EFFECTS OF X-RAY IRRADIATION ON TRYPTOPHAN PYRROLASE INDUCTION *IN VIVO* AND *IN VITRO**

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It is now generally recognized that ionizing radiation has a greater effect on enzyme synthesis than enzyme themselves(1), and studies on the effect of x-ray irradiation on the formation of adaptive enzymes may be of some importance for the elucidation of the underlying biochemical mechanisms of radiation effects.

The majority of the studies hitherto carried out on this subject have dealt with the ability to form adaptive enzymes in microorganisms, which has been found to be unaffected by x-ray irradiation even in lethal doses (2–5). A few reports concerning the effect of radiation on enzyme formation in animal tissues have also appeared (6, 8). However, the observed changes in enzyme levels in animal tissues have been questioned in reference to whether they really reflect the initial effect of radiation, since the time elapsed between radiation exposure and enzyme assays (sampling) was sufficiently long to permit the development of a secondary change in the cell environment (1, 8). Moreover, these studies have been limited to the observations on changes in *in situ* enzyme levels in animal tissues, and little attention has been paid to the effect of radiation on the substrate-induced formation of adaptive enzymes in animal tissues.

Since the activity of tryptophan pyrrolase in liver has been shown to be increased several-fold by the administration of its substrate, tryptophan, *in vivo* (9) as well as by the *in vitro* addition of tryptophan to rat liver homogenate (10), the present studies were undertaken to determine whether the process *per se* of the adaptive synthesis of this enzyme induced by its substrate might be directly affected in some way by x-ray irradiation.

Studies concerning the *in situ* level of TPO[a] in rat liver after x-ray irradiation in terms of time-course changes were also undertaken, since the earlier data presented by Thomson and Mikuta (7), who found that the observed increase in the activity of TPO in rat livers after x-ray irradiation was in large part attributable to the altered hormonal state of the animal evoked by x-ray irradiation, were detailed only

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a The following abbreviations are used: TPO, tryptophan pyrrolase; O. D., optical density; ATP, adenosine triphosphate; DNase, deoxyribonuclease.

in the observation made 4 hours after 1000 r of total body irradiation.

EXPERIMENTAL

Animals-Sprague-Dawley rats of both male and female, bred in this laboratory, weighing 100 to 160 gm. were used in each experiment. Animals were irradiated at a given time with 220 kvp. x-rays (10 ma., 0.5–mm. Cu plus 1–mm. Al filter, 1.5–mm. Cu half-value-layer) at a rate of 27.3 r per minute (measured in air) and target-to-skin distance of 50 cm. A dose of 900 r total body irradiation was used throughout. During the irradiation, groups of 6 rats were held in flat paper containers which fit the size of x-ray machine nozzle and rotated under the x-ray beam. Nonirradiated rats were also held in paper containers for an equal period of time.

Preparation of Enzyme Solution---At the appropriate time, rats were killed by a blow on the head and then decapitated. The livers were quickly removed, washed in ice-cold water, blotted dry, and immediately weighed on a torsion balance. A 12.5% homogenate for the assay of original TPO activity in liver or 25% homogenate for the *in vitro* induction system of TPO was prepared in cold 0.14, KCI solution (preadjusted to PH 7.4 with NaOH) using a Teflon-pestle, glass homogenizer.

In vivo TPO Induction---TPO was induced *in vivo* by intraperitoneal injection of 60 mg. per 100 gm. body weight of L-tryptophan as a 6% aqueous solution. In such experiments, a control group was always prepared into which 1.5 ml. water was injected. At the appropriate time after tryptophan treatment, the animals were killed and the enzyme solution was prepared for TPO assays.

In vitro Induction System of TPO---It has been demonstrated by Gordon et al. (10) that when normal rat liver homogenate is incubated in a complex medium containing glucose, ATP, phosphate buffer solution, $MgCl_2$, amino acid mixture, and an aged liver homogenate (formamidase), the level of TPO activity is increased by the *in vitro* addition of tryptophan to the medium. Early in a series of systematic studies on the in vitro induction system, it was found in this laboratory (11) that the presence or absence of ATP in the supplemented medium of the above formula did not exert any significant effect on the *in vitro* increments of TPO activity by the substrate in rat liver homogenate. Accordingly, in the present studies, the following modified formula of the incubating medium containing no ATP was used: 0.1 ml. of 1.4 M glucose; 0.5 ml. of Krebs-bicarbonate buffer solution, PH 7.4; 0.3 ml. of 0.1 M $MgCl_2$; an amino acid mixture consisting of 0.5 mg. each of glycine and the L-forms of arginine, glutamic acid, histidine, leucine, lysine, methionine, phenylalanine in a 0.5-ml. volume; 0.1 ml. of an aged preparation of 25% whole rat liver homogenate in 0.14 M KCI (containing formamidase but no TPO); 0.4 ml. of 0.1 M L-tryptophan (40 micromoles, replaced by water in the blanks), and water to a total volume of 3.0 ml.

