Effect of Whole Body X-Irradiation on a Natural Inhibitor of Carboxypeptidase.* (20248)

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In the accompanying paper(1), we demonstrated the existence in many tissues of two carboxypeptidases and an inhibitor of one of these. The present paper describes experiments concerning the effect of whole body x-irradiation on these carboxypeptidase factors. It has been found that 400 roentgens significantly, and 600 roentgens totally, destroys the CP*a*I of the blood cells. Since it is probable that all tissue CP*a*I is actually contained in the blood cells present in the tissue at the time of sacrifice, it would be expected that whole tissue CP*a* activity would be increased by whole body x-irradiation. This has been found to be the case. It appears probable that the CP*a*I is contained in the white blood cells, although leukemic blood, like the blood of most other cancer-bearing animals or humans, is low in CP*a*I(2).

Experimental. Details of the assay procedure are given in the preceding paper(1), as are the method of preparation of the tissue for assay, and the definitions of units. Irradiation has been performed with a Keleket x-ray therapy machine operating at 250 KVP and 15 ma. One-half mm of Cu was used as filter. X-ray dose was determined with a Victoreen r-meter, the thimble being placed for calibration in a position corresponding approximately to the mid-section of the animal. The dose rate was generally 30-40 r per minute.

In preliminary studies, the effect of 650 r of whole body x-radiation was determined on 3 different catheptic enzymes in liver, kidney, and intestine of mice. Enzymes tested were the Cathepsins II, III, and IV of Fruton *et al.* (3). For these, the substrates used were, respectively, benzoylargininamide, dl-leucylglycine, and chloracetyltyrosine. Table I indicates that the Cathepsin II of liver, kidney, and intestine, the Cathepsin III of intestine, and the Cathepsin IV (carboxypeptidase) of kidney are all increased by this treatment. The overall catheptic reaction, as measured by Anson's(4) hemoglobin digestion method, was not affected in any tissue.

Whole body x-irradiation might be considered to increase whole kidney carboxypeptidase in either or both of two ways: The enzyme might be increased in activity, or an inhibitor might be destroyed. Other possibilities, such as formation of an activator, seem less likely. The situation is further complicated by the possibility that the x-ray effects are not on the kidney tissue itself, but on the blood present in the kidneys at the time of sacrifice.

These possibilities were simultaneously analyzed by the following set of experiments: a) Kidney perfusion. In each of 4 rats under ether anesthesia, one kidney was thoroughly perfused with 0.9% saline in order to wash out all blood. The enzyme fraction (centrifuged supernatant) was totally unaffected by the perfusion, while the inhibitor activity (centrifuged precipitate) was reduced by an average of 93%. b) Kidney shielding. Four rats were x-rayed (650 r) while firmly restrained within a lead belt which covered the kidney region and effectively reduced the x-ray intensity in air from 35 r/minute outside the shield to 0.5 r/minute within. At the same time, 4 rats were irradiated without being shielded. Twenty-four hours after x-ray, these 2 groups were sacrificed, as were four non-irradiated rats which, like the x-rayed animals, had been starved (water *ad lib.*) for the 24 hours. The kidneys were removed, homogenized, and centrifuged, and the fractions were assayed for CP*a* and CP*a*I. Table II indicates that the enzyme was not affected,

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TABLE I. Effect of 650 r of Whole Body X-Radiation on Proteolytic Activities of Mouse Tissues.

	Liver			Kidney			Intestine		
	Control	X-rayed	Student's t	Control	X-rayed	Student's t	Control	X-rayed	Student's t
Cathepsin II	.2 ± .2	1.1 ± .5	4.8	.4 ± .3	1.2 ± .5	4.1	.8 ± .4	2.8 ± 1.0	5.1
III	.0	.0	.0	1.0 ± .4	1.4 ± .5	1.9	.9 ± .3	1.7 ± .4	4.2
IV	.0	.0	.0	1.9 ± .6	4.4 ± 1.0	6.0	.0	.0	.0
Hemoglobin digestion	1.59 ± .46	1.57 ± .48	.06	1.10 ± .35	1.11 ± .35	.04	1.42 ± .55	1.55 ± .46	.39

For Cathepsins II, III, and IV, values are expressed as milliequivalents increase in alkali titration; for hemoglobin digestion, values are expressed as mols tyrosine solubilized. All are on the basis: per mg protein. Data are mean and average deviation from the mean. Population is 10 mice for each point in the cathepsin series, 7 mice for each point in hemoglobin digestion.

