolism (Goutier, 1961) and even fewer on protein synthesis.

Recent advances in our understanding of protein synthesis at the molecular level are worth reviewing to understand the relationship between physiological radiation damage and protein synthesis. The cell's demand for protein results in the transcription of information coding for the protein from DNA into messenger RNA. This message is then translated into a specific protein in the cytoplasm on ribonucleoprotein particles, the ribosomes. The messenger RNA containing the information for a specific protein is transported into the cytoplasm, where it is bound to the ribosomes and where it directs the placement of aminoacyl transfer RNA's in the proper sequence. An amino acid which has been activated and esterified to a specific species of tRNA

is bound to the ribosomal acceptor site by virtue of codon-anticodon interactions. Peptidyl transferase, an integral part of the ribosome, catalyzes the formation of a peptide bond between the carboxyl group of the nascent peptide and the amino group of the newly attached amino acid. The resultant peptidyl tRNA is translocated to the donor site by a GTP-requiring enzyme, thus freeing the acceptor site for the attachment of the next aminoacyl tRNA (Watson, 1970). Any radiation-induced alterations of chromatin structure, DNA integrity, or DNA replication could lead to an altered gene readout. Eventually, such damage can alter protein synthesis.

Clear-cut demonstrations reflecting the effects of X-irradiation on protein synthesis are scanty. Hevesy (1949) observed an increased incorporation of labeled methionine into proteins of the liver and the small intestine of irradiated animals. The subcellular fractions of liver (nuclei, microsomes, and mitochondria) taken from irradiated animals possessed an increased potential for labeled amino acid incorporation (Butler *et al.*, 1957; Kay and Entenman, 1956). Hedvegi *et al.* (1968), in a study of rats irradiated with a dose between 1000 and 3000 rads, found that the incorporation of labeled amino acid was enhanced by liver polyribosomes. These authors further reported that the protein synthesizing ability of the liver ribosomal systems paralleled the enrichment of polysomes after X-irradiation (Hedvegi *et al.*, 1970).

Hedvegi *et al.* (1968) proposed two possibilities to explain the radiation-induced increase in the protein synthesis of liver cytoplasm. One possibility was that X-irradiation could cause an

increased stability of pre-existing polysomes, perhaps due to certain factors that maintain polysome integrity or to decreased activity of an enzyme involved in the catabolism of polysomes. The other possibility was that irradiated cells contain more messenger RNA and ribonucleoproteins (rRNA) as a result of the increased synthesis of these components. Such an increase in mRNA and rRNA would account for their greater synthesizing capacity.

Mukergee and Goldfeeder (1974b) observed a dose- and time-dependent increase in ^{14}C-Leucine incorporation into the livers of X-irradiated mice, but found no change in polysome distribution one hour after the administration of 2000 rads (Mukergee and Goldfeeder, 1974a, b). These authors thought that the enhanced protein synthesis in X-irradiated liver was due to the increased transport of amino acids through the plasma membrane into the intracellular precursor pool. However, other laboratories have observed that the ribosome content of normal rodent liver responded to whole body doses of ionizing radiation (550-6000 rads) by a shift to heavier polysomes which reached a maximum at 10-15 hours after exposure (Baeyens and Goutier, 1968; Cammarano *et al.*, 1969). This radiation-induced increase in heavy polysomes was accompanied by enhanced incorporation of amino acids into nascent polypeptides on the isolated ribosomes *in vitro* (Corless and Gray, 1967; Metlas *et al.*, 1969; Hedvegi 1970).

Contrary to the above reports, some laboratories have noted conflicting results for cells in culture (Kim *et al.*, 1970; Bases *et al.*, 1970). Kim *et al.* (1970) have shown that irradiation of

asynchronously growing populations of HeLa cells at a dose of 2000 rads reduced protein synthesis by 60% within a two-hour period. They attempted to localize the site of inhibitory action of radiation on protein synthesis by exposing the cells for two or six minutes to labeled Valine. The deoxycholate-treated fraction was centrifuged in a 10-40% linear sucrose density gradient. Specific activity of the polyribosomal fraction was approximately the same in both control and irradiated cells. However, the total radioactive amino acid incorporation into proteins during the two- and six-minute pulse labels was significantly lower in irradiated cells (Kim *et al.*, 1970). A small radiation-induced decrease of polysome content was observed in HeLa cells (Bases *et al.*, 1970). Likewise, no change was seen in the polysome content of Chinese hamster cells at 800 rads (Enger *et al.*, 1973). The reduction in the uptake of labeled amino acid was caused by the reduced rate of release of newly synthesized polypeptides from polysomes after irradiation (Kim *et al.*, 1970).

Attempts have been made to link radiation-induced mitotic delay to effects on protein synthesis. Mitotic delay caused by radiation occurred at the middle of the cell's G2 period in several mammalian cell types (Walters and Peterson, 1968a; Dioda and Okada, 1968; Bachetti and Sinclair, 1971). When Dioda and Okada (1968) found that puromycin induced a mitotic block similar to that caused by X-irradiation in L5178Y cells, they concluded that the block involved interference with the synthesis of a protein or proteins necessary for cell entry into mitosis. A similar conclusion was reached by Walters and Peterson (1968a) in their study of Chinese hamster ovary cells

treated with cycloheximide. Fantoni *et al.* (1971) reported that yolk sac-derived erythroid cells of C_{57}BI/Cas fetal mice irradiated on the tenth day of gestation were mitotically blocked between the twelfth and thirteenth day of gestation. Concurrently, the irradiated cells synthesized hemoglobin at a rate twice that of unirradiated cells. To explain this difference, Fantoni *et al.* (1971) postulated that at day 13 the mitotically-blocked cells maintained a full complement of messenger RNA-ribosomal complexes active for hemoglobin synthesis, but that in normally dividing erythroid cells, these complexes were cleaved in the course of cell division.

In addition to the enhancement and inhibition, the early studies of Hevesy (1949), Altaman *et al.* (1949), Hempleman *et al.* (1950), and Abrams (1941) revealed only small changes or the absence of any effect on protein synthesis in rat tissues after sublethal and lethal doses of X-rays. Thus, there is no satisfactory information regarding the molecular events responsible for, or the effects of, X-irradiation on protein synthesis.

In view of the inconsistencies in the literature, the initial purpose of this thesis was to study the effects of X-irradiation on protein synthesis in two distinct cell types (L5178Y and mouse fetal liver erythroid cells) under similar experimental conditions. Time studies and metabolic inhibitors, such as actinomycin D, were employed in an effort to determine the molecular mechanism of X-irradiation effects on sensitive cells. The fetal mouse liver erythroid cells and L5178Y cells were considered to be suitable for comparative studies because of their different mRNA half-lives. The mRNA in

erythroid cells is stable, even though pool size may be increasing, whereas a nine and one-half-hour half-life of mRNA is characteristic of L5178Y cells.

II. MATERIALS AND METHODS

L5178Y Cells

The murine leukemic lymphoma (L5178Y) cell line and its method of culture in Fisher's medium and 10% horse serum have been described (Yang and Vas, 1970). The studies were performed at a cell concentration of approximately 1 x 10^6 cells/ml.

