9. The Effect of X-rays on the Conjugated Protein *d*-Amino-acid Oxidase

By Walter M. Dale, *From the Christie Hospital and Holt Radium Institute, Manchester*

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In a previous paper [Dale, 1940] the effect of X-rays on crystalline carboxypeptidase and polyphenol oxidase was investigated mainly in regard to the radiosensitivity of these enzymes as a function of their concentration. One of the findings of this investigation was that enzymes are affected by reasonably small doses of X-radiation. Since their high chemical activity allows the measurement of radiation effects on them in concentrations as low as those in which they occur in living matter, they are well suited for a more differentiating examination of their radiosensitivity at such concentrations.

The work of Theorell [1937] and Warburg & Christian [1938] has established that a number of enzymes consist of conjugated proteins, and are separable into a chemical compound of a relatively low molecular weight (prosthetic group sometimes called co-enzyme) and a highly specific protein, both of which are necessary for enzymic activity. There is, therefore, an opportunity of measuring the effect of radiation on these components singly, together, and in combination with the substrate and other compounds.

α -Amino-acid oxidase [Krebs, 1933, 1, 2; 1935], as one of those enzymes whose prosthetic group and specific protein can be separated, has been chosen as the subject of this investigation. Also its prosthetic group, alloxazinadeninedinucleotide, is of nucleotide nature and this forms a first step to the study of the radiosensitivity of nuclear substance on which attention has been focused for a long time.

EXPERIMENTAL

Alloxazinadeninedinucleotide. The Ba salt of the alloxazinadeninedinucleotide (in the following called for shortness 'dinucleotide') was prepared from baker's yeast [Warburg & Christian, 1938]. The fraction containing typical deep-brown globules forming clusters of the shape of grapes was used. Its purity was confirmed by spectrophotometric test kindly carried out by Dr A. S. Gillam of Manchester University. A stock solution of dinucleotide of a concentration of 50 μ g./ml. was kept without any preservative, in the refrigerator in a frozen state, thawed and thoroughly mixed before being used. In the majority of the experiments 0.56 μ g. of dinucleotide saturated the specific protein to about 50 %.

Specific protein preparation. Two types of preparation were available: the highly purified but unstable preparation of Negelein & Brömel [1939], or the less pure preparation of Warburg & Christian [1938]. It was decided to use the latter because for the work described in this paper the greater stability of the preparation outweighs the advantage of increased purity. It was, however, possible in some cases to increase the purity without loss of stability.

The best protein preparation, used in most of the experiments, contained 0.138 mg. protein in 2 mg. of preparation. In the alanine test 2 mg. of the preparation with 5 μ g. of dinucleotide took up 105.5 μ l. O₂ in 10 min. and 1.5 mg. with 0.56 μ g. dinucleotide took up 70.5 μ l. O₂.

The amount of protein preparation used in the experiments was as a rule 1.5 mg. dissolved in 2.0 ml. *M*/20 Na₄P₂O₇ of pH 8.3. The activities of different preparations, of course, varied.

Manometric technique. All measurements were carried out, at 38° in Barcroft-Warburg manometers with conical vessels with side bulb and centre cup for KOH. The various

solutions after their exposure to X-rays were tested for activity by the alanine test, the total volume being made up to 3.0 ml. The thermal sensitivity of the dissolved protein preparation at a temperature of 38° makes it necessary to observe certain important precautions.

(1) The protein preparation was dissolved and filtered as late as possible, i.e. after all preparations for the experiment were completed.

(2) When all flasks were attached to their manometers and charged with O₂ at room temperature, batches of 4 manometers with open taps were immersed *simultaneously* in the water bath and their taps closed after about 7 sec.

(3) The time of waiting until equilibrium of temperature was reached was reduced to about 5 min. Then, before a zero reading was taken the fluid in the side bulb of each manometer was tipped into the main compartment in quick succession and after about 2 min. the zero reading was taken.

Irradiation. The various solutions contained in small pyrex test tubes were irradiated from a 450 kV. Metropolitan Vickers continuously evacuated X-ray tube with a cool window with rays of half value layer 6 mm. Cu. The dosage rate was 500 r. per min. and the total dose was measured by a calibrated Hammer dosimeter.

