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**A HISTOCHEMICAL STUDY
OF LIVER ENZYMES INVOLVED IN
GLYCOGEN METABOLISM
IN THE X IRRADIATED RAT**

**ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Atomic Support Agency
Bethesda, Maryland**

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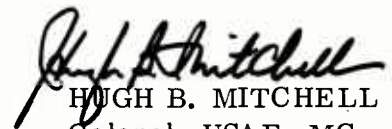
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A HISTOCHEMICAL STUDY OF LIVER ENZYMES INVOLVED IN GLYCOGEN
METABOLISM IN THE X IRRADIATED RAT

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TABLE OF CONTENTS

	Page
Foreword (Nontechnical summary)	iii
Abstract	iv
I. Introduction	1
II. Procedures	1
Cytochemistry	2
Phosphorylase	3
1,4 → 1,6 Transglucosidase	3
UDPG Transglucosylase	3
1,6 Glucosidase	3
Electron microscopy	4
III. Results	4
IV. Discussion	9
References	11

LIST OF FIGURES

	Page
Figure 1. Liver cell of a fed unirradiated rat	5
Figure 2. Liver cell of an unirradiated rat starved for 24 hours	5
Figure 3. Liver cell of an irradiated rat 24 hours after irradiation	5
Figure 4. Liver cell of an irradiated rat 48 hours after irradiation	5
Figure 5. Liver section obtained from an unirradiated rat and incubated in the phosphorylase medium	7
Figure 6. Liver section obtained from an unirradiated rat and incubated in the phosphorylase medium without AMP and glucose-1-P.	7
Figure 7. Liver section obtained from an unirradiated rat and incubated in the 1,4 → 1,6 transglucosidase medium.	7
Figure 8. Liver section obtained from an irradiated rat (24 hours post- irradiation) and incubated in the phosphorylase medium	7
Figure 9. Liver section obtained from an irradiated rat (24 hours post- irradiation) and incubated in the 1,4 → 1,6 transglucosidase medium	7
Figure 10. Liver section obtained from an unirradiated rat and incubated in the UDPG transglucosylase medium	8
Figure 11. Liver section obtained from an irradiated rat (24 hours post- irradiation) and incubated in the UDPG transglucosylase medium	8
Figure 12. Liver section obtained from an unirradiated fed rat and stained in Gram's iodine solution	8
Figure 13. Liver section obtained from an unirradiated fed rat and incu- bated in the 1,6 glucosidase medium	8
Figure 14. Liver section obtained from an irradiated rat (24 hours post- irradiation) and incubated in the 1,6 glucosidase medium	8

FOREWORD

(Nontechnical summary)

Glycogen, sometimes called "animal starch", is a highly branched macromolecule composed essentially of glucose units. It is the main carbohydrate storage material in mammalian systems and constitutes the most important store of energy capable of rapid mobilization. Liver glycogen is reversibly convertible to blood glucose and serves to maintain the blood sugar level when the supply of sugars through intestinal absorption is not adequate.

In vivo, liver glycogen is converted to glucose by means of the enzymes amylophosphorylase and amylo-1,6-glucosidase (debranching enzyme). Its synthesis from glucose is catalyzed by the enzymes UDPG transglucosylase and 1,4 $\rightarrow$ 1,6 transglucosidase (branching enzyme).

Several investigators have reported that the glycogen content of the liver of rats and other animals increases after irradiation. It was therefore of interest to determine if and to what extent radiation affects the activities of the above enzymes. This report describes a cytochemical study of the changes which occur in the activity of these enzymes following whole-body x irradiation of rats. It was found that cytochemically demonstrable changes do occur and that the activities of the enzymes responsible for glycogen breakdown to glucose are markedly reduced by radiation. The same was found for 1,4 $\rightarrow$ 1,6 transglucosidase (branching enzyme). In contrast, the activity of UDPG transglucosylase which is responsible for the in vivo synthesis of 1,4-polysaccharides was found to be enhanced by x irradiation of the animal.