TABLE II. Effect of Kidney Shielding on Carboxypeptidase and Carboxypeptidase Inhibitor of Kidneys of Rats Sacrificed 24 Hours after 650 r X-rays.

Irradiated	Supernatant (enzyme)	Precipitate (inhibitor)
Not (control)	22.7 ± 2.0	20.0 ± 5.1
Whole body	21.4 ± 1.3	13.0 ± 1.1
Whole body, kidney shielded	24.9 ± 2.3	4.1 ± 1.7

Data presented are mean and avg dev. from the mean. Each figure represents avg of 4 rats, expressed as carboxypeptidase units, or carboxypeptidase inhibitor units, per ml of 10% kidney homogenate.

while the inhibitor was reduced by the irradiation. c) Blood cell inhibitor assay. Fig. 1 shows the effect of 650 r of whole body x-irradiation on the blood cell CP*a*I of rabbits. It will be observed that the CP*a*I drops precipitously, reaching a minimum value at 2 days and returning essentially to normal by the seventh day. The decrease in the CP*a*I in the blood of non-irradiated rabbits is probably due to the 48-hour starvation period imposed upon both sets of animals.

The data from these experiments would seem to necessitate the following conclusions: 1) The only CP*a*I found in kidney is that due to the kidney's content of blood at the time of sacrifice; 2) The CP*a* of kidney is not affected by x-irradiation with the doses employed; and 3) the CP*a*I of blood cells is destroyed by whole body x-irradiation.

A crucial experiment to assure the validity of these conclusions would be the whole body x-irradiation of animals, with subsequent assay of the inhibitor of the kidney. If all kidney inhibitor is actually blood inhibitor,

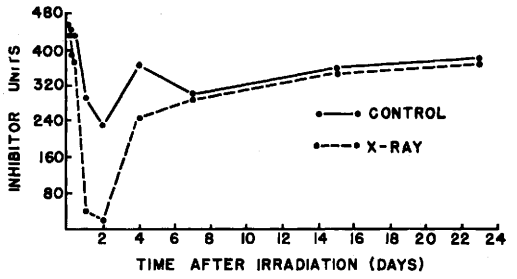

FIG. 1. Effect of 650 r whole body x-irradiation on rabbit blood cell carboxypeptidase inhibitor (CP*a*I). Each datum represents avg of five animals. Inhibitor activity is expressed as units per ml packed blood cells.

TABLE III. Effect of 650 r Whole Body X-Irradiation on Carboxypeptidase *a* (CP*a*) and Carboxypeptidase *a* Inhibitor (CP*a*I) of Rat Kidney.

Rats used				Component assayed	
Strain	Sex	Wt, g	Treatment	CP*a*	CP*a*I
Maguran	♂	250-300	C*	17.8 ± 1.6	11.1 ± .4
			X	20.3 ± 1.8	10.0 ± 1.4
Sprague-Dawley	♀	150-200	C	18.2 ± 3.5	12.6 ± 2.4
			X	13.4 ± .8	3.6 ± .4
" "	♂	250-300	C	16.8 ± .8	12.5 ± .4
			X	16.1 ± .8	2.9 ± 1.5
" "	♀	" - "	C	20.7 ± 1.8	15.0 ± .4
			X	18.9 ± 2.3	3.7 ± 1.3
" "	♂	150-200	C	17.1 ± .7	12.9 ± .5
			X	16.9 ± 1.8	4.1 ± 1.5
Maguran	♂	250-300	C	17.6 ± 1.1	14.0 ± 1.1
			X	17.1 ± 1.1	3.5 ± .5
"	♂	" - "	C†	22.7 ± 2.0	20.0 ± 5.1
			X†	21.4 ± 1.3	13.0 ± 1.1

Data are mean and avg deviation from the mean, expressed as enzyme or inhibitor units per ml 10% kidney homogenate. Each figure represents the avg of 5 or 6 rats in the first experiment and of 4 rats in each succeeding one. Rats sacrificed 24 hr after irradiation.

