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The Mechanism of the Effect of X-rays on Bacterial Toxins

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PART 1. THE SENSITIVITY OF TOXINS TO X-RAYS

Relatively little work has so far been done on the effect of X-rays on bacterial toxins. Gerhartz (1909) studied the effect of X-rays on diphtheria toxin, and Fabre & Ostrovsky (1912) found a partial inactivation of this toxin following the addition of radium to the toxin solution. Ferroux & Muttermulch (1925) detoxified tetanus toxin with radium and observed that the loss of toxicity was accompanied by a loss of antigenicity. Baker (1935) showed that the sensitivity of tetanus toxin to radium was similar to that of Rous sarcoma virus and bacteriophage, and was less marked than the sensitivity of bacteria. Carpenter & Stokinger (1942), working with culture filtrates of *Clostridium botulinum*, *Cl. perfringens*, *Cl. tetani* and *Staphylococcus aureus*, showed that X-rays caused a partial inactivation of each toxin, tetanus toxin being the most resistant. Loiseleur & Crovisier (1943) were able to increase the effect of X-rays on tetanus toxin by bubbling oxygen through the solution during irradiation.

Most of these investigators, however, were able to obtain only a partial destruction of toxins, and the very wide range and unknown degree of purification of the preparations used in all these works make it quite impossible to draw any conclusions as to the actual sensitivity to X-rays and radium of the toxins

used. Moreover, the quantitative results of different experiments cannot be compared and cannot be used as a reliable basis for further investigations. We therefore decided to investigate the conditions favourable for the complete destruction of bacterial toxins by X-rays, the properties of the toxins after irradiation, and the influence of the medium on the sensitivity of toxins to X-rays.

For this purpose two toxins were used, tetanus toxin and *Staph. aureus* haemolysin.

EXPERIMENTAL

Methods

Media used for the production of toxins, and purification of the toxins. Two preparations of tetanus toxin were used.

(1) Crude tetanus toxin. *Cl. tetani* was grown according to Mueller & Miller (1942), and 3% tomato juice was added to the medium. The toxin solution obtained contained 4.5 mg. N/ml., and upon assay on mice was found to contain 1000 minimum lethal doses (Min.L.D.)/ml.

(2) Purified tetanus toxin. This toxin was obtained and purified according to Eaton & Cronau (1938). The purified solution contained 0.14 mg. N and 1000 Min.L.D./ml.

(3) *Staph. aureus* haemolysin. *Staph. aureus* was grown on the medium of Favorite & Haman (1941). The filtrate contained 0.95 mg. N/ml., and up to a dilution of 1:200 was capable of haemolyzing rabbit corpuscles. No purification of the haemolysin was attempted. The haemolysin was assayed as follows: 0.5 ml. of serial dilutions of the

toxin were added to 0.5 ml. samples of a 2% suspension of rabbit red-blood corpuscles in saline, and were incubated for 2 hr. at 37°, after which they were refrigerated overnight. The coagulase activity of the same filtrate was tested according to the method of Williams & Harper (1946).

Irradiation. This was carried out with an Ott tube, operated at a tension of 35 kV., and at a current of 5 ma., with a copper anticathode. The distance between the vessel in which the toxin was irradiated and the focal point was 50 mm., and the intensity of the X-rays was about 90,000 r./min. The window consisted of aluminium foil 30 μ . thick.

In the case of tetanus toxin, 0.1 ml. samples of toxin solution containing 100 Min.L.D. were irradiated in cups 15 mm. in diameter. No visible changes were induced in the solutions by irradiation. A control sample (0.1 ml.) containing 100 Min.L.D., kept near the experimental sample during the irradiation, was serially diluted with saline so that 0.5 ml. of the final solution contained 1-50 Min.L.D. After irradiation, the treated sample was diluted serially in an identical manner. At least four white mice, averaging 20 g. in weight, were inoculated intramuscularly with 0.5 ml. of each dilution of the irradiated and the untreated control toxin.

Irradiation of haemolysin was done under the same conditions as for tetanus toxin; 0.1 ml. was irradiated in the same type of cup, and the assays were performed by using identical serial dilutions of irradiated and control samples.

RESULTS

Tetanus toxin. Purified tetanus toxin is destroyed by irradiation much more readily than crude toxin. Comparison of Tables 1 and 2 shows that a dose of

7 million r., under the conditions of the experiment, completely destroyed the purified toxin, whereas the same dose reduced the activity of the crude toxin preparation by only 20%.

