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On the Effects of X-rays upon Enzyme Activity. III. Several Experiments on Optimum Dose, Blind Point, Inter-relation to Effectors and etc

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Abstract

Recently Kagawa found out that when the enzyme (papayo tin) substrate (gelatin) mixture had been previously irradiated with X-rays the amount of decomposition products varied in comparison with the unirradiated mixture. He thought that such a quantitative difference of fermentative products might be due to the influence of X-rays on the enzyme action. Therefore the author attempted, in the first place, to determine the optimum dose of X-rays in the proteolytic action of papayotin; in the second place, the relation between the effects of activator and inhibitor respectively and of X-rays; and finally the activity of papayotin after the papayotin substrate mixture as well as after the substrate in the mixture was irradiated.

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From the Department of Legal Medicine

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By

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Introduction

Recently *Kagawa*[1] found out that when the enzyme (papayotin) substrate (gelatin) mixture had been previously irradiated with X-rays the amount of decomposition products varied in comparison with the unirradiated mixture. He thought that such a quantitative difference of fermentative products might be due to the influence of X-rays on the enzyme action. Therefore the author attempted, in the first place, to determine the optimum dose of X-rays in the proteolytic action of papayotin; in the second place, the relation between the effects of activator and inhibitor respectively and of X-rays; and finally the activity of papayotin after the papayotin substrate mixture as well as after the substrate in the mixture was irradiated.

Experimental Method and Materials

Experimental materials:

I have used papayotin (1 : 80, Merck) 0.05 g as enzyme, 5% solution of purified gelatin 10 cc as substrate, *Soerensen*'s phosphate buffer solution (pH 5.3) as buffer, l-cysteine 5 mg as activator, 1/100 mol monojodacetic acid 2 cc as inhibitor and 20% trichloracetic acid 5 cc as remover of proteins.

As to the conditions of X-ray irradiation, other factors were 160 KV., 3 mA., 35 cm taget-skin distance., 0.5 mm. Cu, 0.5 mm. Al filter, half value layer Cu 0.85 mm.

Methods for the experiments:

Many studies on papainase have been made.[2) to 7)] In some cases gelatin was used as a substrate and the increased acidity caused by the decomposition of substrate by papayotin was titrated by alkali, or the amount of non-protein nitrogen which increased by the gelatin decomposition is measured. So I dissolved the papayotin in the buffer solution and then the substrate was added to a certain amount of the filtrate. Next, the activator or inhibitor was added; then each mixed solution was irradiated with 60 r, 200 r, 400 r and 1000 r (in air). The non-protein nitrogen in the mixtures was measured immediately, 6 hrs., 12 hrs., 24 hrs., 48 hrs. and 72 hrs. after the irradiation, and the values of the non-protein nitrogen were compared with those in the unirradiated mixtures (controls). Such experiments were repeated a minimum of 3 times for each case, and the average value is shown in the tables. It must be added that, as a method of interrupting the fermentative decomposition, the mixture was boiled and the protein in the mixture was removed by trichloracetic acid.

The Effects of X-rays on Papayotin and their Results

(I) *The optimum dose and "the blind point" of X-rays in the enzyme action:*

X-rays are considered to have sometimes activative, sometimes inhibitive and sometimes destructive effects upon an organism. According to the previous opinion that it is activative with a small dose and inhibitive with a moderate dose, it seems to me that there may be a certain dose of X-rays which has no effect upon an organism. On the other hand, it is also presumed that the optimum dose of X-rays in the enzyme action may exist, such as the optimum pH in it. In Table I the results of irradiation at each augmentation of 20 r from 20 r up to 200 r are shown. As shown in this table, the decomposition of gelatin was maximum at 60 r and equal to control at some dose between 120 r and 140 r. Furthermore, the irradiation was performed in the range from 120 r to 140 r with an interval of 5 r, and it was found, as seen in Table II, that the dose 130 r was the turning point from the activative to inhibitive dose. Therefore this may be the so-called "blind point" where

Table I. The preliminary experiment of the optimum dose and the blind point
(2% gelatin was used in this experiment)

Time \ r	0	20	40	60	80	100	120	140	160	180
Immed.	7.280									
6 hrs.	8.101	8.208	10.082	11.134	11.021	9.714	8.413	8.008	7.925	7.920

(The figure of each table shows non-protein nitrogen in mg)

Table II. The experiment of the optimum dose and

Time \ r	0	40	60	80	100	120	125
Immed.	33.364						
6 hrs.	50.962	51.879	54.611	54.022	53.671	51.943	51.182
12 hrs.	61.114	62.011	64.131	64.041	63.235	62.194	61.719
24 hrs.	63.675	65.043	66.627	66.588	64.921	64.124	63.866
48 hrs.	65.232	67.717	70.099	69.324	68.181	66.932	65.811
72 hrs.	66.721	68.308	71.023	68.947	67.339	66.927	66.784

the influence of X-rays can not be found in spite of the irradiation.