ABSTRACT

The liver enzymes responsible for the breakdown and synthesis of glycogen from glucose have been investigated cytochemically in rats exposed to 1200 rads of x rays. It was found that significant changes occur in their activities and that amylo-phosphorylase and amylo-1,6-glucosidase, both of which are responsible for the conversion of glycogen to glucose, are markedly inhibited by radiation. A significant inhibition of the activity of 1,4 $\rightarrow$ 1,6 transglucosidase (branching enzyme) was also observed. In contrast, the activity of UDPG-glycogen transglucosylase, which is responsible for the in vivo synthesis of 1,4-polysaccharides, was found to be enhanced.

I. INTRODUCTION

Several investigators have reported that the glycogen content of the liver of rats and also of mice increased after exposure of the animals to x rays.^{5,6,16} Radiation-induced glycogen changes have also been demonstrated cytochemically in liver and brain preparations from irradiated rats.^{4,8-10,13} Using biochemical methods we have previously shown that glycogen phosphorylase and amylo-1,6-glucosidase both of which are involved in the breakdown of glycogen to glucose are greatly inhibited by irradiation of the animal.¹ A considerable inhibition in the activity of amylo-(1,4 → 1,6)-transglucosidase was also observed. In contrast, the activity of UDPG transglucosylase which is responsible for the in vivo synthesis of 1,4-polysaccharides was found to be greatly enhanced by radiation.¹ The present study deals with the cytochemical demonstration of radiation-induced changes of the above-mentioned enzymes.

II. PROCEDURES

Female Sprague-Dawley rats of the Charles River strain weighing 90 to 110 grams were used in this study. All animals were kept on a fat-free diet at least 7 days prior to irradiation. Each animal received approximately 12-13 grams of food (dry weight) per day. The entire ration was given at one time and the rats were trained to consume it within 1 to 1-1/2 hours. Radiation exposures started approximately 2 hours after the animals had consumed their food. No food was given to the animals following irradiation. Control rats were also deprived of food over periods of time corresponding to those for the irradiated rats. All animals had free access to water.

This study used a total of 88 rats in 11 experiments. The animals utilized in each experiment were divided into two equal groups, i.e., four rats were irradiated and the remaining four served as unirradiated controls. The experimental animals were exposed to 1200 rads of whole-body x rays. During exposure the animals were individually housed in Lucite boxes which were so arranged in the radiation field that each rat received an equal unilateral exposure. The tissue dose rate at the midline of the animal was 20 rads/min in air. The distance from the x-ray tube to the midline of each rat was 100 cm. The physical characteristics of the exposure source were as follows: 250 kVp, 30 mA, 1.2 mm beryllium and added filtration of 0.95 mm copper (HVL-1.9 mm Cu).

Following irradiation two animals from each experimental group as well as from the unirradiated controls were sacrificed at 24 or 48 hours postirradiation. The livers were quickly excised and pieces approximately 5 x 8 x 3 mm of the left lateral lobe of the liver were removed. These pieces were frozen at -20°C within 1/2 minute after decapitation. When samples for electron microscopy were desired, liver specimens were rapidly cut in approximately 1 mm cubes and placed immediately in fresh 4 percent glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.2.

Cytochemistry. A sliding microtome mounted in a cryostat (International Equipment Co.) maintained at -20°C was used to prepare frozen liver sections 15 μm thick which were then mounted on microscope slides. The slides with the adhering tissue sections were stored in the cryostat (usually less than 10 minutes) until ready for incubation. Plastic cylindrical vials with raised side ridges to hold the microscope slides (Scientific Products) were utilized as incubation containers. Incubations

were carried out for 2 hours at 37°C with gentle shaking. Duplicate specimens were used.

Phosphorylase. Phosphorylase activity was demonstrated in the direction of synthesis of polysaccharide from glucose. Takeuchi and Kuriaki's method²⁰ with minor modifications was used. The incubation medium (10 ml) contained 0.6 mM glucose-1-phosphate (K salt); 3 mM adenosine-5'-phosphate; 0.4 mM NaF; 40 μ M MgSO₄; 1.5 μ M HgCl₂; 3 mg glycogen; and 0.8 mM acetate buffer pH 5.7. Following incubation the slides with the mounted liver sections were rinsed with 40 percent ethanol, followed by water. They were then dried at room temperature, fixed for 3 minutes in absolute ethanol and dried again at room temperature. Staining was carried out by immersing the mounted liver sections for 3 minutes in Gram's iodine solution (I:KI:H₂O 1:2:900).