X-irradiation destroys the toxicity and the antigenicity of tetanus toxin simultaneously. An attempt was made to immunize mice with purified irradiated tetanus toxin. Purified tetanus toxin was irradiated with 7 million r., thereby completely destroying its toxicity. Twelve mice were intramuscularly inoculated with this irradiated toxin, each mouse receiving four injections, at 2-day intervals, of a dose corresponding to 100 Min.L.D. of the original potency. Three weeks after the last injection the mice were divided into three groups, receiving respectively 2, 5 and 10 Min.L.D. of the original unirradiated toxin. A control group of 12 untreated mice received the same doses at the same time.

All the 24 mice both in the control and the experimental groups died, those receiving 10 Min.L.D. after 3 days, those receiving 5 Min.L.D. after 5 days and those receiving 2 Min.L.D. after 6 days, irrespective of whether they had previously received irradiated toxin or not. This clearly demonstrates that X-ray irradiation destroys the antigenicity of tetanus toxin in the same dose that destroys its toxicity.

Staphylococcus haemolysin. Table 3 shows that, under the conditions of the experiment, 5 million r. completely destroyed the activity of our haemolysin

Table 1. *Irradiation of purified tetanus toxin*

		X-ray doses in millions r.																							
		7			5				4				3				2			Control					
Min.L.D.		100	50	20	100	50	20	10	100	50	20	10	50	20	10	5	20	10	5	20	10	5	1		
Days																									
1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	T.	T.	.		
2		.	.	.	T.	.	.	.	T.	.	.	.	T.	T.	.	.	T.	T.	.	.	+	.	T.		
3		.	.	.	+	.	.	.	+	.	.	.	+	T.	.	.	+	.	.	.	.	+	.		
4		.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	+	.	.	.	.	+		
5-10		0	0	0	.	0	0	0	.	0	0	0	.	Δ	.	0	.	.	0	.	.	.	.		
		+ death;				T. tetanus;				Δ recovery;				0 unaffected.											

+ death; T. tetanus; Δ recovery; 0 unaffected.

Table 2. *Irradiation of tetanus toxin prepared in a synthetic, protein-free medium*

		X-ray doses in millions r.																											
		7				5					4					3				2			Control						
Min.	L.D.	100	50	20	10	100	50	20	10	5	100	50	20	10	5	50	20	10	5	20	10	5	20	10	5	20	10	5	1
Days																													
1		.	T.	.	0	.	.	T.	0	0	.	.	T.	0	0	.	T.	.	0	.	T.	.	.	.	T.	0	0	.	.
2		.	+	T.	.	.	.	.	.	0	.	.	.	T.	.	.	+	T.	.	.	.	.	T.	.	T.	T.	T.	.	.
3		.	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	+	.	T.	.	.
4		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5		.	.	T.	0	.	.	.	.	T.	0	.	.	T.	0	.	.	.	T.	0	.	+	T.	.	.	+	.	+	.
6-10		.	.	Δ	.	.	.	.	Δ	.	.	.	.	Δ	.	.	.	.	Δ	.	.	.	.	.	.	.	.	.	.
							+ death;					T. tetanus;					Δ recovery;					0 unaffected.							

+ death; T. tetanus; Δ recovery; 0 unaffected.

preparation, while smaller doses led to only partial destruction.

Destruction of staphylococcus coagulase by X-rays was tested with the same filtrate that was used as haemolysin. It was found that a dose of 3 million r. completely destroyed the coagulase activity.

Table 3. *Irradiation of staphylococcus haemolysin*

Dilutions	X-ray doses in millions r.				
	5	3	2	1	Control
1/10	0	±	++	++	++
1/20	0	±	+	++	++
1/50	0	0	±	+	+
1/100	0	0	0	±	+
1/200	0	0	0	0	±

++ complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

X-rays simultaneously destroy the toxicity and the antigenicity of staphylococcus haemolysin. A rabbit was intravenously inoculated every 2 days with 0.3 ml. of irradiated haemolysin, receiving a total of twelve injections. A control rabbit received an identical series of injections of unirradiated active haemolysin. Three weeks after the last injection serum from each rabbit was tested for its haemolysin-neutralizing power. As a control we also tested normal rabbit serum. The sera were serially diluted up to 1:2000; 0.1 ml. of haemolysin was added to 0.1 ml. of each dilution.

