162. THE EFFECT OF X-RAYS ON ENZYMES

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BECAUSE of the importance of enzymes for cell metabolism many have investigated the action of radiation on crude enzymes but only a few have used purified ones. The general findings arrived at in such investigations can be briefly summarized by repeating the conclusions of a recent review by Scott [1937] of the biological effects of X- and γ -rays: "The evidence shows that X- and γ -rays only influence enzymes when the dose is enormous. Cell division is inhibited by doses which have little or no destructive action on enzymes and therefore the inhibition is produced by a process in which enzymes play no important part."

It has been stated further that X- and γ -rays in doses of the order of 100,000 r. have an indiscriminate destructive effect on all living tissues causing a massive physico-chemical change in the protoplasm. Small doses however are supposed to act in a qualitatively different manner.

This paper deals with the action of X-rays on two enzymes:

(i) The crystalline enzyme carboxypeptidase which was isolated for the first time by Anson [1936-7, 1]. This enzyme is a water-insoluble protein which attacks chloroacetyltyrosine and peptic digests of proteins in the presence of formaldehyde.

(ii) A partly purified sample of polyphenoloxidase.

EXPERIMENTAL

Carboxypeptidase

The enzyme was prepared by the method of Anson with slight modifications. Anson recommends two ways of preparation either from the fluid which exudes when fresh frozen pancreas is allowed to thaw slowly, or by extraction of ordinary commercial pancreas. Since the facilities for the first were not available and the ordinary commercial frozen pancreas obtainable locally proved unsuitable and too expensive, a combination of the two methods was adopted.

Batches of 12 lb. of bovine pancreas were collected weekly, frozen hard immediately after the animal's death, sliced and kept in cold storage at -9° . It was then spread on trays, allowed to thaw slowly in a refrigerator at 5° for 20 hr. and minced. The subsequent treatment followed Anson's instructions as given for ordinary commercial pancreas. The yield of the preparation varies considerably for no apparent reason. It was found several times that the precipitation after dialysis remained incomplete, leaving a large part of the active material in a colloidal state. This difficulty was overcome by adding saturated $\text{Ba}(\text{OH})_2$ until the fluid became just orange to phenol red. At this reaction a precipitate of the active material formed.

The experiments to be described were performed with a uniform batch of white crystals which were obtained after twice recrystallizing an originally good crop of crystals. The crystals were suspended in redistilled water with toluene as preservative and kept as stock suspension in a refrigerator, the activity remaining

unchanged over 12 months. The activity was the same as given by Anson, i.e. the amount of the crystalline enzyme containing 1 mg. N corresponded to 0.103 [C.P.u.]^{P.D.E.} where [C.P.u.]^{P.D.E.} denotes carboxypeptidase units when a peptic digest of edestin was used. With one exception the substrate used throughout the experiment was a peptic digest of edestin (Hopkin and Williams, Ltd.) prepared according to Anson. The digestion took place at 25°, if not stated otherwise.

Titration of enzyme activity. The titrations were carried out with 0.02 *N* NaOH, the burette being protected against CO₂ by soda lime tubes. The procedure as given by Anson [1936-7, 2] was closely adhered to. The pipetting of the substrate solution has been done with the greatest accuracy.

General procedure. The suspension of crystals was thoroughly shaken in order to distribute the fine crystals evenly. The required amount was taken out immediately before the crystals had time to settle, diluted with glass-distilled water to the desired strength and *N*/10 NaOH added drop by drop, pausing for about 1 min. after every two drops and constantly shaking the flask until all crystals were dissolved and the reaction was pink to phenolphthalein. After about 5 min. a water-clear solution of the crystals was obtained. Half of the solution was kept as control, the other half exposed to the X-radiation in flat bottles. When the desired dose of radiation was given a sufficient volume was taken out for 4 titrations of the remaining activity and 4 blank titrations for each point of the curve. In the experiments with highly diluted solutions the number of titrations had to be reduced to 3 or even 2 because the volume of fluid which could be exposed to radiation was limited by the diameter of the X-ray tube window. For the irradiations where high doses or high dosage rates were required, radiation of half value layer 6 mm. Cu from a 450 kV. Metropolitan-Vickers continuously evacuated X-ray tube with a cooled window was used. For the other irradiations X-rays of half value layer 1.7 mm. Cu from a 250 kV. Metropolitan-Vickers tube, as described by Allibone & Bancroft [1934], were used. The dosage rate from the first tube was 1000 r. per hr. whilst from the second it was varied for the different experiments. The total dose was measured in every case by a calibrated Hammer Dosimeter.