1,4 $\rightarrow$ 1,6 Transglucosidase. The incubation medium was the same as for phosphorylase except that no HgCl₂ was added. Following incubation the liver sections were treated for 1 minute at 37°C with an aqueous solution of β -amylase (0.25 percent) buffered to pH 5.7 with acetate buffer (1 part 0.5 M buffer per 100 ml). Staining was performed as described earlier.

UDPG Transglucosylase. Takeuchi and Glenner's method¹⁹ was employed to demonstrate UDPG transglucosylase activity.

1,6 Glucosidase. The incubation medium (10 ml) contained 1 mM potassium phosphate pH 6.8 and 0.05 ml of α -phosphorylase suspension in 0.0015 M EDTA, 0.1 M NaF, 0.00192 M sodium glycerophosphate (pH 6.8; 15.5 mg protein/ml; 1750 units/mg; Sigma Co.), which was added just before starting the incubation. The

mounted liver sections were incubated for 5 minutes at 30°C. They were then fixed and stained as described earlier.

Electron microscopy. The liver specimens were left in the glutaraldehyde fixative overnight. They were then washed in a solution of 0.05 M cacodylate buffer pH 7.2 and postfixed in 1 percent osmium tetroxide.¹¹ Following fixation the tissues were dehydrated in graded ethanol solutions and embedded in Maraglas.¹⁵ The blocks were cut with a Porter-Blum MT2 ultramicrotome and sections mounted on uncoated grids. After staining with uranyl acetate¹⁴ and lead citrate²¹ the sections were examined in the electron microscope (Siemens Elmiskop 1A).

III. RESULTS

Figure 1 shows an electron micrograph of a liver preparation obtained from a fed unirradiated rat and Figure 2 from a rat starved for 24 hours. It can be seen in these figures that the livers of fed animals contain large deposits of glycogen. In contrast, those of starved rats exhibit only small quantities of glycogen since, as expected, their stores were depleted by starvation. Electron micrographs of liver preparations from x irradiated rats 24 and 48 hours after exposure are illustrated in Figures 3 and 4, respectively. As shown in these figures, large deposits of glycogen are still present although the animals did not receive any food after irradiation.

Figure 5 shows a liver section obtained from a control (unirradiated) rat and incubated in the phosphorylase medium. It exhibits large deposits of polysaccharide synthesized by the action of phosphorylase. That this polysaccharide was actually synthesized and not already present is illustrated in Figure 6 which shows a similar liver section incubated in the same medium but without glucose-1-phosphate and