The enzyme solutions (25% liver homogenate) in a 1.0-ml. volume were added

last to the incubation medium, which was contained in 20-ml. incubation flasks, after preliminary temperature eqilibration. The incubations were run in a Dubnoff metabolic shaker at 37°C in air for 30 minutes after the addition of the enzyme solution.

Enzyme Assay---The original TPO activity in livier was assayed immediately after livers were removed according to the method of Knox (12) with slight modifications in gas phase and of deproteinization procedure. The *in vitro* induced TPO activity after 30-minutes incubation was also assayed by the same method employed for the original TPO activity as follows: At the end of 30-minutes incubation, 2.0-ml. aliquots of the enzyme-medium mixture were removed and transferred to separate 20-ml. vessels containing the TPO assay medium of Knox (1.0 ml. of 0.2 M phosphate buffer, PH 7.0; 0.3 ml. of 0.03 M L-tryptophan (9 micromoles); and water to make a final volume of 4.0 ml. after transfer of the above mixture), and were shaken again in the Dubnoff shaker in air for 60 minutes. The reaction was stopped by the addition of 2.5 ml. of 5% zinc acetate and 3.5 ml. of 0.18 M NaOH, and the mixture was centrifuged for the determination of kynurenine formed.

After deproteinization with zinc acetate and NaOH, the amount of kynurenine in the clear, protein-free supernatant was measured spectrophotpmetrically at 365 mm using the value of 0.454 for the optical density of a 1.0 micromole/10ml. of kynurenine solution. Enzyme activity is expressed in terms of the micromoles of kynurenine formed per ml. of enzyme preparation per hour, and the specific activity in terms of g. protein, as calculated:

$$\frac{\text{Test O. D. minus Blank O. D.}}{0.454} \times \frac{1000}{\text{mg. protein in 1.0 ml. of 12.5 \% liver homogenate}}$$

$$= \mu\text{moles kynurenine/g. protein/hr.}$$

protein was determined by the biuret method of Gornall et al. (13)

RESULTS

Time-Course Observation on Changes in TPO Activity of Rat Liver after Total Body X-ray irradiation.

Fig. 1 shows the changes in the activity of TPO of rat liver observed 2 to 72 hours after 900 r of whole body x-ray irradiation. The increase in TPO activity was evident as early as 2 hours after irradiation, and it can be seen that the increase reached maximum 2 to 4 hours after exposure and then gradually declined toward plateau levels still higher than normal values at 12 to 72 hours postradiation. Thomson et al. (7) reported about two-fold increase in TPO activity 4 hours after 1000 r of total body irradiation. Data presented here show, however, a nearly 4-fold increase 4 hours after 900 r. Additional mention should be also made here of the plateau level

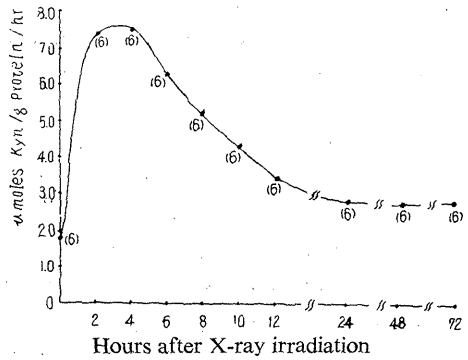

Fig. 1. Time-Course changes in the activity of Tryptophan pyrrolase in rat livers after whole Body x-ray irradiation each point represents the average activities of six x-ray irradiation rats.
Radiation Dose: 900 r.

of about 2-fold increase which was still maintained even at 72 hours postradiation, as contrasted with the report of Thomson et al. that normal ranges were reached 48 to 72 hours after exposure (7).