The same stock suspension of crystals was used for further dilutions throughout. The estimation of the activity for the various dilutions was carried out under the conditions laid down by Anson. The digestion time of the substrate and the amount of enzyme solution used was adapted to the concentration of the enzyme solution of each particular experiment (see Table 1, columns 5 and 6). The lower the concentration of the enzyme solution the more the digestion time and the volume of the enzyme solution in the digestion titration had to be increased. The volume of the carboxypeptidase solution, however, never exceeded 2.5 ml. in order to keep the total volume of the digestion titration within the prescribed limit. When the volume of the carboxypeptidase solution for titration was under 2.5 ml. it was made up to 2.5 ml. by adding glass-distilled water.

Effects of X-radiation on carboxypeptidase solutions of different concentrations

The results of these experiments are shown in Figs. 1-4 and in Table 1 giving the experimental details and the calculations.

In calculating the carboxypeptidase units of the solution the values corresponding to the number of ml. of 0.02 *N* NaOH used in the titration (Table 1, columns 3 and 4) are read from the standard curve of Anson's paper, and then divided by the number of ml. of CP solution used (Table 1, column 6), and by the digestion time (Table 1, column 5).

The absolute number of the carboxypeptidase units inactivated by irradiation is found by converting the ml. of NaOH corresponding to the original activity (column 3) and to the activity left after irradiation (column 4) into carboxypeptidase units (columns 7 and 8), and then taking the difference between these two values (column 9).

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 1. Exp. A of Table 1.

Fig. 2. Exp. B of Table 1.

Fig. 3. Exp. C of Table 1.

Fig. 4. Exp. D of Table 1.

Abscissae: Dose in roentgen. Ordinates: Activity in ml. N/50 NaOH.

The reaction of the enzyme with its substrate is not strictly linear, i.e. decreasing the amount of enzyme or the digestion time leads to a comparatively smaller decrease in the activity of the enzyme. Therefore all four curves show a diminishing slope and the dilution of the enzyme by volume is not quite proportional to the dilution as indicated by the values of the enzymic activity (Table 1, columns 1 and 2). Because of this it is necessary when comparing the four curves with each other to choose a point on each curve representing the same percentage of destruction of the original activity. In Table 1, the points chosen for the curves A to D are where 25 or 50 % of the original activity is lost.

Table 1. *Inactivation of carboxypeptidase solutions of different concentration*

Exp.	1 Dilution by volume	2 Dilution by units	3 Activity unirradiated ml. 0.02 N NaOH	4 Activity left after inactivation ml. 0.02 N NaOH	5 Digestion time min.	6 C.P. solution used per titration ml.
For 25% inactivation						
A	$\times 1$	$\times 1$	0.69	0.52	1×10	0.2
B	$\times 62$	$\times 62.5$	0.68	0.51	6×10	2.0
C	$\times 172$	$\times 210$	0.46	0.35	12×10	2.5
D	$\times 345$	$\times 884$	0.34	0.26	30×10	2.5
Exp.	7 Column 3 in $10^6 \times \text{C.P.u.}/$ ml./10 min.	8 Column 4 in $10^6 \times \text{C.P.u.}/$ ml./10 min.	9 Difference between columns 7 and 8	10 X-ray dose in r. inactivating units of column 9	11 $10^6 \times \text{C.P.u.}/$ ml. inacti- vated per 1000 r./ 10 min.	
A	8000	5400	2600	60,000	43	
B	128	88	40	800	50	
C	31	23	8	145	55	
D	9.1	6.8	2.3	45	50	
Exp.	1 Dilution by volume	2 Dilution by units	3 Activity unirradiated ml. 0.02 N NaOH	4 Activity left after inactivation ml. 0.02 N NaOH	5 Digestion time min.	6 C.P. solution used per titration ml.
For 50% inactivation						
A	$\times 1$	$\times 1$	0.69	0.35	1×10	0.2
B	$\times 62$	$\times 62.5$	0.68	0.34	6×10	2.0
C	$\times 172$	$\times 210$	0.46	0.23	12×10	2.5
D	$\times 345$	$\times 884$	0.34	0.17	30×10	2.5
Exp.	7 Column 3 in $10^6 \times \text{C.P.u.}/$ ml./10 min.	8 Column 4 in $10^6 \times \text{C.P.u.}/$ ml./10 min.	9 Difference between columns 7 and 8	10 X-ray dose in r. inactivating units of column 9	11 $10^6 \times \text{C.P.u.}/$ ml. inacti- vated per 1000 r./ 10 min.	
A	8000	3450	4550	185,000	25	
B	128	57	71	2,500	28	
C	31	15.7	15.6	420	37	
D	9.1	4.7	4.4	125	35	