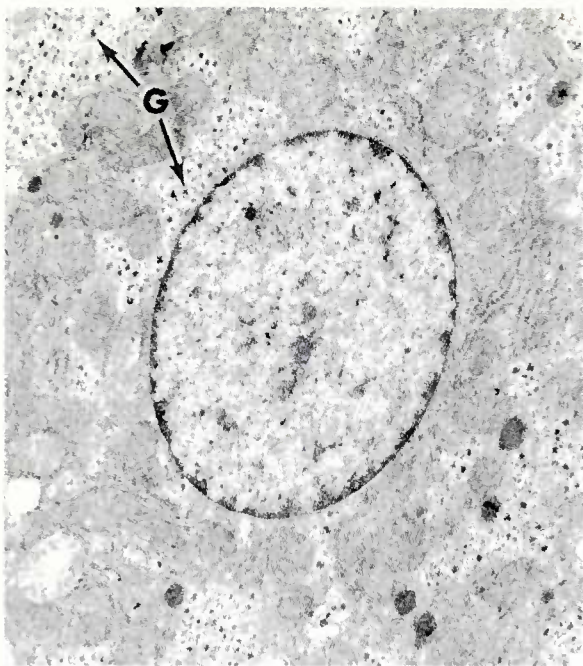


Figure 1. Liver cell of a fed unirradiated rat.
X 8,000 G = glycogen

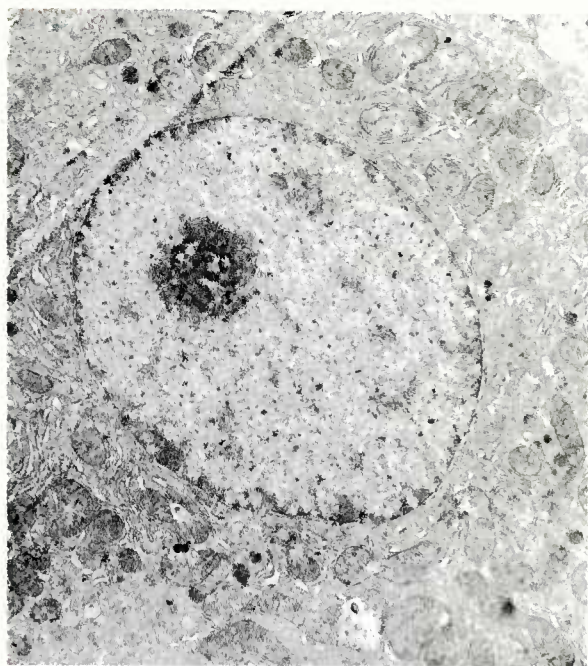


Figure 2. Liver cell of an unirradiated rat
starved for 24 hours. X 10,000

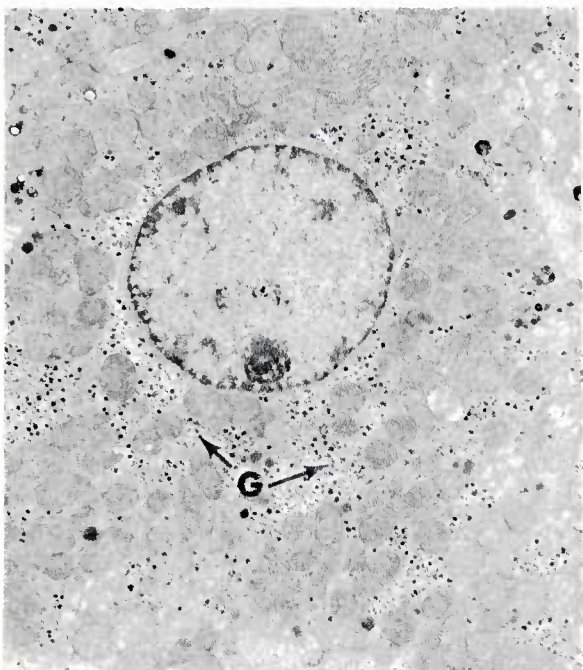


Figure 3. Liver cell of an irradiated rat
24 hours after irradiation.
X 4,800 G = glycogen

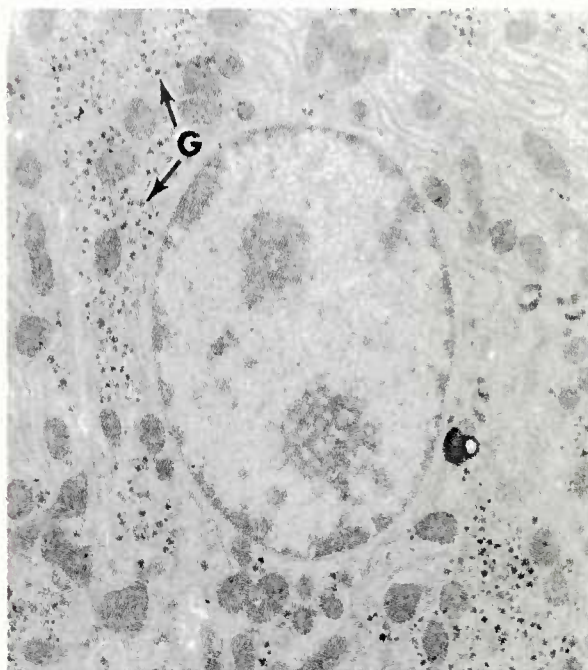


Figure 4. Liver cell of an irradiated rat
48 hours after irradiation.
X 9,000 G = glycogen

adenosine-5'-phosphate. No deposits of polysaccharide can be detected. A liver section obtained from a control (unirradiated) rat and incubated in the 1,4 $\rightarrow$ 1,6 transglucosidase medium is shown in Figure 7. The presence of polysaccharide deposits indicates significant transglucosidase activity.