The effect of radiation on alloxazinadeninedinucleotide

A solution of the dinucleotide containing 0.56 μ g./0.8 ml. of water was exposed to increasing doses of X-rays. Samples of 0.56 μ g. taken after the delivery of 2250, 4750 and

Fig. 1.

10,000 r. were tested by the alanine test in presence of 1.5 mg. of protein preparation. The results are given diagrammatically in Fig. 1, curve 2.

There is a decreasing rate of inactivation with increasing X-ray dose. The decreasing slope of the curve resembles that obtained in the previous paper on carboxypeptidase. This point will be discussed later.

The effect of radiation on the specific protein

Two examples of curves are given in Fig. 1. Curve 1 is obtained with 5.6 μ g. of dinucleotide added to 1.5 mg. of protein preparation and curve 3 with 0.56 μ g. of dinucleotide. Thus the protein also undergoes inactivation by the action of radiation, the curves showing the same shape as in the case of irradiated dinucleotide.

The effect of radiation on the dinucleotide-protein system

For clearness abbreviations will be used to denote the various components. The complete system of the alanine test consists of dinucleotide (D), protein preparation (P) and the substrate alanine (A). If one or more components have been irradiated the system has to be completed by the addition of the remaining unirradiated components before the activity test can be carried out.

In Table 1, describing a comparative experiment, the symbols in black type surrounded by a bracket and marked by an asterisk, represent the irradiated substances and indicate whether they were irradiated singly or mixed with one another. The result is that dinucleotide (Man. 1) or protein (Man. 2) or dinucleotide + protein (Man. 3) are affected by radiation to about the same extent whereas the irradiation of dinucleotide and protein carried out separately (Man. 4) causes the highest degree of inactivation, indicating an additive type of effect.

Table 1

X-ray dose 4500 r.; 0.56 μ g. dinucleotide; 1.5 mg. of protein in each manometer.

Manometer	Contents	μ l. O ₂ in 1st 10 min.
1	[D]* + P + A	20.7
2	[P]* + D + A	20.6
3	[D + P]* + A	18.2
4	[D]* + [P]* + A	5.6
5	D + P + A	59.1

Table 2 analyses still further the effect of radiation on a mixture of protein and dinucleotide.

Table 2

X-ray dose 7000 r. D = 0.56 μ g., 2D = 1.12 μ g. dinucleotide.

Manometer	Contents	μ l. O ₂ in 1st 10 min.
1	[P + D]* + A	27.2
2	[P + D]* + P + A	51.6
3	[P + D]* + D + A	26.4
4	P + D + A	46.6
5	P + 2D + A	53.9
6	[P]* + D + A	25.3
7	[D]* + P + A	18.5

Here P and D were irradiated separately and as a mixture as in the previous experiment (Table 1), but on adding P to [P + D]* the O₂ uptake is raised above that in the control in manometer 4 thus making good the loss in activity of P whereas adding D to [P + D]* leaves the drop in O₂ uptake unaltered, i.e. no restoration of the activity lost takes place. Doubling D in the control very slightly raises the O₂ uptake which is already near its maximum.

Therefore the inactivation by irradiation of the mixture P + D is entirely due to the destruction of P whereas D remains unaffected. We can call this prevention of inactivation of dinucleotide by the presence of protein a 'protective' effect of the protein with regard to dinucleotide without, however, stipulating any particular mechanism by using this term.

This protective effect of the protein suggests that the isolated example of a protective effect of the substrate on carboxypeptidase noted in the previous paper is part of a more

general phenomenon. Consequently a number of substances were examined as to their protective action. First alanine itself, then other amino-acids structurally related to alanine but known to be unaffected by the amino-acid oxidase, were found to have a protective effect. The underlying mechanism was first thought to be a competitive inhibition by the formation of a compound between substrate and enzyme. This assumption had to be abandoned when it became clear that many substances not structurally related to the substrate could inhibit the radiation effect.