Fetal Mouse Liver Erythroid Cells

Livers were obtained from the embryos of C3H mice at 13-14 days of gestation. Cells were disaggregated by repeated, gentle aspiration through a pasteur pipette to provide a separated cell suspension in Eagle's essential medium + 5% fetal calf serum (Dunn and Napier, 1976). The cell suspensions were diluted to 2.4 x 10^6 cells/ml.

In individual experiments, 3.0 ml aliquots (L5178Y) and 1 ml aliquots (fetal mouse liver erythroid cells) were removed from the main culture flasks to individual test tubes for short-term studies on protein synthesis. The radioactive precursors ^{3}H-Leucine and ^{3}H-Uridine (5 mCi/0.013 mg; 5 mCi/0.030 mg; New England Nuclear, Boston, MA) were used at a final concentration of 1.6 μCi/10^6 cells. Dibutyryl cAMP and actinomycin D were purchased from Calbiochem, Los Angeles, CA. Actinomycin D was used to inhibit DNA-dependent RNA synthesis. Reactions were terminated by transferring 0.5 ml (L5178Y) and 1 ml (fetal mouse liver erythroid cells) aliquots at the designated times to tubes containing 5 ml ice-cold physiological

saline. The cells were pelleted by centrifugation and then resuspended in 1.0 ml of water. Total protein and RNA was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA). The precipitates of protein were heated to 90° for 20 minutes to disrupt amino acid-tRNA complexes. The RNA precipitates were cooled at 0° for 20 minutes.

After cooling, the precipitates were collected on Millipore filters and washed repeatedly with 5% TCA. The amount of radioactivity incorporated was determined in a Packard liquid scintillation counter after the protein and RNA had been dissolved with 0.2 ml of 88% formic acid and Scintiverse (Fisher Scientific) was added.

In another set of experiments, fetal mouse liver erythroid cells were incubated overnight after irradiation. The cells were counted and protein synthesis measured, as mentioned previously.

The rates of synthesis of individual hemoglobin polypeptides were determined in a third set of experiments. Peptides were separated, as described by Clegg *et al.* (1966). Reactions were terminated by adding excess amounts of ice-cold isotonic saline. The cells were pelleted and lysed in distilled water, and hemoglobin carrier was added. Globin was collected by centrifugation. The protein was redissolved in 5 ml of water and lyophilized overnight. Approximately 35 mg of free-dried globin was then dissolved in a starting buffer (pH 6.8) consisting of 0.01 M phosphate, 0.048 M mercaptoethanol, and 8 M urea, which had been deionized by passage through a column of mixed bed resin (Ag 501 x 8D). The redissolved globin was dialyzed for two hours against 100 ml of starting buffer (SB). The

SB was changed after one hour to ensure the correct ionic strength of the sample. The sample was applied to a 12 ml column of carboxymethyl cellulose (Whatman CM 23), which had been equilibrated in SB for one hour. After each sample had been washed into the resin with 80 ml SB, the globin polypeptides were eluted with a linear gradient of 0.01-0.035 M phosphate. Elutions were monitored every 60 seconds at 280 nm by a Gilford model 2000 spectrophotometer. The fractional volumes containing globin were pooled and desalted by passage through Sephadex G-25. The absorbance of eluates was determined at 280 nm with a Zeiss spectrophotometer. A 1-ml aliquot from the peak tube was counted directly in Scintiverse, and the specific activity was calculated.

Hyperthermia

Sample tubes were heat-shocked by immersion in a water bath calibrated for temperature. The large quantity of water (35 liters) in the bath helped to minimize fluctuations in temperature to $\pm 0.1^{\circ}$ of that desired.

Irradiation Techniques

The cells in plastic suspension culture tubes were irradiated with 250 KV X-rays. A constant potential X-ray therapy unit (GE Maximizer 250 III) operated at 15 mA, with 1 mm of Al filtration with a half-value layer (HVL) of 0.14 mm of copper. The dose was delivered at the desired rate, as measured with a Victoreen 250R chamber.

III. RESULTS

Effect of X-Irradiation on Protein Synthesis in L5178Y Cells

Table 1 and Figure I show the incorporation of ^{3}H-Leucine in asynchronously growing L5178Y cells that had been irradiated at doses ranging from 500-3000 rads. There was no significant change in incorporation during the five-hour post-irradiation period. The rate of incorporation (slope of the line) in irradiated cells was essentially the same as in the control cells. Protein synthesis was not significantly altered when dose rates were changed from 38 to 608 rads/min (Table 2).

Williams (1976) has reported that resting or non-cycling cells, as typified by plateau-phase cultured cells, have additional recovery capacity beyond that of exponentially growing cells. Suspensions of plateau and exponential phase cells were exposed to 1000 rads. As shown in Figure II, no change in the incorporation of labeled amino acid into proteins of cells with different growth rates was demonstrated. Though irradiated cells appeared to have a tendency for inhibition, the values presented are statistically insignificant.

Subsequently, the effect of X-irradiation in conjunction with metabolic inhibitors was examined (Table 3). The literature contains sufficient evidence to establish that hyperthermia increases radiation sensitivity of a wide variety of biological systems (Overgard and Overgard, 1972; Mukle and Dickenson, 1973). Therefore, the cell suspensions were exposed to 44° for ten minutes immediately after irradiation. The data in Table 3 show the absence of a radiation

TABLE 1

EFFECT OF DIFFERENT DOSES OF X-IRRADIATION ON PROTEIN SYNTHESIS IN MURINE LEUKEMIC LYMPHOBLASTS (L5178Y)*

Dose	^{3}H-Leucine Incorporation (% of Control)			
	60 Minutes	120 Minutes	240 Minutes	300 Minutes
Control	100	100	100	100
500 rads	102 ± 3.08	104 ± 3.85	98 ± 7.9	95.9 ± 2.6
1000 rads	92 ± 3.2	103 ± 3.7	95 ± 3	100 ± 3.6
2000 rads	99 ± 2.2	102 ± 1.88	104 ± 3.7	101 ± 2.39
3000 rads	99 ± 2.01	102 ± 2.7	104 ± 3.07	99 ± 3.3

*After irradiation ^{3}H-Leucine was added, and all suspensions were incubated at 37°. Aliquots were taken at indicated intervals, and radioactivity in acid precipitable material was determined. Results of the different incubations (N=4) are expressed as percentage of controls of their respective reactions. The figures represent mean and standard error of the mean.