Direct Effect of Whole Body X-ray irradiation on the in vivo TPO Induction by Substrate

This was studied by inducing the TPO *in vivo* prior to irradiation, by intraperitoneal injection of 60 mg. per 100 gm. body weight of L-tryptophan immediately fol-

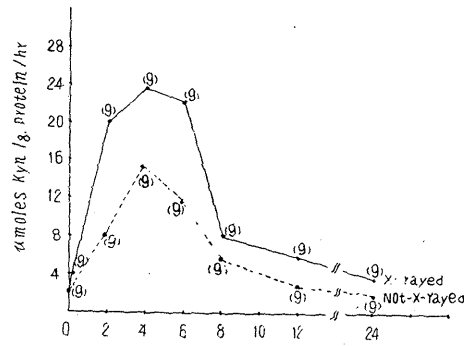

Fig. 2. Effect of whole Body x-ray irradiation on the In Vivo Tryptophan Pyrrolase Induction in Rats.
Each point represents the average activities of nine x-ray irradiated rats, and line above and below it denotes standard error of the mean. All rats were injected intraperitoneally with 60 mg/100gm Body weight of L-Tryptophan prior to x-ray irradiate and immediately following the injection x-rayed group was exposed to 900 r of irradiation at the appropriate time after Tryptophan injection as indicated, TPO was assayed.
Not-x-rayed group were similarly treated with tryptophan without subsequent x-ray irradiation.

lowed by 900 r of irradiation and killing the rats for TPO assays at 2, 4, 6, 8, 12, and 24 hours after tryptophan injection.

As shown in Fig. 2, an about 4-fold increase in TPO activity of control (not-x-rayed) rat livers was noted 2 hours following the in vivo tryptophan treatment, and the maximal increase (about 8-fold) 4 hours after tryptophan injection. The activity thereafter decayed gradually reaching the normal range 12 to 24 hours after tryptophan treatment. The pattern of induction and decay course of TPO following tryptophan treatment as observed in not-x-rayed rats was not appreciably altered by x-ray irradiation which was applied immediately following the tryptophan treatment. However, it should be noted that the absolute levels of the induced TPO in irradiated rats were significantly higher than those of not-x-rayed rats in each TPO assay time after tryptophan injection. Thus, except 4 and 8 hours after tryptophan treatment, nearly 2-fold increases over the induced levels of TPO in not-x-rayed rats were brought about by x-ray irradiation in every TPO assay time.

Effect of x-ray irradiation on *In vitro* TPO Induction by Substrate

The results of the previous experiment suggessts that the increase in substrate-induced TPO levels observed in irradiated rats might be secondary to an altered hormonal state of animals by x-irradiation as is the case with the increased level of TPO in liver *in situ* after exposure, and it was desirous to study the effect of x-irradiation on the system, if any, of TPO induction which will not be subject to hormonal controls. Since the *in vitro* induction system by substrate of TPO in liver homogenate can afford a possible experimental approach to the exclusion of hommonal influences apt to be present in *in vivo* studies, use was made here of this *in vitro* TPO induction system.

Rats were irradiated prior to *in vitro* induction, and at the appropriate time after

TABLE 1. Effect of whole Body X-ray irradiation On the *In vitro* Increments of Tryptophan Pyrrolase in Rat Liver Homogenate*

Time After X-radiation	TPO Activities in Original Homogenate	TPO Activities Induced In vitro	% Increase Over Original Activity
	μ moles Kyn/g. Protein/hr.		
Not-X-rayed	1.76±0.19 (6)	6.80±1.13 (6)	386
24 Hours	3.63±0.94 (6)	6.90±3.97 (6)	190
48 Hours	3.69±0.62 (6)	9.12±1.76 (6)	247
72 Hours	3.32±0.55 (6)	5.83±2.32 (6)	176
96 Hours	4.3 (1)	10.90 (1)	254

* Activities are the averages from separate homogenates, Standard error of the mean. The number of animals tested is in brackets.
In vitro induction of tryptophan pyrrolase was performed by adding 40 micromoles of L-tryptophan to induction medium. Induction period, 30 minutes. Other experimental procedures are described in the text.

* P values of original TPO activity in not-X-rayed rats against 24 hrs after irradiation $0.1 > P > 0.05$: against 72hrs after X-rayed rats $0.05 > P > 0.01$.

irradiation TPO was induced *in vitro* by adding directly 40 micromoles of tryptophan to liver homogenate and the activity thus induced *in vitro* was compared to that of the original homogenate. The results are given in Table 1.

As can be seen from Table 1, the addition of 40 micromoles of L-tryptophan to the homogenate obtained from normal (not-x-rayed) rats resulted in a nearly 4-fold increase in activity. In liver homogenates from x-ray irradiated rats, however, the relative increments of the induced TPO activity over original activities were significantly, reduced regardless of the time elapsed after x-ray irradiation, although the absolute activities induced *in vitro* in liver homogenate of irradiated rats were not appreciably changed from those observed in normal liver homogenates. Careful examination of the data reveals that the reduction in the relative *in vitro* increment of TPO observed in x-ray irradiated rats was in reality brought about by the almost doubled increase over normal levels in original TPO activity alone which was not paralleled by the same order of magnitude of increase in the *in vitro* induced activities as observed in normal (not-x-rayed) rats.