Column 9 gives the absolute amounts of enzyme in carboxypeptidase units/ml. which have been inactivated by irradiation. Column 10 gives the corresponding dose of inactivating X-rays and column 11 the absolute amount of enzyme in carboxypeptidase units/ml. inactivated per 1000 r.

This column shows that the number of carboxypeptidase units/ml. inactivated by a given number of roentgens and for a given percentage of inactivation is a constant figure for a wide range of X-ray doses, and from columns 2 and 10 it is evident that the same percentage inactivation is obtained by a wide range of X-ray doses depending on the concentration of the carboxypeptidase solution. This applies to 25% and also to 50% inactivation as the constant in column 11 shows. That this constant is lower for 50% than that obtained for 25% inactivation (Table 1) follows from the non-linearity of the enzyme reaction.

*Effect of X-radiation on carboxypeptidase solution
acting on its substrate during radiation*

The concentration of the enzyme solution was C.P.u./ml. = $10^{-6} \times 59.4$ in this experiment (2.6 ml. enzyme for titration, digestion time = 12×10 min.): irradiation of this solution by 10,000 r. reduces the activity to $10^{-6} \times 7.7$ u./ml., whereas the enzyme acting on its substrate during irradiation had an activity of $10^{-6} \times 67.4$ u./ml. which is even slightly higher than the activity of the original enzyme solution. The temperature during irradiation was 20°.

The substrate irradiated alone in control experiments did not show any change.

The carboxypeptidase solution of $10^{-6} \times 59.4$ u./ml. would be inactivated to about 77% by a dose of about 1200 r. which was delivered in the first 3 min., during which time the enzyme could not have reacted to any extent since the digestion time for this concentration was 2 hr. To be sure of the ineffectiveness of the radiation on the enzyme whilst mixed with its substrate, radiation was continued up to a total of 10,000 r. This experiment showed clearly that no inactivation takes place whilst the enzyme is acting on its substrate during irradiation.

The same type of experiment with chloroacetyltyrosine as substrate had an analogous result. The figures are:

Activity of the original enzyme solution [C.P.u.] _{ml} ^{CT}	= $10^{-6} \times 16.7$
Activity after 10,000 r.	= $10^{-6} \times 4.67$
Activity after 10,000 r. irradiated whilst mixed with substrate	= $10^{-6} \times 16.7$

Effect of X-rays on polyphenoloxidase

It was of interest to test whether the results obtained on carboxypeptidase would also hold for an oxidizing enzyme containing a heavy metal. The sample of purified polyphenoloxidase, made from mushrooms by the method of Keilin & Mann [1938], was kindly given to me by Dr H. Jackson of Manchester University. The course of reaction of polyphenoloxidase with its substrate pyrocatechol falls off very rapidly after a short linear start. This can be improved to a certain extent by adding aniline which removes the reaction product of the enzymic activity by forming insoluble dianilo-*o*-quinone. In spite of this the necessity of extending the reaction time between enzyme and substrate with increasing dilution of the enzyme solution soon leads to such a diminution of enzymic activity that the limit of the method for estimating the activity is reached. It is, however, possible to demonstrate that polyphenoloxidase behaves in an analogous way within the range of 20,000–2500 r.

The estimation of the activity was carried out according to Keilin & Mann [1938] in Barcroft-Warburg manometers with conical vessels equipped with side bulb and inner compartment. The various dilutions used in the experiments were made up with distilled water from a stock solution of polyphenoloxidase of a Q_{O_2} value of 8461, and a dry weight of 1.04 mg./ml. The conditions of the experiment (Table 2) were as follows:

The main chamber of the manometers received 2.6 ml. of 0.03 *M* phosphate buffer solution (*pH* 7.3), of which 30 ml. contained 100 mg. pyrocatechol and two drops of aniline. The side bulb contained 0.5 ml. enzyme solution, the inner compartment 0.2 ml. of a 20% KOH solution soaked into a roll of filter paper. The temperature of the water bath was 20°.