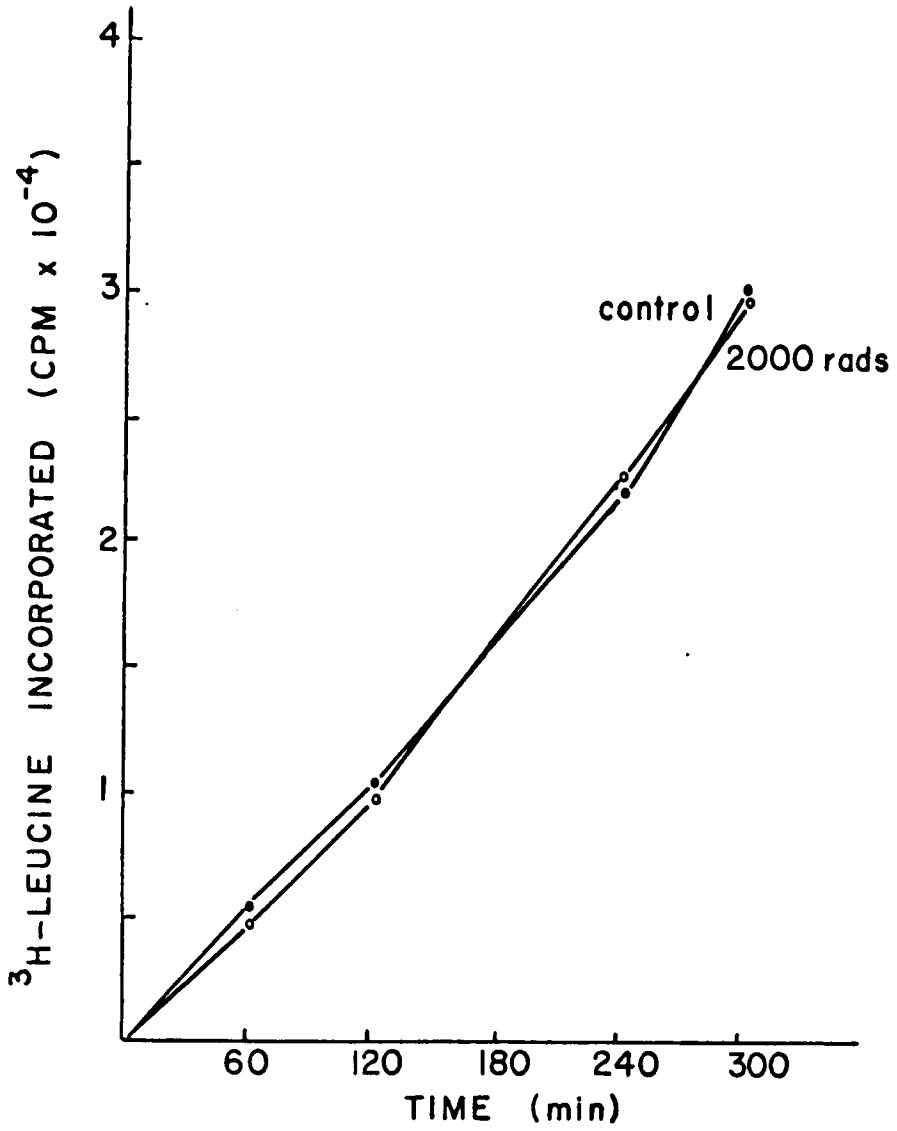


Figure I. Time course of protein synthesis in L5178Y cells after X-irradiation.*

*Murine lymphoma (L5178Y) cell suspensions were exposed to X-irradiation. Following irradiation ^{3}H-Leucine was added, and all suspensions were incubated at 37°. Aliquots were taken at indicated intervals, and radioactivity in acid precipitable material was determined.

TABLE 2

EFFECTS OF DIFFERENT DOSE RATES OF X-IRRADIATION ON PROTEIN SYNTHESIS IN L5178Y CELLS*

	^{3}H-Leucine Incorporation (% of Control)			
Dose Rate	60 Minutes	120 Minutes	240 Minutes	300 Minutes
Control	100	100	100	100
38 r/min	98 ± 7.8	99 ± 2.1	95 ± 4.8	96.6 ± 1.21
163 r/min	93 ± 1.6	100 ± 1.24	92 ± 1.02	96.6 ± 3.0
350 r/min	99 ± 3.25	97 ± 1.3	96 ± 8.3	97 ± 1.07
608 r/min	102 ± 2.27	94 ± 1.5	97 ± 1.21	97 ± 8.8

*Cell suspensions were irradiated at a constant dose (1000 r) with different dose rates. After irradiation ^{3}H-Leucine was added. Aliquots were taken at indicated intervals, and radioactivity in acid precipitable material was determined. Results of the different incubations (N=3) are expressed as percentage of controls of their respective reactions. The figures represent mean and standard error of the mean.

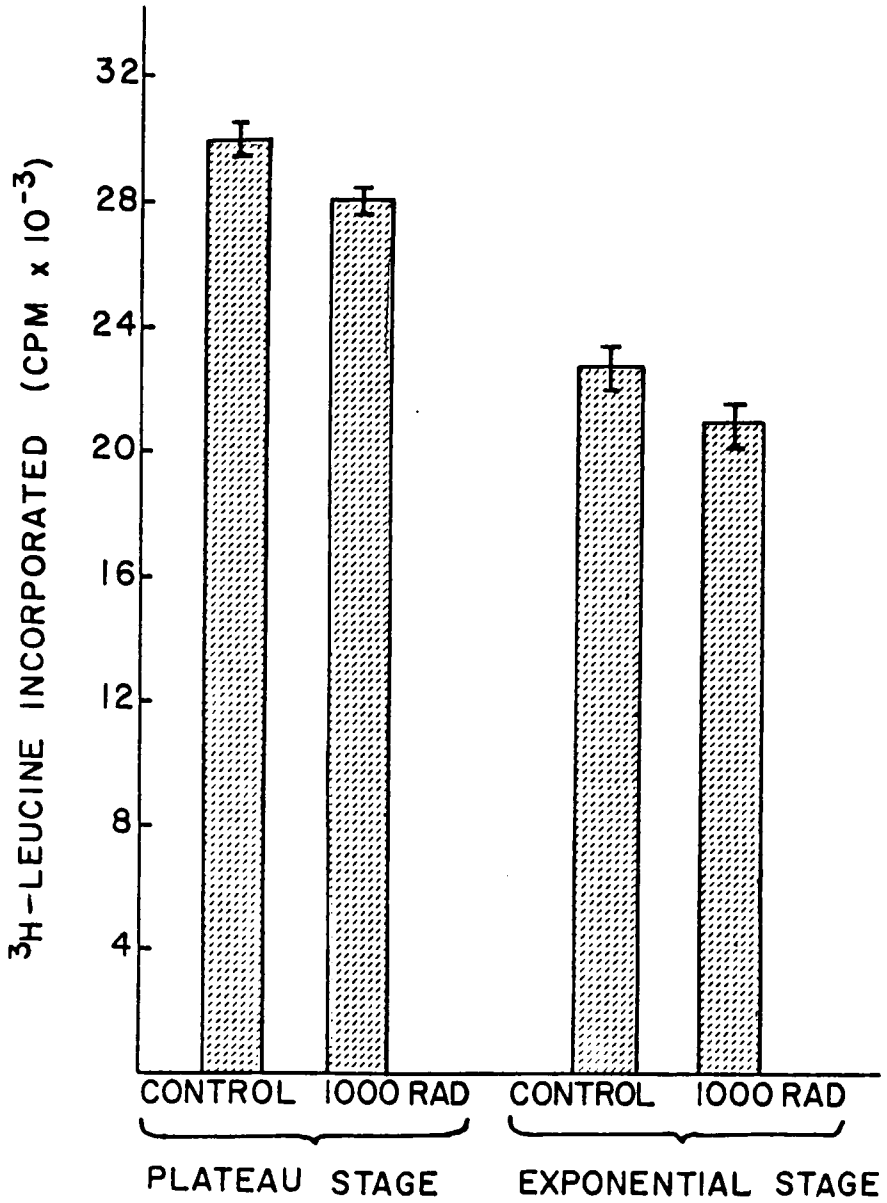


Figure II. Effect of X-irradiation on protein synthesis in murine leukemic lymphoblasts (L5178Y) at different growth rates.*

*The cell suspensions of exponential and plateau stages were exposed to X-irradiation (1000 rads). ^{3}H-Leucine was subsequently added, and all suspensions were incubated at 37° for five hours. Aliquots were then taken and assayed for radioactivity in acid precipitable material. Each bar and line represents the mean and standard error of the mean of two different experimental determinations of ^{3}H-Leucine incorporation.