The amounts of polysaccharide synthesized by the above two enzymes in liver sections from rats exposed to 1200 rads of x rays and sacrificed 24 hours later are illustrated in Figures 8 and 9. It can be seen in these figures that only small amounts of polysaccharide are present which indicates that the activities of both enzymes, i.e., phosphorylase and 1,4 $\rightarrow$ 1,6 transglucosidase are markedly reduced by irradiation of the animal. UDPG transglucosylase activities in the livers of control (unirradiated) rats and animals exposed to x rays and sacrificed 24 hours after exposure are shown in Figures 10 and 11, respectively. As shown in these two figures the amounts of polysaccharide synthesized indicate that the activity of this enzyme is stimulated by x irradiation.

Figure 12 illustrates a liver section obtained from an unirradiated fed rat and stained in Gram's iodine solution. Figure 13 shows a similar liver section which, prior to fixing and staining, was incubated in the 1,6 glucosidase medium. It can be seen that the amount of unhydrolyzed glycogen is greatly reduced indicating significant 1,6 glucosidase activity. Figure 14 shows a liver section obtained from an irradiated rat sacrificed 24 hours after exposure and incubated in the 1,6 glucosidase medium. It is obvious that the amount of unhydrolyzed glycogen is larger than that in the liver section from the unirradiated control (Figure 13). This indicates that the activity of this debranching enzyme has been impaired by radiation. Results similar

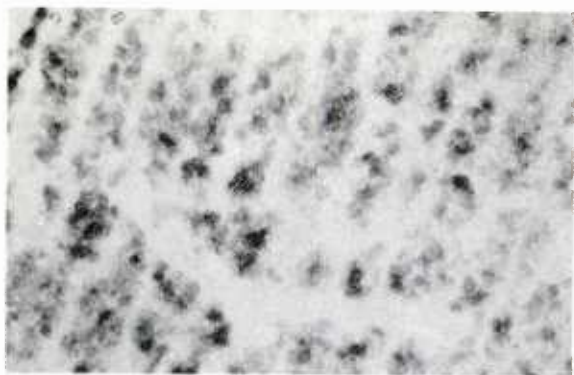


Figure 5. Liver section obtained from an unirradiated rat and incubated in the phosphorylase medium. (Stained in Gram's solution.)



Figure 6. Liver section obtained from an unirradiated rat and incubated in the phosphorylase medium without AMP and glucose-1-P. (Stained in Gram's solution.)

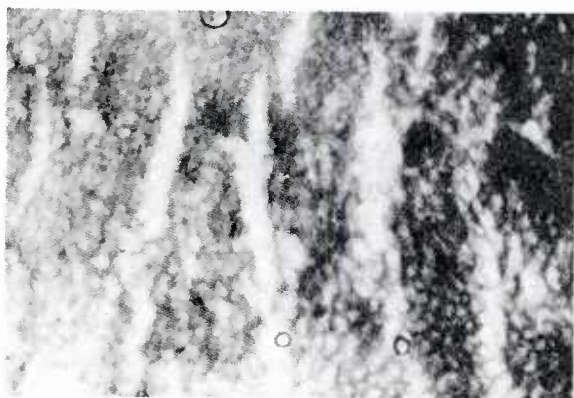


Figure 7. Liver section obtained from an unirradiated rat and incubated in the 1,4 to 1,6 transglucosidase medium. (Stained in Gram's solution.)



Figure 8. Liver section obtained from an irradiated rat (24 hours postirradiation) and incubated in the phosphorylase medium. (Stained in Gram's solution.)

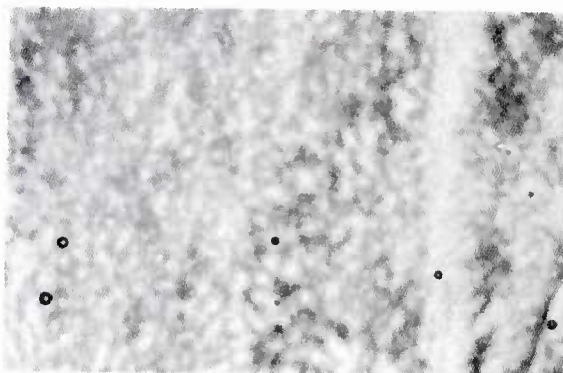


Figure 9. Liver section obtained from an irradiated rat (24 hours postirradiation) and incubated in the 1,4 to 1,6 transglucosidase medium. (Stained in Gram's solution.)