Table 4. *Action of immune sera (staphylococcus haemolysin) on rabbit corpuscles*

Serum dilutions	Serum of animals immunized with irradiated haemolysin	Serum of animals immunized with active haemolysin	Normal serum control
1/10	0	0	0
1/20	0	0	±
1/50	+	0	+
1/100	++	0	++
1/200	++	0	++
1/500	++	0	++
1/1000	++	0	++
1/2000	++	+	++

++ complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

Table 4 shows that both normal serum and serum from the rabbit that had been inoculated with irradiated haemolysin prevented haemolysis up to a dilution of 1:20, whereas serum from the rabbit which had been inoculated with active, unirradiated haemolysin prevented haemolysis up to a dilution of 1:2000. Thus, irradiation of staphylococcus haemolysin destroyed its antigenicity together with its haemolytic power.

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PART 2. THE INFLUENCE OF ACCOMPANYING SUBSTANCES IN THE MEDIUM ON THE SENSITIVITY OF BACTERIAL TOXINS TO X-RAYS

The mechanism of the effect of irradiation on toxins and other biological materials was first studied by investigating the effect of the concentration of the biological substance on the ease with which it was destroyed. It was very quickly observed that the substances in the medium accompanying the active biological material were of the greatest importance in determining the sensitivity of the material to irradiation.

As early as 1909, Courmont & Nogier showed that the inactivation of tetanus toxin by ultraviolet rays proceeded much more rapidly if they diluted the medium with distilled water rather than with bouillon. They explained this effect by assuming that the bouillon contained substances which protected the toxin from the effect of ultraviolet radiation. Friedewald & Anderson (1941), working with Shope papilloma virus, showed that the purification of extraneous proteins from the medium greatly decreased the resistance of the virus to X-rays, whereas the addition of protein to the purified virus solution increased its resistance to X-rays. Dale (1940, 1942) and Tytel & Kersten (1941) found that the sensitivity of enzymes to X-ray irradiation was dependent on the degree of purification of the preparation, and at low concentrations on the concentration of the enzyme in the medium. Similar results were obtained by Goldhaber & Leibowitz (1943) with ultraviolet radiations, and by Luria & Exner (1941) in the action of X-rays on bacteriophage. Evans, Slaughter, Little & Failla (1942) carried out detailed experiments on the influence of foreign proteins and a peptide on the X-ray sensitivity of the fertilizing power of *Arbacia* spermatozoa. Lea, Smith, Holmes & Markham (1944) noted the protective effect of gelatin on the inactivation of tobacco mosaic virus by X-rays.

All these studies make it quite clear that proteins, their degradation products and other organic compounds protect a wide range of biologically active materials from the destruction normally caused by electromagnetic radiations. It seemed interesting, therefore, to determine whether the same effect could be demonstrated with bacterial toxins, and whether any light could be thrown on the mechanism of such an effect.

EXPERIMENTAL

Materials used. Proteins: crystalline egg albumin, bovine serum globulin, casein, gelatin, protamine, edestin.

Peptones: albumin, serum globulin, casein peptones.

Protein hydrolysates were prepared from egg albumin, serum globulin and casein by autoclaving for 10 hr. with

25 % (v/v) sulphuric acid. The latter was removed as BaSO₄, and the filtrate neutralized with NaOH.

Amino-acids: the mixture of amino-acids used was prepared in the proportions in which they are found in egg albumin (Schmidt, 1938). Separate amino-acids were also tested.

Reducing agents: ascorbic acid, thioglycollic acid and glutathione.

Oxidizing agents: cystine and maleic acid.

Procedure. Each of the above substances was added in varying concentrations to the toxin preparations which were then irradiated with a dose sufficient to destroy the toxin in the absence of any additions. Careful controls were set up to ascertain whether the added substance by itself in any way affected the activity of the toxin, as for example by a change of pH, oxidation, etc. After irradiation the toxin was assayed in order to determine the lowest concentration of the added substance that would protect the toxin from X-ray inactivation, under the conditions of the experiment.

RESULTS

The protective effect of proteins

Tables 5 and 6 show that tetanus toxin was protected by egg albumin in a concentration as low as 0.5 %, while haemolysin was protected by a concentration of 2.5 %. In the case of serum globulin, a concentration of 2.5 % was insufficient to protect tetanus toxin, but haemolysin was protected in a concentration of 1 % (see Table 6).

Protamine was tested only on tetanus toxin, and was found to protect it in a concentration as low as 0.5 %. Casein, gelatin and edestin were tested up

to a concentration of 2 % on tetanus toxin and on staphylococcus haemolysin, and were found to have no protective effect whatsoever.