TABLE 3

EFFECT OF X-IRRADIATION ON PROTEIN SYNTHESIS IN L5178Y CELLS EXPOSED TO HYPERTHERMIA, ACTINOMYCIN D, AND DB cAMP*

Dose	^{3}H-Leucine Incorporation (CPM)			
	30 Minutes	60 Minutes	120 Minutes	240 Minutes
Control	1614	2725	5965	9989
1000 rads	1683	3309	7135	10381
Actinomycin D	1321	2331	3854	5202
1000 rads + Actinomycin D	1013	2496	3968	4766
db cyclic AMP	1941	3632	6521	10731
1000 rads + db cyclic AMP	1987	3304	7037	11006
Hyperthermia	730	1464	3994	10102
1000 rads + hyperthermia (44°, 10 min)	675	1254	3517	9450

*After irradiation, cell suspensions were heat-shocked for 10 min at 44°. ^{3}H-Leucine was then added, and all suspensions were incubated at 37°. Aliquots were collected at indicated intervals, and radioactivity in acid precipitable material was determined. Cells were incubated for 20 min at 37° in the presence of the compounds listed above before X-irradiation and the addition of ^{3}H-Leucine.

effect on protein synthesis in heat-shocked cells. Cell suspensions were also incubated with actinomycin D, an inhibitor of DNA-dependent RNA synthesis, for 20 minutes before irradiation. X-irradiation had no effect on protein synthesis in actinomycin-inhibited cells. Yang and Wang (1974) reported that db cAMP induced an increase of viral particles in this cell line. In view of their studies, the possibility that radiation stimulated viral particles and virus-directed protein synthesis was examined. Cell suspensions were incubated with db cAMP for 20 minutes and were exposed to X-irradiation. The results presented in Table 3 demonstrate no effect on db cAMP action.

RNA synthesis in irradiated cells was examined in an attempt to evaluate the molecular events responsible for radiation resistance of protein synthesis. As shown in Table 4, there was no significant change in the incorporation of ^{3}H-Uridine.

Effect of X-Irradiation on Protein Synthesis in Fetal Mouse Liver Erythroid Cells

^{3}H-Leucine incorporation into proteins was significantly inhibited in day 13 fetal mouse liver erythroid cells within two hours after irradiation (Figure III). As the dose of X-irradiation was increased, protein synthetic activity was further inhibited. Leucine incorporation was inhibited to 57, 51, and 43% of control values at 100, 500, and 1000 rads, respectively, during a four-hour post-irradiation period. In order to determine the long-term effects of radiation, the cell suspensions were cultured for 24 hours after irradiation. The labeled precursor was then added and the cells

TABLE 4

EFFECT OF X-IRRADIATION ON RNA SYNTHESIS IN MURINE LEUKEMIC LYMPHOBLASTS (L5178Y)*

	^{3}H-Uridine Incorporation (CPM)			
Dose	30 Minutes	60 Minutes	120 Minutes	240 Minutes
Control	1572 ± 5.4	5158 ± 3.1	7214 ± 5.0	17416 ± 7.6
500 rads	1733 ± 4.6	5141 ± 3.8	7209 ± 3.0	17195 ± 5.1
1000 rads	1609 ± 3.7	5225 ± 3.9	6866 ± 1.2	16835 ± 4.7
2000 rads	1579 ± 3.5	4846 ± 2.4	6398 ± 4.1	18093 ± 1.2

*After irradiation ^{3}H-Uridine was added, and all suspensions were incubated at 37°. Aliquots were taken at indicated intervals, and radioactivity in acid precipitable material was determined. Figures represent the mean and standard error of mean of triplicate determinations of ^{3}H-Uridine incorporation.

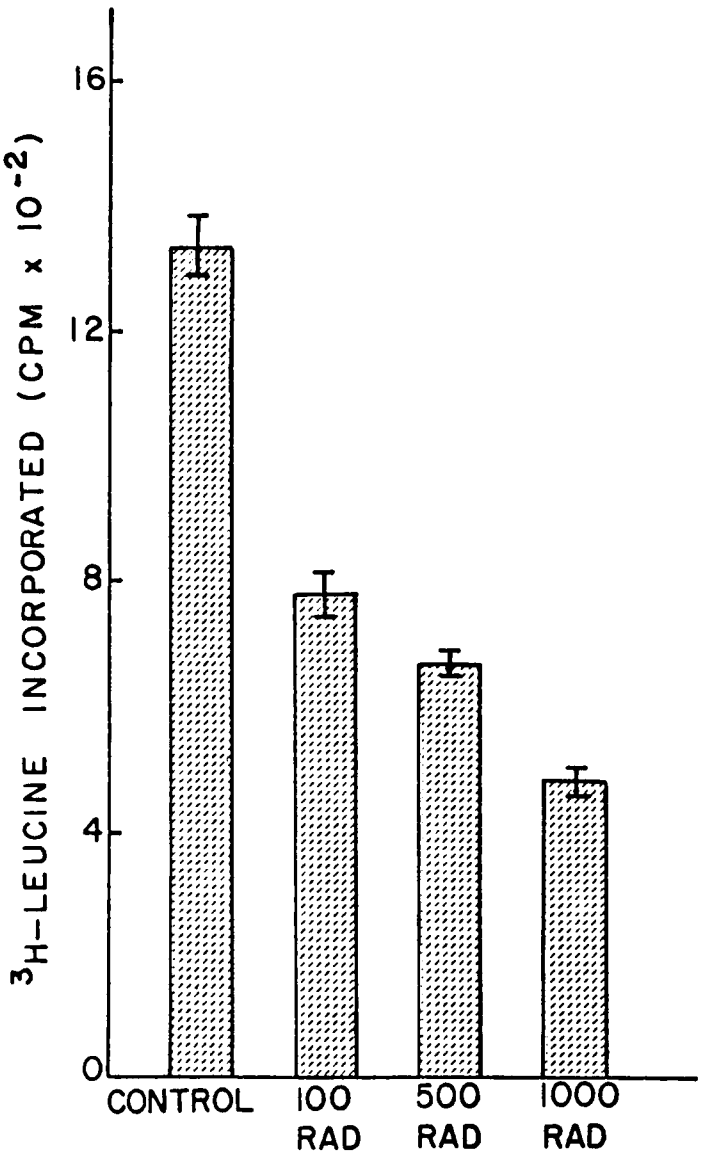


Figure III. Effect of X-irradiation on protein synthesis in fetal mouse liver erythroid cells incubated at 37° for four hours.*

*After irradiation ^{3}H-Leucine was added, and all suspensions were incubated at 37° for four hours. Aliquots were then assayed for radioactivity in acid precipitable material. Each bar and line represents the mean and standard error of the mean of triplicate determinations of ^{3}H-Leucine incorporation.

incubated for an additional two-hour period. Protein synthesis was inhibited by 57.6, 50.3, and 40% below control values at the doses of 100, 500, and 1000 rads, respectively (Figure IV).