Rats were manifestly moribund 96 hours postradiation, and only one rat could be tested in this respect.

Delayed Effect of x-ray irradiation on *In vivo* TPO Induction and on *in vitro* Increment of TPO in Trytophan-pre-treated Rat's Liver Homogenate

In order to learn the delayed effect of x-ray irradiation on *in vivo* TPO induction by substrate, and also to investigate the possibility that prior *in vivo* treatment of animals with the substrate, tryptophan, might provide a protection against the reduction in the relative *in vitro* TPO inducibility by x-ray irradiation which has been demonstrated in the previous experiment, a study was made of the activity increment of TPO induced *in vitro* by substrate addition in liver homogenate obtained

TABLE II. Delayed Effect of X-ray irradiation on *In vivo* and *In vitro* Tryptophan Pyrrolase Induction*

Time After Tryp. Inj.	In viro Induction	In vitro Induction	% Increase Over Original Activity©
	μ moles Kyn./g. Protein/hr.		
2 Hours	18.0±6.8 (6)	27.9±7.8 (6)	155
4 Hours	50.4±3.7 (6)	74.7±6.7 (6)	148
6 Hours	8.1±2.7 (6)	18.2±5.7 (6)	200

* Rats were exposed to 900 r. of x-ray irradiation 72 hours prior to *in vivo* and *in vitro* induction. Rats were killed 2, 4 and 6 hours after the injection of 60 mg./100 gm. of L-tryptophan, and the *in vivo* induced TPO activity in original homogenate was immediately assayed. An aliquot of liver homogenate made from the same rat was incubated for 30 minutes in the incubating medium containing 40 micromoles of L-tryptophan and then analyzed for the *in vitro* induced TPO activity.

© % increase of *in vitro* induced activity over original activity induced *in vivo*.

Each figure represents Mean. Standard Error of the mean. Figures in parenthesis are number of the animals tested.

from 72 hours-post-irradiated and tryptophan-pretreated animals.

As shown in Table II, animals were still able to respond to the tryptophan injection in their *in vivo* TPO inducibilities even when 72 hours had elapsed after exposure. The pattern of TPO induction and decay course after tryptophan treatment was similar to those observed immediately after x-ray irradiation. However, the absolute levels of the induced TPO were much higher (about twice) at 4 hours and lower (nearly half) at 6 hours after tryptophan injection than those observed immediately after x-ray irradiation (cf. Fig. 1).

Although much marked increases in the absolute levels of *in vitro* induced TPO were resulted when animals had been pre-treated with irradiation plus tryptophan injection, *in vitro* increments of TPO activity relative to original activity were also similarly decreased than normal by x-ray irradiation even under the tryptophan pretreatment as was the case with not-tryptophan-treated rat's liver homogenate. Therefore, it may be stated that the pretreatment of animals with the substrate, tryptophan, prior to *in vitro* induction does not lend any protection against the radiation-caused decrease in the *in vitro* induction of TPO.

DISCUSSION

The results presented here show that while x-ray irradiation augments the *in vivo* TPO induction by substrate administration not only within a few hours but also as late as 72 hours after x-ray irradiation, the *in vitro* increment of TPO activity by the addition of tryptophan directly to liver homogenate is appreciably interfered with by x-ray irradiation. This difference in response to x-ray irradiation between *in vivo* and *in vitro* TPO induction might be related to the presence or absence of hormonal influences caused by x-ray irradiation upon *in vivo* and *in vitro* induction systems of TPO. Thus, it seems highly probable that higher absolute levels of TPO observed after tryptophan plus x-ray irradiation treatments than single same dose of tryptophan treatment alone might be resulted from the summation of the primary substrate effect plus radiation-induced secondary hormonal effect. Evidences pointing to this inference have been provided by the finding of Knox et al. (14) that in adrenalectomized rats the increase in TPO activity produced by the combined treatments with tryptophan and hydrocortisone could be accounted for by the summation of the activities found after each inducer was administered alone, and by the already cited report of Thomson and Mikuta (7) that x-ray irradiation produced the increase in *in situ* level of TPO indirectly through adrenal gland stimulation.