Table 3

<i>dl</i> -Alanine	Na oxalate	α -Methyl- <i>d</i> -glucoside
β -Alanine	Na formate	NaCl
Leucylglycine	Na nucleate (yeast)	KCNS
Glycine	Na nucleate (thymus)	$K_3Fe(CN)_6$
Na anilinoacetate	Glucose	$K_4Fe(CN)_6$
Na hippurate	Fructose	$NaNO_2$
Na benzoate	Sucrose	$NaNO_3$

The substances (Table 3) were added one at a time to the dinucleotide before irradiation in such a way that 1 ml. of the final mixture contained $0.56 \mu g. = 0.61 \times 10^{-9}$ mol. of dinucleotide and 10^{-4} mol. of the substance. A protective effect of Na benzoate, Na anilinoacetate and $K_3Fe(CN)_6$ could not be examined because 10^{-4} mol. of these substances proved to be inhibiting the amino-acid oxidase in the control. With the exception of NaCl which showed no protective effect whatsoever, all the other substances have a protective effect of 90–100 %, when exposed to an X-ray dose of 8000 r. If, however, the concentration of the protective substance is reduced by stages the protection becomes less and less as is shown, e.g. for leucylglycine in Table 4.

Table 4

X-ray dose = 8000 r. L = Leucylglycine.

Manometer	Contents	$\mu l. O_2$ in 1st 10 min.
1	D + P + A	28.6
2	[D + 10^{-4} mol. L]* + P + A	27.1
3	[D + 10^{-6} mol. L]* + P + A	19.3
4	[D + 10^{-7} mol. L]* + P + A	12.8
5	[D + 10^{-8} mol. L]* + P + A	10.0
6	[D]* + P + A	10.7

For some of the substances of Table 3 the concentration affording about 50 % protection has been determined; the values are given in Table 5.

Table 5

50 % protection by:

10 ⁻⁶ mol./ml.:	Glycine	10 ^{-7.5} mol./ml.:	Glucose
	Na oxalate		Sucrose
	$NaNO_3$		KCNS
			Na formate
10 ⁻⁶ mol./ml.:	Leucylglycine	10 ⁻⁸ mol./ml.:	Fructose
	Alanine		$NaNO_2$
	$K_3Fe(CN)_6$		Na nucleate from yeast
	$K_4Fe(CN)_6$		Na nucleate from thymus
10 ⁻⁷ mol./ml.:	Na hippurate		

None of these substances will protect in a lower concentration than that of the dinucleotide itself; their chemical structure gives no clue as to why some of these substances act protectively in so much lower concentration than others.

The concentration for about 50 % protection has been determined also for glucose (10^{-4}), glycine ($10^{-4.5}$), alanine (10^{-5}), and leucylglycine (10^{-5} mol./ml.) when mixed, one at a time, with the protein. It can be seen from these figures that the protective concentrations are invariably higher for the protein than for the dinucleotide.

DISCUSSION

The experiments show that the prosthetic group, as well as the protein part of the enzyme, is radiosensitive. But this radiosensitivity is in an additive direction only when the two components are mixed after having been irradiated separately, and is modified as soon as they are irradiated as a mixture. This phenomenon of interference by a second partner during irradiation is not confined to the components of the enzyme but obtains also in varying degree for many other substances. It is probable that all substances of organic nature, and inorganic substances which are less inert than, for example, NaCl, will produce this effect. Some investigators of radiation effects on substances other than enzymes have made similar observations [Stenstrom & Lohmann, 1931; 1933; Fricke & Brownscombe, 1933; Fricke & Washburn, 1932]. This inhibition of the radiation effect by the presence of other substances, i.e. a protective effect, may be explained in the following way by taking into account results of the previous paper.

The effect of radiation on substances dissolved in water must be an indirect one because it is independent of the concentration of these solutions. The radiation causes some change in the water and the product thus formed in turn acts on the solute. If there is more than one solute present each one will share in the reaction with this intermediate product according to its respective amount and affinity, i.e. leaving a larger or smaller share for its partner. Consequently some solutes are not protecting at all, as, for instance, NaCl; some others, e.g. glucose, fructose and nucleic acids, are 1000 times more effective than, e.g., NaNO_3 .