(II) *The relationship of X-ray irradiation to the effectors in the enzyme action:*

An investigation of the effective substances, which have some activative or inhibitive influence upon enzyme action, might help us to make suitable classifications and to explain the mechanism of the enzyme action as well as the chemical structure of the enzyme, especially the chemical composition of its active radicals. Therefore, in order to investigate the changes in the enzyme activity under certain conditions the following experiments were performed in which the enzyme substrate solution was irradiated after adding the effector.

(1) The irradiation of the mixture with activator.

There are two kinds of activators: (A) the specific activator, without which the enzyme can not become active and (B) the general activator which accelerates the enzyme activity. Furthermore, the former (A) is divided into 3 groups: a) the general

irreversible specific activator which we find in connection with secretion enzymes, such as hydrochloric acid with pepsin[8] and enterokinase with trypsine[9], b) the activator which accelerates the combining power between the active group and the carrier group of an enzyme, such as iron or copper with the dehydrase[10], and c) the reversible activator which is the so-called complement[11) 12)], such as galactose diphosphoric acid with phosphoglucomutase. The latter (B) is called, in general, activator, except the specific ones.

In this experiment l-cysteine was used as an activator after confirming that it activates the papayotin sufficiently (Table III) and the results of the experiment are seen in Table IV and V.

the blind point

130	135	140	160
50.962	50.960	50.824	50.824
61.127	60.943	60.921	60.844
63.677	63.631	63.630	63.511
65.232	65.113	65.009	64.312
66.733	66.710	66.520	66.522

(2) The irradiation of the mixture with inhibitor.

The inhibitors are likewise divided into 2 groups: (A) the irreversible destructive inhibitor in the enzyme action, namely, a) the substances which decompose the protein, the carrier groups of an enzyme[13], b) the denaturators of protein[14], and c) the substances which influence mainly the active groups of an enzyme[15]. (B) The reversible inhibitor, namely, a) the absolute inhibitor in the enzyme action[16]. b) An inhibitor which enfeebles the enzyme activity[17] or prevents the combination of the enzyme with substrate by absorbing the enzyme[18]. I have carried out the irradiation experiment in which monojodacetic acid was used as an inhibitor (Table VII), after confirming the fact that the substance has a sufficient inhibitive action (Table VI).

Table III. The experiment in which an activator was added
(In this experiment 2% gelatin was used)

Cyst. \ Time	Immed.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
With	7.285	9.947	10.437	10.788	11.068	11.488
Without		8.055	8.571	9.814	10.850	11.212

Table IV. The irradiation experiment in which an activator was added

r	Cyst. \ Time	Immed.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
0	With	33.263	56.174	66.304	66.781	70.245	71.121
	Without		51.542	61.321	63.785	65.193	66.146
60	With		59.080	66.421	67.084	70.913	71.425
	Without		55.782	64.625	66.723	70.086	70.923
200	With		54.731	65.823	66.435	70.271	70.861
	Without		50.164	60.363	62.067	65.741	66.230
400	With		53.997	65.764	66.393	70.301	70.888
	Without		50.043	60.020	62.339	64.999	65.907
1000	With		60.021	66.881	66.921	70.904	71.325
	Without		55.112	65.333	67.372	70.713	70.864

Table V. Ratio of hourly increases of non-protein nitrogen in experiments where some activator was added

r	Cyst. \ Time	0–6 hrs.	6–12 hrs.	12–24 hrs.	24–48 hrs.	48–72 hrs.
0	With	3.818	1.688	0.039	0.144	0.036
	Without	3.046	1.629	0.205	0.058	0.039
60	With	4.032	1.223	0.055	0.159	0.021
	Without	3.753	1.473	0.174	0.140	0.034
200	With	3.578	1.848	0.051	0.159	0.024
	Without	2.816	1.699	0.142	0.153	0.020
400	With	3.455	1.961	0.052	0.162	0.024
	Without	2.796	1.662	0.193	0.110	0.037
1000	With	4.459	1.143	0.003	0.165	0.017
	Without	3.641	1.703	0.169	0.139	0.006

(III) *The activity of papayotin solution after irradiating and boiling:*

All kinds of enzymes which have colloidal nature are usually unstable against heat and most enzymes thereby are decomposed. But the experiment performed by *J. Bodnár*, and *J. Villányi*[19] tells us that an enzyme in an aqueous solution, which had lost its activity by being heated to 100°C, again becomes active.