The protective effect of peptones

From Tables 6 and 7 it can be seen that albumin peptone protects tetanus toxin in a concentration of 2 %, and haemolysin in a concentration of 5 %; that globulin peptone completely protects tetanus toxin and haemolysin in a concentration as low as 2 %. Casein peptic, tryptic and papain peptones were all found to have no protective effect in a concentration as high as 5 %.

Methylation of the albumin peptone used above with diazomethane did not decrease its protective effect, demonstrating that the free carboxyl groups are not essential for this property.

Comparing the protective effects of proteins and peptones, it is clear that the latter are considerably more effective than their corresponding proteins. This is particularly clear in the case of serum globulin, which does not protect tetanus toxin at all up to a concentration of 2.5 %, whereas globulin peptone completely protects it at a concentration of 2 %.

The protective effect of amino-acids

In order to determine whether the total molecular structure of the proteins and peptones or the amino-acid building blocks were the agents responsible for the effects studied, acid hydrolysates of proteins, various amino-acid mixtures and single amino-acids were tested.

Table 5. *Effect of egg albumin on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

		Albumin																						Control						
		10%					5%				2%			1%			0.5%			0.1%										
Min.	L.D.	50	10	5	2	1	50	10	5	2	50	10	5	50	10	5	50	10	5	50	10	5	50	10	5	50	10	2	1	
Days																														
1		+	+	+	T.	T.	+	T.	T.	0	T.	.	.	T.	.	.	0	.	.	0	0	0	+	+	T.	T.	.	.	.	
2		.	.	.	+	.	.	+	T.	.	+	T.	0	T.	T.	.	T.	.	.	.	.	.	.	.	.	.	.	+	.	.
3		.	.	.	.	+	.	.	+	T.	.	+	0	+	T.	0	T.	T.	.	.	.	.	.	.	.	.	.	.	.	+
4		.	.	.	.	.	.	.	.	T.	.	.	.	.	.	.	+	T.	.	0	0	0	.	.	.	.	.	.	.	.
5-10		.	.	.	.	.	.	.	.	Δ	.	.	.	.	Δ	.	.	Δ	.	.	.	.	.	.	.	.	.	.	.	.
							+ death;					T. tetanus;					Δ recovery;					0 unaffected.								

Table 6. *Effect of proteins and peptones on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

Haemolysin dilution	Albumin (%)			Serum globulin (%)			Albumin peptone (%)					Globulin peptone (%)				Control		
	5	4	2.5	4	2.5	1	5	2	1	0.5	0.1	2	1	0.5	0.1	.	.	.
1/10	++	++	0	++	+	±	++	++	++	+	0	++	++	+	±	.	++	.
1/20	++	+	0	+	+	0	++	++	+	±	0	++	+	+	±	.	++	.
1/50	+	0	0	+	±	0	++	+	0	0	0	+	+	0	0	.	++	.
1/100	+	0	0	±	-	0	+	0	0	0	0	±	0	0	0	.	+	.

Table 7. *Effect of peptones on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

Min.L.D. Days	Albumin peptone (%)																Control			
	2				1				0.5				0.1				50	10	5	2
1	+	T.	T.	0	T.	T.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	T.	T.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	+	.	T.	T.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	+	+	.	.	.	.	.	.	.	.	.	.	+
5-10	.	.	.	Δ	.	.	Δ	Δ	.	Δ	0	0	0	0	0	0	.	.	.	.

Min.L.D. Days	Globulin peptone (%)																Control			
	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
1	+	T.	T.	.	T.	.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+
5-10	.	.	.	.	.	.	Δ	.	+	0	0	0	0	0	0	0	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Acid hydrolysates of egg albumin, serum globulin and casein were studied and were found to be much less effective than the original proteins and peptones. Thus, albumin and globulin hydrolysates showed partial protection of both toxins at 5%, very little at 2%, and none at all at 1%. Casein hydrolysate did not protect at all, up to 5%. An amino-acid mixture, in the proportions present in egg albumin, was approximately as effective as were the acid hydrolysates, and no more. It should be noted that the mixture contained cystine, and not cysteine.

Of all the amino-acids tested singly, only tryptophan, cysteine and methionine were effective as protective agents, while all the others, singly or in groups, were ineffective. Thus, almost complete protection of haemolysin was obtained with cysteine at

0.1%, with tryptophan at 0.5% and with methionine at 2%. Tyrosine could not be tested because at pH 7 it was insoluble in adequate concentrations. Glutathione was an effective protective agent in a concentration of 0.5%. Cystine on the other hand, was totally ineffective.