Table 2. *Effect of X-rays on various concentrations of polyphenoloxidase*

Time min.	Unirradiated	X-ray dose 10,000 r.	X-ray dose 20,000 r.	ml. enzyme: ml. H ₂ O
5	5.5	5.3	2.8	0.1:20
10	11.5	8.6	6.1	
20	15.7	13.9	6.9	
	Unirradiated	5000 r.	10,000 r.	0.1:40
10	8.7	2.4	0	
20	11.3	4.2	0	
40	12.5	5.4	0	
	Unirradiated	2500 r.	5000 r.	0.1:80
20	5.6	2.1	0	
40	7.0	3.8	0	

It can be seen from Table 2 that for a dilution of the enzyme solution of 1:200, 10,000 r. reduce the original activity slightly, and 20,000 r. to about one-half. For a dilution of 1:400, 5000 r. inactivate about 65% and 10,000 r. inactivate it completely. This shift of the inactivating X-ray dose to lower levels continues on doubling the dilution again. In other words, the dependence of the percentage inactivation on the dilution for a given dose of X-rays is principally the same as the effect of radiation on carboxypeptidase though it is not possible to work out a constant for the reasons mentioned above. Experiments in which the dilution was once more doubled showed that no O₂ consumption took place even in the unirradiated control, making it impossible to perform experiments with doses of X-rays still lower than 2500 r.

DISCUSSION

The reason why earlier investigators have found either no effect of radiation on enzymes or only after the application of enormous doses is that they did not use sufficiently low concentrations of enzymes and also probably that they used too impure enzymes.

The fact that a constant amount of enzyme is inactivated by a given dose of radiation independent of the concentration recalls results by Fricke & Hart [1935], when they obtained oxidation of ferrocyanide to ferricyanide by irradiating their aqueous solution. They found the number of equivalents oxidized per litre and per 1000 r. to be constant and independent of the concentration, and concluded that these transformations are due to "X-ray-activated" water molecules. This is only another way of expressing the same facts and we refrain from assuming any hypothetical mechanism of how the X-rays act on enzymes.

The fact, however, that the two different types of enzymes examined are affected similarly may justify the assumption that many more may respond in a similar way.

In view of the results obtained with dosages ranging from 400,000 r. to values well below those normally used in radiotherapy there is now no reason to assume that only very big doses have an effect on enzymes or that big doses act qualitatively differently from very small ones, nor is it justified to exclude enzymes from playing an important part in explaining the radiation effects on living tissues. On the contrary the concentration of the enzyme and its state during irradiation will be a factor in the action of X-rays on living tissue, the importance of which will vary with the degree to which a particular enzyme is a limiting factor in cell metabolism. Those cells, or even certain phases in their life (e.g.

mitoses) for which the action of certain enzymes is the limiting factor would then be spoken of as showing greater radiosensitivity than other cells. It does not seem to be without significance in this connexion that the increase of the percentage inactivation of enzymes with increasing dilution coincides with the well-established fact of a higher water content and greater radiosensitivity of embryonic tissues compared with the lower water content and lower radiosensitivity of adult tissues. Finally the ineffectiveness of X-rays on carboxypeptidase irradiated in the presence of its substrate may, if it can be generalized, furnish an explanation of the so-called delayed action of X-rays in the sense that the reserves of unused enzyme in the cell are inactivated by radiation, whilst that part of the enzyme already in action remains unaffected by radiation.

SUMMARY

1. The effects of X-rays on crystalline carboxypeptidase and on partly purified polyphenoloxidase have been examined.

2. The percentage inactivation of each of these enzymes is a function of the concentration of the enzyme for a given dose of radiation.

It can be shown for carboxypeptidase that a definite amount of radiation energy absorbed corresponds to a constant amount of enzyme inactivated. In consequence the inactivating dose of X-rays decreases with decreasing concentration of the enzyme for a given percentage inactivation to levels far below those normally used in radiotherapy.

3. X-rays have a qualitatively uniform effect on carboxypeptidase over the whole range from very small doses to doses 1000 times as large.

4. Inactivation of carboxypeptidase does not take place when the enzyme is acting on its substrate during irradiation, whereas the enzyme irradiated without its substrate is inactivated to 85 %.

5. The biological significance of the experimental results is discussed.

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