* C = control; X = x-rayed.

† Data repeated from Table II.

TABLE IV. Effect of Whole Body X-Radiation at Various Dose Levels on Blood Cell Carboxypeptidase Inhibitor of Rabbits, Guinea Pigs, and Rats 24 Hours Later.

Species	Approx. LD_{50} of 250 KVP X-rays (roentgens)	X-ray dose, roentgens							
		0	175	200	225	400	600	650	800
Man	400 (est.)*	656±36 (19)							
Rat	650	184±18 (23)		186±15 (3)		126±30 (3)	2±3 (3)		1± 2 (3)
Guinea pig	225	350±16 (4)	384±20 (4)		378±24 (4)			46±22 (3)	
Rabbit	790	310±66 (45)				256±13 (4)			26±20 (4)

Data given are mean and avg deviation from the mean, expressed as inhibitor units per ml packed blood cells. Figures in parentheses indicate number of individuals.

* Ref. 6.

and if all blood inhibitor is destroyed by whole-body x-irradiation, then it follows that whole body x-irradiation should destroy kidney inhibitor. The data are presented in Table III, which indicates this conclusion to be valid.

The effect of whole body x-irradiation in reducing the blood cell CP*a*I is of particular interest as a possible biological "dosimeter," a means of measuring the dose of ionizing radiation received by an individual human or animal. Since such dosimeter may measure dose either as a function of dose lethality or as a measure of absolute amount of radiation received, several animal species of widely varying sensitivity to x-rays have been tested at a variety of doses. The data of Table IV indicate decisively that the blood cell CP*a*I test measures absolute amounts of radiation received, rather than measuring the percentage of lethal dose.

Evidence indicates the white blood cell to be the source of the inhibitor. In one experiment, we repeatedly washed, by centrifugation, a tube of packed rabbit blood cells, trying with each washing to suck off as much as possible of the "buffy layer" of white cells. After three washings, the packed cells gave a

negligible inhibitor activity, while the washings, containing the buffy layer, showed inhibitor activity. In another experiment, packed cells were laked with 20 volumes of water, then centrifuged. A slight precipitate, presumably rich in the debris of difficultly lysed white cells, appeared. When this precipitate was resuspended and assayed for inhibitor, the bulk of the initial inhibitor activity was found in this fraction; the supernatant was devoid of activity. That the inhibitor is not merely washed off or out of the red cells is indicated by the fact that our routine assay of inhibitor involves repeated washings of the red cells with saline, care being taken, however, not to remove the buffy layer. It is therefore concluded that the white cells are the source of the carboxypeptidase inhibitor.

Discussion. Although as yet not decisively proven, it appears probable that the source of the inhibitor is the white blood cell. The relatively great activity of the inhibitor as compared to most tissue enzymes would argue for the source being the erythrocyte, but the experiments cited above indicate otherwise. In addition, some theoretical considerations favor the white cell as the source. We have shown that whole body x-irradiation reduces the blood cell inhibitor greatly by the second day, with return to normalcy at about a week. This would correspond to the life span of, say, a polymorphonuclear leukocyte, but is much too fast an action to be explained on the basis of an effect on newly formed red cells. Jacobson and co-workers(5) have shown that after LD_{50} doses of whole body x-rays, a lymphopenia appears in a few hours, a granulocytopenia in less than a week, and red cell effects only after 4-6 weeks. It is possible, of course, that the actual source of the inhibitor is the platelet or some plasma-insoluble substance.

Although the reduction by whole body x-irradiation of the blood cell CP*a*I does not measure the administered radiation as a per cent of the lethal dose, it still seems to us to be of great interest and potential value in estimating the lethality of a dose received by a human being. In this connection, it is a fortunate circumstance that the dose range measured by this test is one of practical interest in the study of man. If we assume an LD_{50} for man of 400 r(6), and further assume that the test applies to man in a fashion similar to the rodents tested, then it follows that a determination of the blood cell carboxypeptidase inhibitor will indicate at least whether or not the individual has received a lethal dose of radiation.