The synthesis of individual polypeptide chains of hemoglobin was examined to determine whether radiation exhibited any selectivity of inhibition. Fetal mouse liver erythroid cells were exposed to 500 rads. After incubating the cells for four hours with ^{3}H-Leucine, globin was prepared and separated into individual polypeptide chains, as described in Section II. In Figure Va and b, the separation of globin polypeptide chains by ion exchange chromatography is shown. The broken line represents the absorbance at 280 nm of material continually eluting from a typical column at increasing ionic strengths. The solid line represents the amount of radioactive leucine in each polypeptide chain. As indicated in the figures, the radioactive precursor was incorporated into polypeptides of both control and irradiated samples. However, the amount of incorporation was less in the irradiated samples than in control cells. These results confirm the validity of the faster assay used above and indicate that ^{3}H-Leucine was incorporated into completed globin chains. After comparison of specific activities of the peak tubes (Table 5), no selectivity of irradiation-induced inhibition on polypeptide chains was observed.

The time course of radiation-induced inhibition of protein synthesis can be evaluated by the results from a typical experiment presented in Figure VI. The protein synthetic activity in cells exposed to X-irradiation, either with or without actinomycin D, was compared to the activity in unirradiated cells containing actinomycin

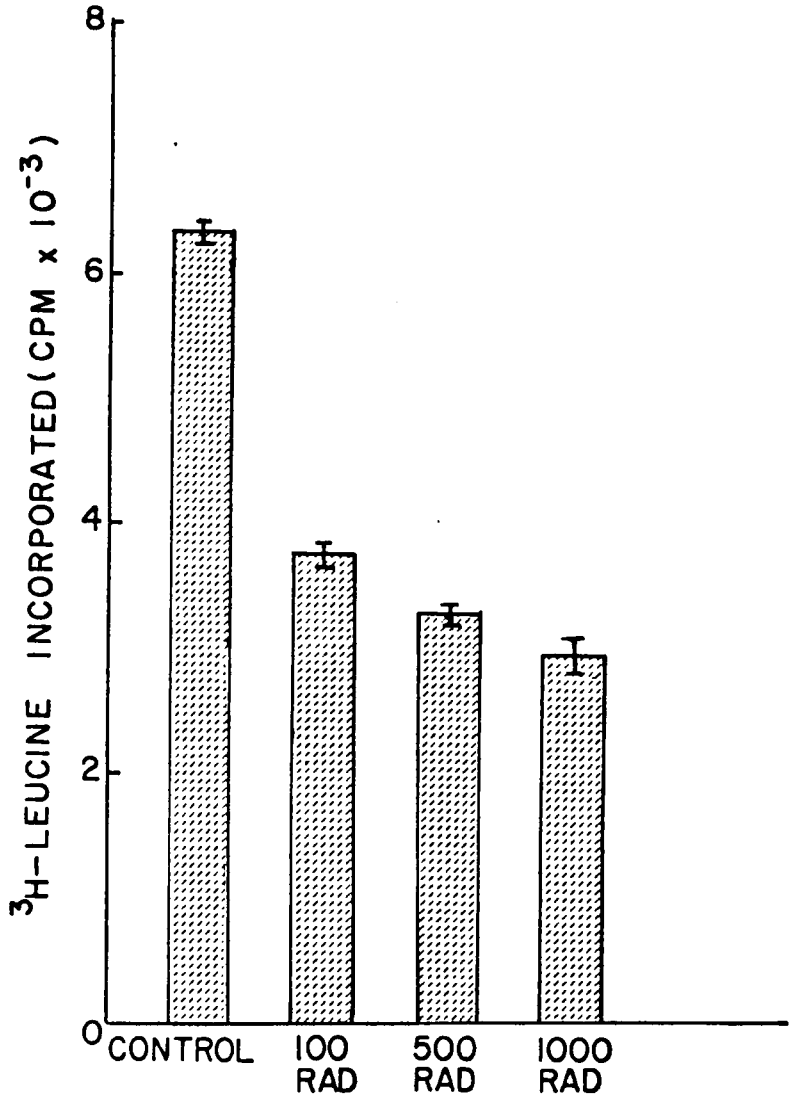
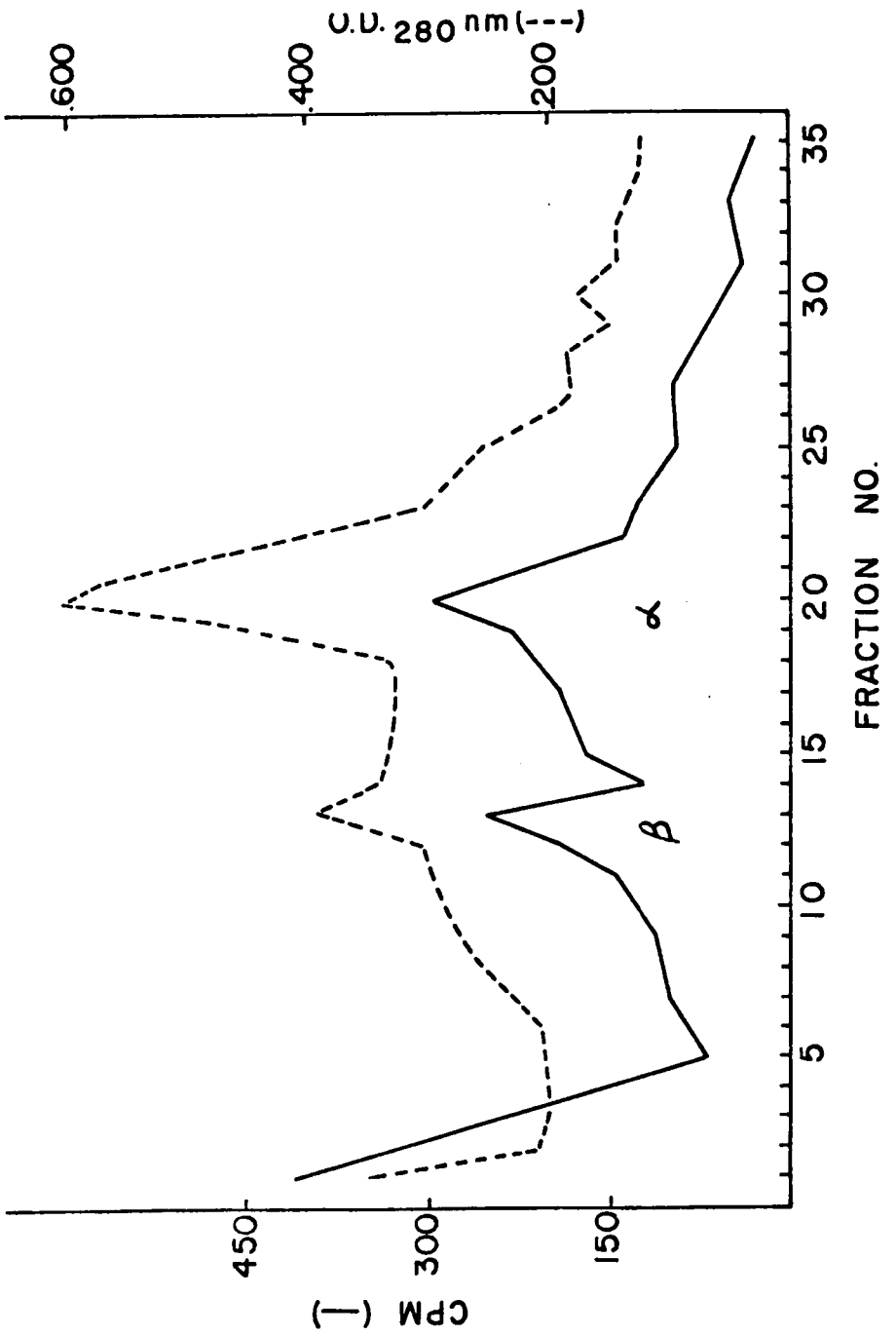