At this point it became obvious that the effective amino-acids were those that had reducing properties, and additional reducing agents were therefore tested.

Reducing and oxidizing substances

The results with these substances are summarized in Tables 8, 9 and 10. Thioglycollic acid was found to afford protection of haemolysin in a concentration of 0.5% and of tetanus toxin in a concentration of

Table 8. *Effect of reducing substances upon the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

Haemolysin dilutions	Cysteine (%)			Cystine* (%)		Glutathione (%)			Thioglycollic acid (%)			Methionine (%)			Tryptophan (%)			Control		
	1	0.1	0.05	0.5	0.1	0.5	0.1	0.05	0.5	0.1	0.05	2	0.5	0.1	1	0.5	0.1	.	.	.
1/10	++	++	+	±	-	++	+	-	++	++	±	++	++	±	++	++	+	.	++	.
1/20	++	+	±	±	-	±	±	-	++	+	±	+	+	-	+	+	±	.	++	.
1/50	+	+	-	-	-	-	-	-	±	±	-	±	-	-	±	±	-	.	+	.
1/100	±	-	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	.	+	.

* Present in the form of a precipitate.

Table 9. *Effect of reducing substances on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

Min. L.D. Days	Cysteine (1%)				Cysteine (0.5%)				Thioglycollic acid (1%)				Thioglycollic acid (0.5%)				Control			
	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
1	+	.	.	T.	.	.	.	.	+	.	.	.	T.	.	.	.	+	+	.	.
2	.	T.	.	.	.	.	.	.	T.	.	.	.	+	.	.	.	.	.	T.	.
3	.	.	T.	.	+	T.	.	.	.	T.	.	.	T.	.	.	.	.	.	.	T.
4	.	+	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	+	.
5-10	.	.	Δ	0	.	Δ	0	0	.	.	Δ	0	.	Δ	0	0	.	.	.	+

Table 10. *Effect of ascorbic acid on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

Haemolysin dilutions	Ascorbic acid (%)				
	0.25	0.05	0.01	0.005	Control
1/20	++	+	+	±	++
1/50	+	+	±	-	+
1/100	±	±	-	-	±

1%. Since a number of the effective substances contained SH groups an attempt was made to demonstrate a protective effect with H_2S . This was not possible, however, since H_2S always destroyed the toxin.

As an example of an entirely different type of reducing agent, ascorbic acid was tested and found to protect haemolysin in a concentration of 0.05%. Thus, ascorbic acid was by far the most effective protective agent of all the substances tested. It should be noted that tetanus toxin was destroyed by ascorbic acid, and it could not therefore be tested on this toxin. All attempts to reactivate the X-ray-destroyed haemolysin with ascorbic acid were entirely unsuccessful, thus suggesting that the effect of X-rays on haemolysin cannot be reversed by reduction.

Oxidizing substances such as cystine and maleic acid were totally ineffective as protective agents.

DISCUSSION

The experiments reported above in Part 1 provide an additional example of a phenomenon repeatedly observed with biological materials, namely, that as long as the substance studied is not absolutely pure, it is impossible to draw any conclusions as to its specific sensitivity to X-rays. Thus, partially purified tetanus toxin was found to be far more sensitive to X-irradiation than a crude preparation, 7 million r. destroying 100% of the purified toxin, but only 20% of the crude toxin. Moreover it seems probable that further purification would have increased the sensitivity of the toxin even more. These results are very similar to those of Friedewald & Anderson (1941) working with Shope papilloma virus, of Dale (1940, 1942) and Tytel & Kersten (1941) working with various enzymes and of Luria & Exner (1941) working with bacteriophage, etc.

Comparing the toxins we have studied, it is probable that the haemolysin is more sensitive to X-rays than the tetanus toxin, since the purified tetanus toxin solution containing 0.14 mg. N/ml. was approximately twice as resistant as the crude haemolysin solution containing 0.95 mg. N/ml.

In the case of both of these two toxins, the antigenicity was found to be destroyed simultaneously with the toxicity, thus precluding the use of X-rays

for the production of toxoids. This phenomenon is not a general one, as it was found by various authors that toxicity and infectivity were sometimes more easily destroyed by X-rays than were antigenic properties (Friedewald & Anderson, 1941; Moore & Kersten, 1936).