It is improbable that the x-ray effect on blood cell CP*a*I is directly related to the lethal effects of the radiation. This conclusion is drawn from two facts: First, the reduction by whole body x-irradiation of blood inhibitor is dependent on the absolute amount of radiation received, not on the degree of lethality in the particular species. For example, it is possible to irradiate guinea pigs at lethal levels without affecting the blood cell CP*a*I. Second, we have been unable to detect any CP*a*I whatsoever in the shed blood of mice or dogs. This is of particular interest because, in the mouse at least, whole body x-irradiation does increase the whole kidney carboxypeptidase activity. The difference between this mechanism in the rat and the mouse has not yet been elucidated.

Summary. Whole body x-irradiation of mice, rats, and rabbits at the LD_{50} range increases the activity of certain tissue cathepsins. Liver, kidney, and intestine were the tissues tested, and it was found that one of 3 liver cathepsins, 2 of the same 3 cathepsins in kidney, and 2 of the 3 in intestine were increased in activity by the radiation. This appears due, at least in the case of rat kidney carboxypeptidase, to the destruction by the x-rays of an enzyme inhibitor. It is possible that the source of all inhibitor found in tissues and blood alike is the blood cell, probably the leukocyte. The possibility is suggested that the reduction of carboxypeptidase inhibitor in blood cells may serve as a basis for determining, within 24 hours of the exposure, whether or not an individual has received a lethal dose of ionizing radiation.

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Carboxypeptidase in Mammalian Tissue.* (20249)

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The term "carboxypeptidase" is used in two distinctly different senses. On the one hand, it refers specifically to the enzyme entity first isolated in crystalline form from pancreas by Anson(1). On the other hand, it is used in more general reference to an enzyme or group of enzymes found in any of several tissues and classified by Fruton, Irving, and Bergmann(2) as Cathepsin IV, and later by Tallan, Jones, and Fruton(3) as carboxypeptidase. The pancreatic carboxypeptidase is usually assayed at pH 7.3-7.8 in the absence of cysteine, which Smith and Hanson(4) found actually to inhibit this enzyme. The carboxypeptidase (Cathepsin IV) of spleen and kidney was measured by Fruton, Irving, and Bergmann(2) at pH 5.0-5.1, and 0.01 M cysteine was used for maximum activity.

Work in this laboratory has demonstrated the existence in a wide variety of tissues of both types of carboxypeptidase. We have designated as carboxypeptidase *a* (CP*a*) that enzyme which requires cysteine for activity and which has optimal activity at pH 5.5-6.5 and 8.0-8.5. This enzyme has not previously been detected in many tissues because, first, it is much less active in the neighborhood of pH 7.5, and second, because most tissues also contain a cellular inhibitor of this enzyme. We have designated as carboxypeptidase *b* (CP*b*) that enzyme which has pH optima of 5.5 and 7.5 and is active in the absence of cysteine; in fact, the cysteine concentration used in the assay of CP*a* completely inhibits CP*b*. The natural tissue inhibitor of CP*a* is without effect on CP*b*. The inhibitor is apparently protein in nature, is generally found with the particulate matter in centrifuged homogenates, and inhibits the enzyme in a non-competitive fashion.

Experimental. Animals used were normal, Sprague-Dawley or Maguran rats and Carworth Farm mice. In general, CP assays were carried out by incubating a given tissue fraction with chloracetyl-L-tyrosine, with or without cysteine, for 10 minutes at 37°C. The chloracetyl-L-tyrosine was present in a final concentration of 0.0088 M. Tissue preparation, substrate, and cysteine were each brought to pH 5.5 before the assay. Aliquots were removed immediately after mixing, and again exactly ten minutes later, into 10 volumes of absolute alcohol. The 2 alcoholic solutions were then titrated with alcoholic KOH, approximately 0.01 N, using thymolphthalein as indicator. The titration thus resembles that of Grassmann and Heyde(5). That the titration increment is directly proportional to the amount of enzyme source added is demonstrated by the data of Fig. 1. Protein was determined by the method of Robinson and Hogden(6), as modified by Keyser and Vaughn(7).

Results. Efforts were first directed at the detection of CP in a variety of tissues. When whole homogenates (9 ml H_2O per g wet tissue) were used with 0.0088 M cysteine present, enzyme activity was detected in rat kidney but not in liver or intestine. The

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