Table VI. The experiment in which an inhibitor was added
(In this experiment 2% gelatin was used)
M. J. means the monojodacetic acid

M. J. \ Time	Immed.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
With	7.291	7.907	8.207	9.734	10.805	11.115
Without		8.174	8.593	9.826	10.912	11.334

Table VII. The irradiation experiment in which an inhibitor was added

r	M. J. \ Time	Immed.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.
0	With	32.949	44.372	50.039	55.437	58.278	59.717
	Without		51.538	61.321	63.655	65.283	66.378
60	With		50.721	58.345	61.044	65.017	65.949
	Without		55.766	64.625	66.613	70.245	70.954
200	With		43.118	49.721	57.818	58.801	61.234
	Without		50.121	60.325	62.125	65.740	66.222
400	With		43.089	49.902	58.024	58.992	61.043
	Without		50.125	60.020	62.327	64.987	65.973
1000	With		50.443	57.911	62.324	65.219	66.305
	Without		55.164	65.342	67.385	70.722	70.875

In this experiment also the activity of papayotin which was irradiated and heated changed in some degree with the lapse of time. Of course, this experiment was performed with careful consideration for the influence of bacteria, etc. The results of this experiment are shown in Table VIII.

(IV) *The activity of papayotin added to the substrate which was previously irradiated:*

Kagawa[1] has reported that X-rays had some influence upon papayotin itself, but it is questionable that the excited energy occurs in the substrate by irradiation and that consequently the substrate has aquired a tendency to be easily decomposed. Therefore the following three cases should be examined.

Table VIII. The experiment in which the fermentative was boiled

Time / r	Immed.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.
0	33.264	42.371	48.297	50.288	53.047	56.722	56.81!	56.811	57.029
60		45.352	51.444	52.471	56.392	58.021	58.323	58.334	58.341
200		42.307	47.947	50.285	54.761	56.722	56.744	56.752	56.739
400		42.371	48.037	50.089	54.821	56.745	56.745	56.752	56.793
1000		46.002	51.473	52.556	56.173	58.090	58.355	58.355	58.357

Table IX. The experiment of X-ray irradiation in the substrate only

Gel.	Pp.	Pp. in Pb.	Immed.	6 hrs.	12 hrs.	24 hrs.
(+)	(+)		33.071	55.780	64.633	66.749
(−)	(−)			51.540	61.332	63.791
(−)	(+)			55.777	64.633	66.750
(+)	(−)			51.536	61.335	63.788
(+)		(+)		55.782	64.635	66.751
(−)		(−)		51.535	61.340	63.788
(−)		(+)		55.777	64.632	66.748
(+)		(−)		51.540	61.343	63.790

Gel.……Gelatin. Pp.……Papayotin powder. Pb.……Phosphate buffer solution. (+)……Irradiated. (—)……Unirradiated.

(1) Papayotin and phosphate buffer solution were added to the substrate which was previously irradiated.

(2) The substrate and phosphate buffer solution were mixed with the papayotin which was previously irradiated.

(3) The substrate was mixed with the papayotin, which was dissolved in the phosphate buffer solution and irradiated.

The results of these experiments are given in Table IX.

Discussion

(I) It is quite clear that specific relations exist between the enzyme and the substrate, and further that enzyme activity is in-

fluenced greatly by its surroundings, especially by pH. For instance, it showed maximum activity at pH 6.95 – 8.0 in the case of pancreaslipase of man and dog[21] and at pH 5.4 in the case of cathepsine[22]. Such optimum pH of many kinds of enzyme has been studied in detail[23)-25)]. Considering that the effects of X-rays upon the activity of enzymes differ from each other with respect to the dose of X-ray irradiation, it seems to me that there should exist the optimum dose of X-rays in the enzyme action. That is why I made the above experiments. As already mentioned, the substrate (gelatin) was maximally decomposed by the papayotin irradiated with 60 r. It means that 60 r has to be the optimum dose of X-rays for papayotin. And then the important problem is raised as to why the X-ray irradiation of this dose stimulated the papayotin activity maximally.

W. Roman, who discovered that the activity of the dehydrase irradiated with visible light increased, discussed the possibility that the atom arrangement or the electron state of the enzyme might be brought into the most favourable condition for its catalytic activity by irradiation. I presume also, according to his hypothesis, that in my experiment the papayotin irradiated with 60 r might result in a condition similar to the case of the above mentioned dehydrase, but I have no method to affirm this presumption at present.

(II) Next, let us turn to a discussion of the so-called "blind point" of X-ray irradiation. With reference to the previous opinion that X-ray irradiation in small dose acts activatedly, in moderate dose inhibitedly and in large dose destructively, *Jungling*[28] has explained that the small dose of irradiation activates the active factors and the moderate dose activates the inhibitive factors in the cell. I think there must exist a turning point from the activation to inhibition in a dose of X-rays, where no influence of X-ray irradiation can be seen. My experiment has demonstrated that such a turning point, or blind point, actually exists at the dose of 130 r where the amount of non-protein nitrogen nearly equals that of the controls (unirradiated papayotin substrate mixture) as shown in Tables I and II. According to the opinion of *Jungling*, it can be explained that this phenomenon occured because of the irritation of active and inhibitive factors in equal amounts.