But the same mechanism applies to the inactivation of one substance in solution, this in fact being only a special case of the more general statement just made. The radiation energy converted into the intermediary product is at the start of the irradiation fully available for the inactivation of the solute and becomes less and less so as more and more of the solute is inactivated, since it is now shared by the inactivated fraction. Thus the inactivated solute plays the same protective part as a second substance would do if added to the dinucleotide or the protein before irradiation. Hence one arrives at an explanation of the diminishing slope of the activity/X-ray dose curve common to all experiments in the present and in the previous paper. In the latter the amount of enzyme destroyed per 1000 r. over a wide range of concentration was found from the data for 25 and 50 % destruction. In both cases this amount was independent of the concentration but dependent on the percentage destruction. For 25 % destruction 1000 r. were found to destroy about 50 carboxypeptidase units whilst for 50 % destruction only 30 units were destroyed. It was originally thought that these results were due to the non-linearity of the enzyme reaction, whereas they, and hence the diminishing slope, can now be explained more fully on the basis of the protective effect described above.

It follows finally that the protein component of the enzyme must be of greater radiosensitivity than the prosthetic group as may be seen from the following considerations: the protein preparations consist of a mixture of ammonium sulphate, inactive protein and active protein. Our best protein preparation contained in 1.5 mg. of preparation 105 μg . of protein of which about 20 μg . were active protein of the purity obtained by Negelein & Brömel [1939]. The mol. wt. of the active protein can be assumed to be 70,000 [Warburg, *et al.* 1935; Warburg & Christian, 1936; Negelein & Brömel, 1939; Broda & Goodeve, 1941]. Therefore 20 μg . represent 3×10^{-10} mol. of active protein which is of the same order as the amount of dinucleotide employed in the present experiments. The amount of inactive protein accompanying the active portion in all preparations will act protectively

against the action of radiation. If, therefore, in spite of this protection, the protein is destroyed in preference to dinucleotide, in a mixture of protein and dinucleotide, one must assume a higher degree of radiosensitivity per mol. of protein. The same conclusion may be drawn from the fact that the concentrations of other substances needed to protect the protein to the same extent as the dinucleotide are higher than in the case of the dinucleotide.

The greater radiosensitivity of the protein, as compared with its prosthetic group, is noteworthy in view of the fact that it is also the more specific of the two since it determines the type of reaction which the total enzyme catalyses.

In conclusion one can say that the radiosensitivity of an enzyme not only depends on the amounts originally present as pointed out in the previous paper but is also modified by the amounts and radiosensitivities of other substances present.

SUMMARY

The relative sensitivity to X-radiation of the components of the conjugated protein, *d*-amino-acid oxidase, has been studied under varying conditions with the following results:

(1) Not only is the 'specific' protein part of the enzyme radiosensitive but its prosthetic group alloxazinadeninedinucleotide as well.

(2) The radiosensitivity is greatest when the dinucleotide and the 'specific' protein are first irradiated separately and then added to the substrate for the enzymic reaction to take place (additive effect).

(3) The radiosensitivity of the system protein-dinucleotide is equal to, or slightly less than, that of the protein irradiated by itself and almost entirely due to the inactivation of the protein since the dinucleotide is 'protected' by the presence of the protein.

(4) This 'protective' action of the protein is not confined to it, but is exhibited by a great variety of compounds less inert than NaCl.

(5) Different amounts of various substances are needed to inhibit the destruction of 50% of the dinucleotide by a given X-ray dose, nucleic acids belonging to the most effective group. None of the substances examined will protect in lower concentrations than that of the dinucleotide itself.

(6) Larger amounts than those mentioned in (5) are needed for the protection of the protein.

(7) The nature of this protection is discussed and from it an explanation derived for the diminishing slope of all activity/X-ray dose curves in this and the previous paper and for the greater radiosensitivity of the protein as compared with the dinucleotide.

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ERRATUM

On p. 1368 of the previous paper [Dale, 1940], 1000 r./hr. should read 1000 r./min.