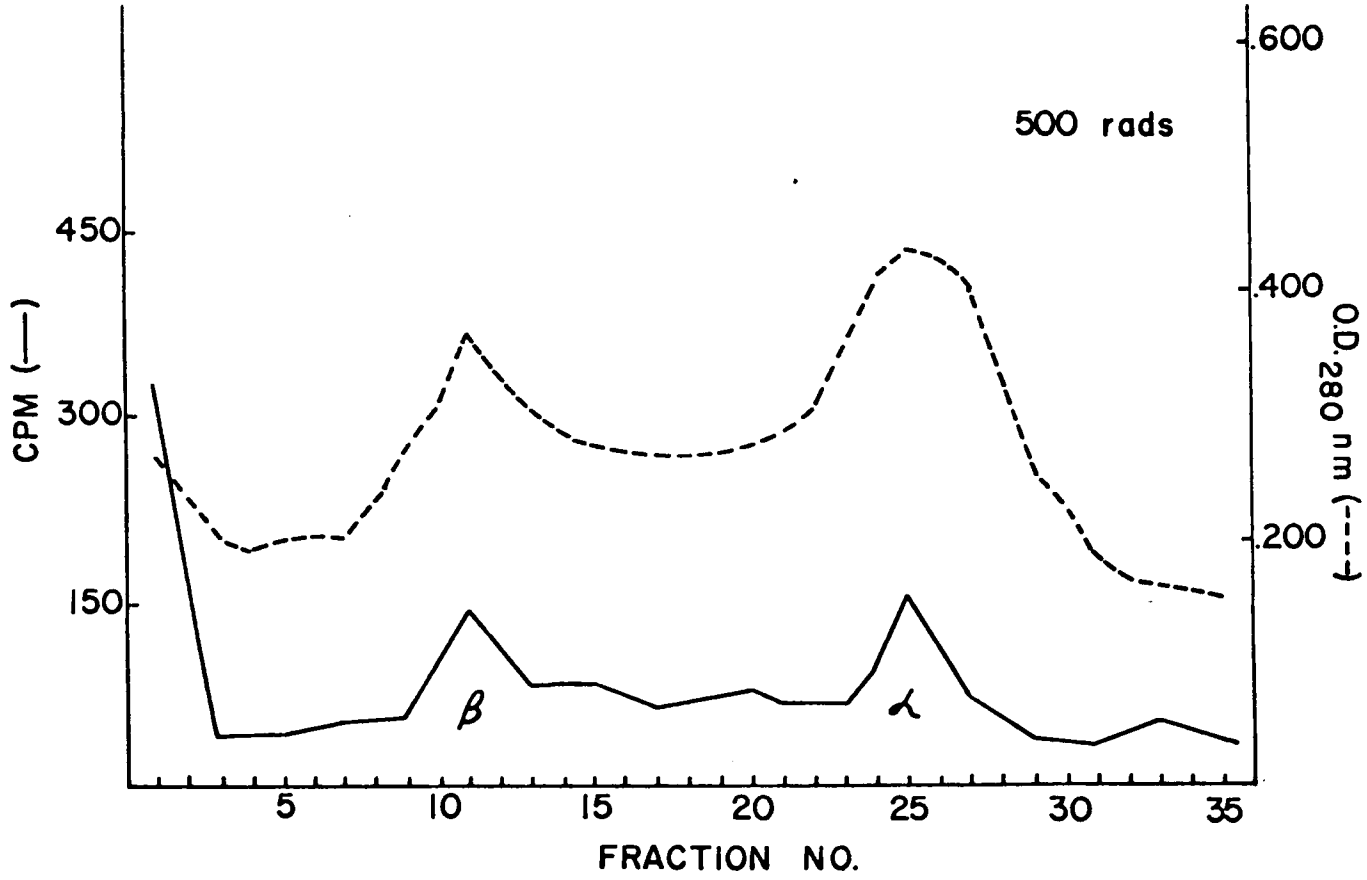
Figure IV. Effect of X-irradiation on protein synthesis in fetal mouse liver erythroid cells cultured 24 hours at 37°.*

*After irradiation the cells were cultured 24 hours at 37°. ^{3}H-Leucine was then added, and the reaction proceeded for two additional hours. Aliquots were then taken and assayed for radioactivity in acid precipitable material. Each bar and line represents the mean and standard error of the mean of triplicate determinations of ^{3}H-Leucine incorporation.



a. Control

Figure V. Separation of globin polypeptides by ion exchange chromatography.*



b. 500 rads

Figure V. (Continued)

*Cell suspensions were exposed to 500 rads. ^{3}H-Leucine was subsequently added, and all suspensions were incubated at 37° for 240 min. Globin was then prepared and separated into individual polypeptide chains, as described in Section II. The broken line (--) represents the absorbance of material continually eluting from a typical column at increasing ionic strenghts. The solid line represents the amount of incorporation of radioactive precursor into each polypeptide chain during the 240-min incubation following irradiation.

TABLE 5

EFFECT OF X-IRRADIATION ON THE SYNTHESIS OF INDIVIDUAL HEMOGLOBIN POLYPEPTIDES*

	Specific Activity (CPM/OD280)		
Dose	β	α	α/β
Control	1402	1272	0.90
500 rads	622	649	1.04

*Fetal mouse liver erythroid cell suspensions were exposed to 500 rads. Cell suspensions were incubated in the presence of ^{3}H-Leucine for 240 min. Globin was then prepared and separated into individual polypeptides, as described in Section II. Peak tubes from CMC-urea column were pooled and desalted by passage through Sephadex G-25. The absorbance of void volume eluates was determined at 280 nm. One ml aliquots were counted directly in Scintiverse, and specific activities were calculated.