On the other hand, the decreased *in vitro* inducibility of TPO in liver homogenate of irradiated rats indicates that prestimulated hormonal influences caused primarily by x-ray irradiation is no longer operating in the *in vitro* induction system, and also suggests that some rate limiting steps involved in the *in vitro* induction system are interfered with by radiation to such a extent that the inducibility is lowered than

normal without being completely lost.

Although there have been several demonstrations (15-17) in favor of the view that the increased activity of TPO induced *in vivo* by substrate or cortisone administration reflects *de novo* enzyme protein synthesis, direct evidences are still lacking, especially of the *in vitro* increment of TPO activity. Gordon et al. (18) ascribed the *in vitro* increment of TPO activity by substrate addition in liver homogenate to the permeability function of mitochondria on the basis of the observation that mitochondria are required for the *in vitro* increment effect, and that disruption of these particulates reduces the *in vitro* increment, but isotonic solutions containing substances which change the mitochondrial permeability promote such increments.

A considerable body of evidence (8,19) has been accumulated during the past few years to indicate that ionizing radiation causes sensitive structural changes in mitochondria of radiosensitive tissues, which has been incriminated as a cause of the reduced oxidative phosphorylation in spleen or thymus mitochondria (20) or of the changes observed in DNase 11 activity (21) shortly after exposure. Although liver mitochondria are known to be more resistant to radiation than those of spleen or thymus, the possibility of liver mitochondrial damage by x-ray irradiation was suggested by the work of Fritz-Niggli (22. 23), who studied the oxidation of various members of the Krebs cycle by rat liver mitochondria. The mitochondria were prepared in mannitol solution and were irradiated at 0. A dose of 50 r was suffieient to inhibit pyruvate oxidation by 30% and citrate by 10 to 15%. With succinate, on the other hand, more than 1000 r was required to show an effect. In later experiments, with reduced mannitol concentration, a dose as small as 0.1 r gave a 60 % inhibition of oxygen uptake 30 to 60 minutes postradiation, and the inhibition was not greatly altered when 500 times the exposure was applied.

In view of the evidences cited above, therefore, it seems likely that the relative reduction in the *in vitro* induction of TPO in liver homogenate from x-ray irradiated rats might be the result of a break in continuity of mitochondrial structure evoked primarily by radiation.

However, there still remains the possibility that the *in vitro* induction of TPO might be directly affected in some other way than mitochondrial damage by radiation. This possibility is implicated in the finding that the activity of catalase, which contains a porphyrin prosthetic group like tryptophan pyrrolase,was markedly reduced in mouse liver after whole body x-ray irradiation (24), and, perhaps more strongly, in the recent report of Dancewicz and Lipinski (25) that the activity of delta-amino-levulinic acid dehydrase, one of the sulfhydryl enzymes which catalyzes the conversion of delta-amino-levulinic acid to porphobilinogen, the monopyrrole precursor of porphyrin and heme, was decreased in beef liver homogenate by x-ray irradiation *in vitro*, and changed *in vivo* in the direction of either increase or decrease depending on radiation dosage and also on the organs of rat from which the enzyme originated.

In this connection, it is of interest that Auerbach et al. (26) have shown that 3-amino-1,2,4-triazole, a specific inhibitor of delta-amino-levulinic acid dehydrase, caused decreases in both of the substrate-induced activity and *in situ* activity of TPO in rat liver.

The consideration of this possibility, therefore, proposes the need of further investigations on the underlying mechanisms of the reduced *in vitro* inducibility of TPO by x-ray irradiation.

SUMMARY

Effects of whole body x-ray irradiation on the induction of tryptophan pyrrolase by tryptophan administration and also on the *in vitro* increment of tryptophan pyrrolase activity in rat liver homogenate incubated in tryptophan-containing complex medium were studied.

900 r of x-ray irradiation caused the increase in the substrate induced enzyme activity immediately as well as at 72 hours after exposure. The pattern of induction and decay course of tryptophan pyrrolase after tryptophan treatment, however, was not appreciably altered by x-ray irradiation.

On the other hand, *in vitro* increments of tryptophan pyrrolase activity relative to original activity were significantly reduced in liver homogenate from x-ray irradiated rats, and this reduction in the *in vitro* inducibility by substrate addition in irradiated rat's liver homogenate was not prevented by prior treatment of irradiated animals with tryptophan.

Possible roles of hormonal influences on the increased *in vivo* tryptophan pyrrolase induction after exposure and mitochondrial damage by x-ray irradiation relevent to the reduced *in vitro* inducibility are discussed.

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