Figure 10. Liver section obtained from an unirradiated rat and incubated in the UDPG transglucosylase medium. (Stained in Gram's solution.)

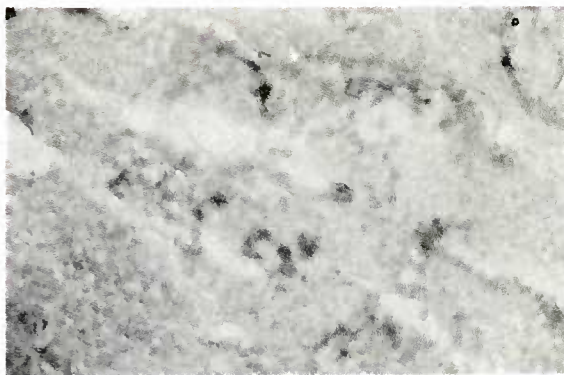


Figure 11. Liver section obtained from an irradiated rat (24 hours postirradiation) and incubated in the UDPG transglucosylase medium. (Stained in Gram's solution.)



Figure 12. Liver section obtained from an unirradiated fed rat and stained in Gram's iodine solution.



Figure 13. Liver section obtained from an unirradiated fed rat and incubated in the 1,6 glucosidase medium. (Stained in Gram's solution.)



Figure 14. Liver section obtained from an irradiated rat (24 hours postirradiation) and incubated in the 1,6 glucosidase medium. (Stained in Gram's solution.)

to those described above for rats sacrificed at 24 hours were obtained for animals sacrificed at 48 hours postirradiation.

IV. DISCUSSION

It is known that the glycogen content of liver and other tissues is affected by the dietary condition of the animal and that starvation results in depletion of the glycogen stores. Electron micrographs taken of liver preparations from fed and starved rats (Figures 1 and 2) verify this effect. It has been shown also that, as seen in the electron microscope, the deposits of glycogen in the liver do not decrease following irradiation of the animal although no food was available between exposure and sacrifice (Figures 3 and 4). To clarify these observations a cytochemical study of the activity changes of enzymes responsible for the breakdown and synthesis of glycogen from glucose was undertaken. It was found that under our experimental conditions the activities of the enzymes amylophosphorylase and 1,6 glucosidase are markedly decreased by x irradiation of the animal. Both these enzymes are involved in the breakdown of glycogen to glucose units. It was also found that the activity of 1,4 $\rightarrow$ 1,6 transglucosidase (branching enzyme) is impaired by radiation. In contrast, the activity of UDPG transglucosylase which is responsible for the synthesis of 1,4-poly-saccharides⁷ is stimulated by irradiation of the animal.

To avoid the possibility of reactivating phosphorylase previously inactivated by irradiation and therefore interfering with the effect of radiation, cyclic-3',5'-AMP, a cofactor which mediates the conversion of inactive phosphorylase b to its active form a,³ was omitted from the incubation medium when phosphorylase activity was to be determined. For the same reason glucagon or epinephrine was not added to the

incubation medium since these hormones act by enhancing the production of cyclic-3',5'-AMP.^{2,12,17} The omission of these compounds, in all probability, would displace the corresponding enzymic reactions from their optimal conditions. However the differences in the amounts of polysaccharide synthesized were distinct enough to show clearly the effect of radiation on the enzymes under study.

To demonstrate phosphorylase activity in the presence of 1,4 → 1,6 transglucosidase, HgCl₂ was added to the incubation medium since this compound is known to inhibit the activity of the latter enzyme.¹⁸

As mentioned earlier, rats sacrificed at 48 hours after irradiation were also studied in these experiments. However, only the results obtained with liver sections from animals sacrificed 24 hours after exposure have been presented here since no detectable differences were observed between these two sets of animals. These results indicate that, under our experimental conditions, glycogen breakdown is markedly inhibited by irradiation of the animal, whereas the rate of synthesis of polysaccharide is enhanced. On the basis of these findings the accumulation of glycogen, which has been observed to occur in the liver of the irradiated animal and which has been verified by the electron micrographs shown, could be easily explained.

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