(III) With reference to the influence of the activator upon papayotin, many investigations were made, in which hydrogen

sulphide[29], l-cysteine[30], reduced glutation[31], sodium sulphite[32], sulphurous anhydride[4] etc. were used. In my experiment, l-cysteine was used as an activator under the above mentioned conditions, as shown in Table IV : the result thereof shows that the activity of papayotin was increased by adding l-cysteine and by irradiation with either 60 r or 1000 r. In this case the activity of papayotin accelerated by adding 3 mg l-cysteine only is greater than that by mere irradiation with 60 r as well as 1000 r.

The activity of papayotin which has been irradiated with 200 r as well as 400 r and accelerated by l-cysteine is smaller than that of acceleration by cysteine only, but larger than that of irradiation with 200 r as well as 400 r only. Therefore the papayotin activity in this case is activated by cysteine on the one hand, and inhibited by X-ray irradiation on the other hand where the influence of the inhibiting power is stronger than that of the activating power.

(IV) Quinone[32], diazomethan[15], monojodacetic acid[5, 33, 34], etc. have hitherto been used as inhibitors of papayotin activity. As above mentioned, I also used monojodacetic acid as an inhibitor in the experiment. Contrary to the result of the experiment in which the activator was used, 200 r as well as 400 r irradiation, and monojodacetic acid inhibited the papayotin action, but 60 r as well as 1000 r irradiation in the case of adding monojodacetic acid inhibited the papayotin activity slightly in comparison with the control, because the amount of non-protein nitrogen, which increased by 60 r as well as 1000 r irradiation, is smaller than that which decreased by the addition of monojodacetic acid as shown in Table VII.

(V) *J. Pace*[35] has thought that the inactivation of an enzyme could be caused by the heat denaturation of the protein part of the enzyme, the carrier group of the enzyme, but as already explained the reversible reaction of trypsine was observed by *Bodnár* and *Villányi*, and that of amylase by *Northrop* and *Kunitz*. As seen in Table VIII, in which the result of the experiment performed with papayotin is shown, the non-protein nitrogen increases gradually with the elapsing time after boiling. From this we can acknowledge the fact that the heat denaturation of papayotin is also reversible as well as that of trypsine or amylase. Therefore we must pay attention to the fermentative action of papayotin which has been made inactive by boiling while in an aqueous solution.

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Moreover, with regard to the fact that the amount of non-protein nitrogen in the case of the irradiation with 60 r and 1000 r was greater than that in the case of unirradiation or irradiation with 200 r and 400 r, future investigations must be made.

(VI) The relation between enzyme and substrate is of the most specific nature in the enzyme action, and now is thought to be founded upon the atom arrangement of the substrate. Concerning this important problem *Kagawa*, who had observed that papayotin powder irradiated with X-rays changes its activity activatedly or inhibitedly for a considerable time, inferred that, in the irradiation of the enzyme substrate system, X-rays have influence directly on the enzyme itself. In the experiment which I have also made to determine whether X-rays influence enzyme or substrate, no influence could be found on the substrate in the case of the irradiation with 60 r, as shown in Table IX. Consequently we can decide that X-ray irradiation directly influences papayotin itself, and not the substrate.

Summary

(I) The activity of papayotin increases or decreases according to the dose of X-ray irradiation, and the irradiation with 60 r is thought to cause the maximum activation of papayotin.

(II) It can be supposed that there exists a so-called "blind point" in the dose of X-ray irradiation, at which point no influence of X-rays can be found.

(III) It can be affirmed that in the case of adding an activator (l-cysteine), the activity of papayotin is additionally stimulated with 60 r as well as 1000 r irradiation and stimulated slightly with 200 r as well as 400 r ; and in the case of adding the inhibitor (mono-jodacetic acid), the activity of papayotin is inhibited with 200 r as well as 400 r and inhibited slightly with 60 r as well as 1000 r.

(IV) In experiments in which activators were added, the quantity of the non-protein nitrogen discovered in those groups that have been irradiated with 60 r or 1000 r is greater than that of other groups, which fact may be ascribed to the elevated activity of papayotin in the beginning of the reaction ; for, in the first six hours only, differences could be found in those values which are obtained by dividing the amount of non-protein nitrogen present by

the time intervals required in each group to produce the nitrogen: after that, almost no difference can be seen in each group.

(V) Papayotin is thought to belong to enzymes which show reversible reaction against heat.

(VI) No influence could be found on the activity of papayotin in the reaction system, when only the substrate was irradiated with X-rays.

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