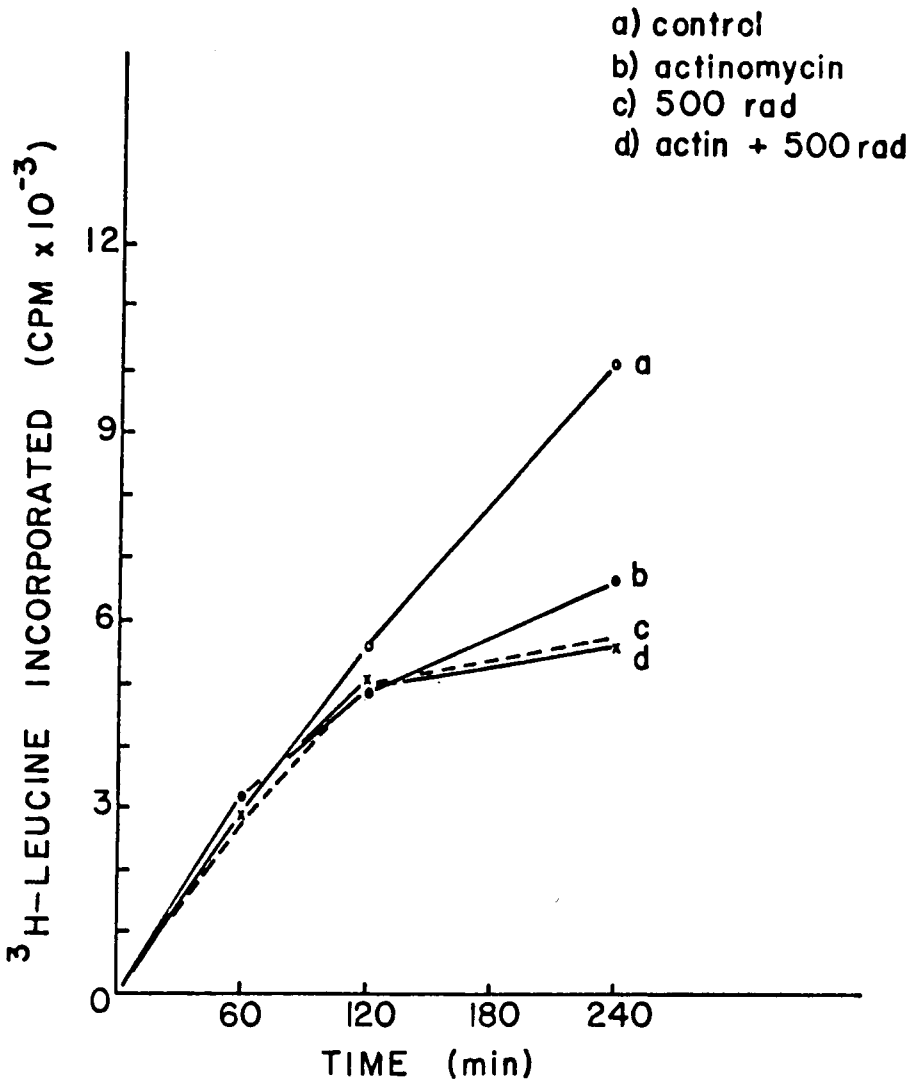


Figure VI. Time course of protein synthesis in fetal mouse liver erythroid cells after X-irradiation.*

*Fetal mouse liver erythroid cell suspensions were exposed to 500 rads and or Actinomycin D (4 ug/ml). ^{3}H-Leucine was subsequently added. Aliquots were taken at indicated intervals for the determination of radioactivity in acid precipitable material.

D. By 100 minutes after irradiation, the rate of incorporation (slope of the line) in the cells, with or without actinomycin D, was essentially the same as in the control cells. At 240 minutes, however, protein synthesis was reduced to almost 50% of control values in irradiated cells with or without actinomycin D (lines d and c). After 240 minutes, actinomycin D reduced protein synthesis in the unirradiated cells by 36%. Although a slight decrease in incorporation was noted in cells which had been both irradiated and treated with actinomycin D, the inhibition was not significantly different from the level observed in cells treated with actinomycin alone.

In an attempt to determine the role of RNA synthesis in the radiation-induced inhibition of protein synthesis, separate cell suspensions were incubated with ^{3}H-Leucine and ^{3}H-Uridine. Aliquots were collected at indicated intervals. The ^{3}H-Uridine incorporation was reduced by 18% of the control values, while ^{3}H-Leucine incorporation was unchanged 60 minutes after irradiation. However, at 120 minutes RNA synthesis was reduced by 40% and protein synthesis by 10% of control values. The rate of inhibition (slope of the line) of RNA synthesis was more rapid than the inhibition of protein synthesis (Figure VII). However, 180 minutes after irradiation, both RNA and protein synthesis reached a plateau of inhibition.

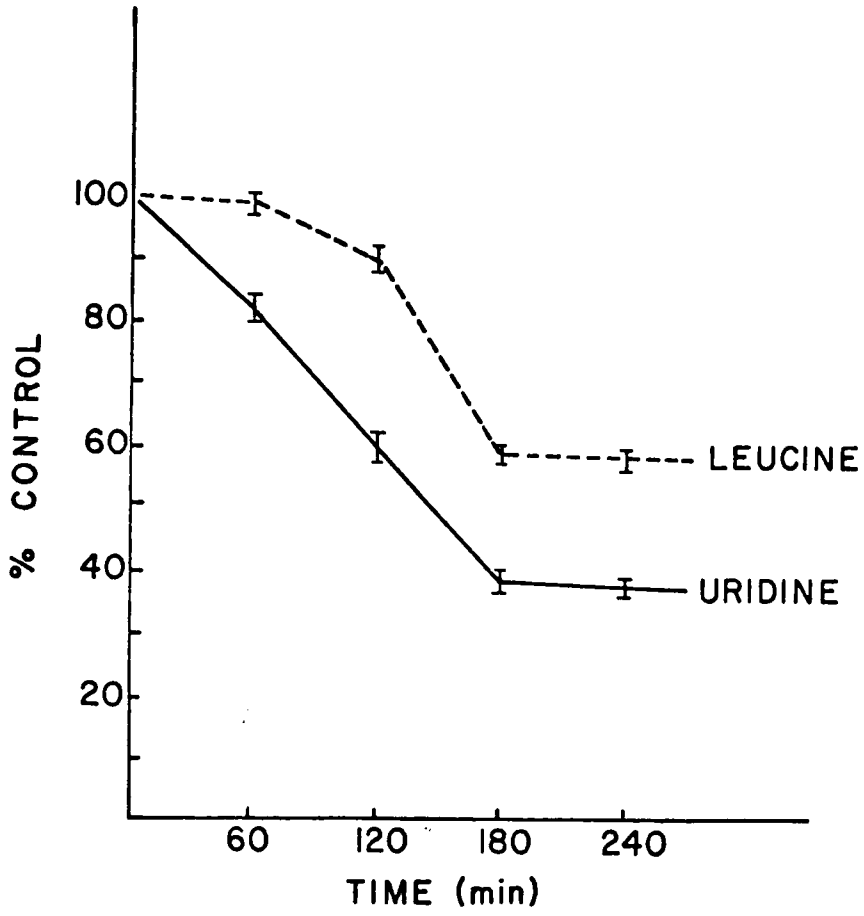


Figure VII. Effect of X-irradiation on ^{3}H-Leucine and ^{3}H-Uridine incorporation by fetal mouse liver erythroid cells.*

*After irradiation at 500 rads, ^{3}H-Leucine and ^{3}H-Uridine were added. Aliquots were taken at indicated intervals, and radioactivity in acid precipitable material was determined. Values are expressed as percent of controls. Each bar and line represents the mean and standard error of the mean of triplicate determinations of ^{3}H-Leucine and ^{3}H-Uridine incorporation.

IV. DISCUSSION

The results presented here demonstrate the radiation resistance of protein synthesis in L5178Y cells and the radiation sensitivity in fetal mouse liver erythroid cells. X-irradiation apparently produced irreversible and dose-dependent inhibition of protein synthesis in fetal mouse liver erythroid cells.