Turning to the mechanism of the action of X-rays on toxins (Part 2), it is now generally accepted that the effect of X-rays on substances in dilute aqueous solution is indirect, the radiation causing ionization and other dissociations of water molecules, which produce OH^- and H^+ ions, H_2O_2 molecules, H and HO_2 radicals and other active groups. The end result will depend on whether the substance in solution is more easily destroyed by oxidation or by reduction (Fricke, 1935; Weiss, 1944).

In the case of simple organic substances it is possible to tell by analysis whether the effect of X-rays has been one of oxidation or reduction. In the case of complex, biologically active materials this is obviously impossible, and it is therefore necessary to resort to the study of the protective effects of added extraneous substances.

In the case of the toxins here employed the conclusion that X-ray destruction in dilute aqueous solution is brought about by oxidation of the toxin fits all the observed facts well. Indeed, the ability of a substance or a mixture to protect the toxin from destruction by X-rays was in direct proportion to the magnitude of its reducing properties.

(1) The only amino-acids that were effective, cysteine, methionine, and tryptophan, were those known to have reducing properties or that could acquire them on irradiation (Mirsky & Anson, 1936). Glutathione evidently acted in the same way.

(2) Reducing substances entirely unassociated with proteins, such as thioglycolic acid and ascorbic acid, were among the most effective protective agents.

(3) Oxidizing substances, such as cystine and maleic acid, had absolutely no effect.

It is very interesting to note that Forsberg (1947), working with solutions of pure catalase, has observed the opposite effect, namely, that the destructive effect of X-rays on catalase in dilute aqueous solution is prevented by oxidizing substances, such as cystine, maleic acid, oxidized glutathione, etc., and enhanced by reducing agents such as cysteine, glutathione, etc. The obvious conclusion is that catalase was sensitive to reduction whereas the toxins used in this study were sensitive to oxidation.

On this basis, it is possible to explain a number of observations that would otherwise be very obscure. The peptones were found to be more effective than the corresponding proteins, since reducing groups are presumably set free during partial hydrolysis. The total acid hydrolysates of the proteins were less effective than the proteins themselves, since tyrosine

and tryptophan are destroyed and cysteine may be partially oxidized during the process.

It is possible to explain the mechanism of the protective effect of proteins in one of several ways: (1) The protein molecules in solution may act by screening the substance in solution from the destructive effect of X-rays, i.e. passive protection; (2) active protection may be induced, whereby the protein molecules actually intervene in the reaction between the active groups produced in the water and the substance in solution, either by combining with the substance and thus stabilizing it (Tytel & Kersten, 1941) or by reacting with the active groups thus inactivating them.

The experiments reported above clearly show that the protective effect of the proteins cannot be explained by the first mechanism, since it is not dependent on the molecular size and shape or on the amount of extraneous materials in the solution. Indeed, a number of proteins, such as casein, gelatin and edestin, and many amino-acids singly or in combination are completely inactive even in very high concentrations, although other proteins, such as egg albumin and serum globulin, and some amino-acids very similar in their general molecular structure to the inactive ones, are very active in low concentrations. It is also extremely improbable that the same toxin can combine with such a wide variety of molecules as were found to be effective protective agents, thus excluding the explanation that the protective agents react with the toxin and stabilize it.

We are thus left with the last possibility, namely, that the protective agents combine with the active groups formed in the water and destroy their activity. The native proteins here tested show no reducing properties, but it is known that denaturation sets free reducing groups, e.g. SH groups

(Mirsky & Anson, 1936) and, since X-rays cause denaturation of proteins, it is reasonable to believe that the protective effect of proteins was due to the reducing power of their denatured products, just as in the case of the reducing amino-acids.

Gelatin has only very slight reducing properties, and it was found to have no protective effect on the toxins tested. It is noteworthy that gelatin acts as a very effective protective agent for tobacco mosaic virus against X-rays (Lea *et al.* 1944). The mechanism in that case must be different from the one which is effective for tetanus toxin and staphylococcus haemolysin.

SUMMARY

1. Tetanus toxin and staphylococcus haemolysin were destroyed by X-rays. Purification of tetanus toxin greatly increased its sensitivity to X-rays.

2. In both cases the antigenicity was destroyed simultaneously with the toxicity.

3. Certain proteins, their partial and total hydrolysis products, reducing amino-acids, glutathione, thioglycolic and ascorbic acids were found to be effective as protective agents, whereas oxidizing substances were ineffective.

4. All the experimental observations support the theory that the mechanism of the effect of X-rays on tetanus toxin and staphylococcus haemolysin is an indirect one, the radiation causing the formation of active oxidizing radicals in the water, which in turn destroy the toxin by oxidation.

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