The results of experiments by some other workers do not agree with the findings in this study regarding L5178Y cells. Dioda and Okada (1969) have conjectured that X-irradiation induced a mitotic delay in L5178Y cells in the middle of G2 stage and that this delay involved the inhibition of protein synthesis. The delay in the initiation of division may not be a reflection of a deficiency in the mechanism of protein synthesis. These authors based their conclusions on radioautographic measurements that could not be confirmed by biochemical methods. Moreoever, during division delay, total protein synthesis is inhibited either in terms of quantity (Bachetti and Sinclair, 1971; Enger *et al.*, 1973; Kovacs and Vant Hof, 1972; Noland *et al.*, 1974) or quality (Tobey *et al.*, 1969, 1970). However, one cannot exclude transient or specific effects on total protein synthesis activity during or immediately following X-irradiation or on the synthesis of specific proteins that only comprise a few percent of the total protein synthesis.

However, the results found in fetal mouse liver erythroid cells agree with the observations reported by several investigators. Kim *et al.* (1970) have shown that protein synthesis in HeLa cells was

reduced within a two-hour period after irradiation. A log linear reduction in erythropoeitin-stimulated hemoglobin synthesis was also reported in bone marrow cells that had received increasing doses of ionizing radiations (Brown and Adamson, 1971).

In contrast, other laboratories have reported that the ribosome content of normal rodent liver responds to whole body doses of ionizing radiations by a shift to heavier polysomes. This shift is accompanied by enhanced incorporation of amino acids into nascent polypeptides on isolated ribosomes *in vivo* (Corless and Gray, 1967; Hedvegi *et al.*, 1970). A dose of 120 rads to mice on the tenth day of gestation prevents the final mitotic division of yolk sac erythroid cells. This was accompanied by a two-fold rate of hemoglobin synthesis in mitotically-blocked cells compared to that of control cells (Fantoni *et al.*, 1972).

When intact animals are irradiated, it is difficult--if not impossible--to completely control such variables as nutritional state and hormone levels. One cannot determine the mechanism of radiation effects on the physiologic state of the animals because these effects are reflected hours or days later in altered polysome levels and protein synthesis.

The specific activity of separated globin polypeptides of fetal mouse liver erythroid cells found in the present study suggests that X-irradiation has a direct effect upon erythropoiesis. Erythropoeisis is a process regulated by a series of biochemical steps involving cell proliferation and the initiation of hemoglobin synthesis. Erythropoiesis in the fetal mouse is first recognized in the yolk sac blood

islands, where a population of nucleated red blood cells synthesizing embryonic hemoglobin appear during the seventh gestational day. These erythrocytes are released into the circulation before day 10, mature to nucleated erythrocytes without cytoplasmic ribosomes or mitochondria while circulating in the blood, and then disappear by day 16. Between the tenth and eleventh gestational days, a second population of erythrocytes differentiates from hepatic mesenchyma (Rifkind, Chui, and Epler, 1969). In this site proerythroblasts develop sequentially into basophilic, polychromatophilic, and orthochromatic erythroblasts synthesizing adult type hemoglobin (Kovach, Martes, Russel, and Epler, 1967). On day 12, proerythroblasts and basophilic erythroblasts constitute a large proportion of the erythroid cell precursor population. Later, more mature forms, principally polychromatophilic and orthochromatic erythroblasts, are the major components of this population (Rifkind *et al.*, 1969; Fantoni *et al.*, 1968).

In view of these observations, the fetal liver erythroid cells (13- to 14-day) used in the present study would be composed predominantly of proerythroblasts and basophilic erythroblasts, and these, most likely, are the target cells for radiation effects. It has been shown that reticulocytes (the penultimate stage in the development of the erythrocyte), which contain all the enzymes necessary for hemoglobin synthesis, do not respond to X-irradiation (Barcos and Goldwasser, 1976). Therefore, inhibition at a translational step is most unlikely, because radiation-induced inhibition was not observed before two hours after irradiation. Moreover, the absence of an additive effect in irradiated cells, with or without actinomycin D (an

inhibitor of transcription), also tends to rule out the possibility of a translational step, rather than a transcriptional event.

In immature fetal liver erythroid precursors, proerythroblasts, and basophilic erythroblasts, hemoglobin synthesis is either directed by a relatively unstable messenger RNA, or these cells are in the stage of acquiring a full complement of mRNA in such large amounts as not to be limiting for protein synthesis until the terminal stages of maturation (Rifkind *et al.*, 1964). This later interpretation is consistent with the hypothesis that immature erythroid cell precursors may have a relatively unstable hemoglobin-synthesizing capacity. The transition from unstabilized to stabilized hemoglobin synthesis involves a decreased requirement for a rate-limiting species of RNA (Djaldetti, 1970). Furthermore, the results presented in this paper (Figure VII) demonstrate that radiation-induced inhibition of RNA synthesis is evident within one hour, while inhibition of protein synthesis appears later. This finding is consistent with the dependence of protein synthesis on RNA synthesis. Therefore, it is most probable that the radiation effects in fetal liver erythroid cells involves the inhibition of RNA synthesis and a probable disruption of the transition from an unstabilized to a stabilized protein-synthesizing mechanism.

In a comparison of actinomycin D sensitivity of protein synthesis in L5178Y and mouse fetal liver erythroid cells, the most striking observation was the two- to three-fold greater sensitivity in L5178Y cells. This finding might suggest the rapid synthesis of actinomycin-sensitive molecules, presumably RNA. Such an occurrence

may be correlated with the short half-life in fetal mouse liver erythroid cells. In contrast, L5178Y cells exhibited radio-resistance, while in fetal mouse liver erythroid cells, X-irradiation inhibited RNA synthesis and was followed by the inhibition of hemoglobin synthesis.

The long half-life of globin mRNA enables the cell to synthesize hemoglobin in the absence of continuous RNA synthesis. This event would account for the decreased actinomycin sensitivity in fetal mouse liver erythroid cells. The higher actinomycin sensitivity in L5178Y cells would also correspond to the short half-life of mRNA which requires the rapid synthesis of these molecules in order to maintain the pool size. Thus, it is possible that relatively abundant actinomycin sensitive sites, presumably transcriptional units, may exist. If this is the case, the target theory would predict damage to very few of these sites; redundancy of these sites would preclude measurable effects on protein synthesis.

Although an attempt has been made to explain the difference in radiosensitivity of protein synthesis, more information is needed for the mechanism to be understood at molecular levels. The results of this study indicate that total Uridine incorporation in L5178Y cells was unaffected by irradiation. Future studies, however, may be profitably directed toward examining the effects of irradiation upon specific types of RNA (e.g., messenger RNA). It would also be interesting to study X-irradiation effects under the influence of radiosensitizing agents.

In summary, the radiosensitivity of protein synthesis is different in the two cell types examined in this study. X-irradiation inhibited RNA synthesis within 60 minutes, while hemoglobin synthesis was inhibited at 240 minutes in the fetal mouse liver erythroid cells of 13-14 days of gestation. Immature proerythroblasts and basophilic erythroblasts are believed to be the targets for radiation effects. Since these immature erythroid cells synthesize hemoglobin dependent upon continuous RNA synthesis, it is postulated that radiation inhibited the transcription of messenger RNA required for the stabilization of hemoglobin synthesis.

Radio-resistance of protein synthesis in L5178Y cells is hypothesized to be due to a relative abundance of transcriptional units to produce messenger RNA to cope with the short half-life of mRNA and to maintain the pool size. Therefore, in view of the target theory, it is speculated that redundancy of the units might preclude a measurable radiation effect on protein synthesis.

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