THE EFFECT OF IRRADIATION WITH ULTRAVIOLET LIGHT ON VARIOUS PROPERTIES OF TYPHUS RICKETTSIAE[1]

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Several properties of typhus rickettsiae including toxicity, hemolytic activity, and respiration appear to be associated intimately with the infectivity of these organisms (Gildemeister and Haagen, 1940; Bengtson *et al.*, 1945; Clarke and Fox, 1948; Bovarnick and Snyder, 1949). For purposes of further study with purified toxin, hemolysin, or respiratory activity, it would be important to know whether this association with infectivity and with each other is essential. If so, then the highest degree of purity that could be achieved with respect to preparations showing any one property would be that of the whole rickettsiae, which also would show all the other properties and would make impossible any study of isolated properties. That this might be so has been suggested not only by the fact that toxicity, hemolytic activity, and respiratory activity accompany infectivity during all stages of purification, but also by the loss of each of these activities brought about even by mild procedures used to reduce infectivity, such as short heating at 56 C or more prolonged incubation at 34 C or 0 C.

However, the association cannot be functionally obligatory since two of the activities, hemolytic and respiratory, take place *in vitro* in the absence of any rickettsial multiplication. The toxicity for mice also appears to be independent of multiplication, first because of the short time factor, and secondly, because it is exhibited as strongly by the epidemic strains of typhus which cannot multiply in this host as by the murine strain which can. Therefore it seemed probable that the association between infectivity and the other properties is coincidental rather than obligatory, and that dissociation might be achieved with proper means. It has been found now that ultraviolet light can accomplish such a dissociation. With properly chosen amounts of ultraviolet irradiation it is possible to lower the infectivity of rickettsiae by a factor of at least 10,000 with little loss in respiration, hemolytic activity, or toxicity.

It was hoped that ultraviolet irradiation also might allow separation of the other three properties. This, however, was not achieved. While there are differences in the rates of loss of toxicity, hemolysin, and respiration on prolonged irradiation, the differences are not sufficient to allow clear-cut separation of any one of these properties from the other two.

METHODS

Rickettsiae. The Madrid E and Breinl strains of typhus rickettsiae grown in the yolk sacs of embryonated eggs (Cox, 1941) were used in these experiments. The rickettsiae were purified partially by using a slight modification of the previously described procedure (Bovarnick and Miller, 1950). Infected yolk sacs which had been homogenized in a Waring blendor with one volume of a buffered isotonic salt solution and quickly shell-frozen in an alcohol-dry ice mixture were used as starting material. A portion of such suspensions was diluted with two volumes of the same salt solution and centrifuged at 5,000 rpm in an angle centrifuge for 45 minutes. The precipitate was resuspended in the same volume of buffered isotonic sucrose solution containing 0.6 per cent added bovine albumin, treated with 1 g celite for each 6 g yolk sac, and centrifuged at 2,000 rpm for 10 minutes. The supernatant was centrifuged again at 5,000 rpm for 60 minutes, and the final precipitate resuspended to a concentration equivalent to 2 g original yolk sac per ml. The isotonic salt solution used above contained KCl, 0.118 M; NaCl, 0.0072 M; Na_2HPO_4,

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0.0076 M; KH_2PO_4, 0.004 M; potassium glutamate, 0.0049 M; pH 7.0. The isotonic sucrose solution contained sucrose, 0.218 M; KH_2PO_4, 0.0036 M; K_2HPO_4, 0.0071 M; potassium glutamate, 0.0049 M; pH 7.0. Rickettsiae prepared by this method contained only one-fourth as much contaminating yolk sac material, as estimated by complement fixation with antiyolk sac serum, as did the precipitate of rickettsiae obtained by the earlier method, in which isotonic salt solutions were used throughout.

Irradiation. Two ml aliquots of the suspension of washed, concentrated rickettsiae were placed in a 60 mm petri dish. The vessels were shaken on a rotary shaker at 75 rpm and were irradiated for the indicated time intervals at a distance of 4.5 inches from a General Electric 15 watt germicidal bulb. Immediately after irradiation the suspensions were transferred to chilled tubes and were kept at 0 C until assayed.

Methods of assay. Toxicity. Toxicity was estimated by the intravenous injection of serial 3-fold dilutions of the rickettsial suspensions into white mice. Four mice were used for each dilution (Bengtson *et al.*, 1945). Deaths were counted 24 hours after injection, and 50 per cent end points were estimated by the method of Reed and Muench (1938).

Hemolytic activity. This was measured as previously described (Snyder *et al.*, 1954; Bovarnick *et al.*, 1953).

Respiration. This was measured by the usual Warburg technique at 34.3 C. Each flask contained 0.2 ml 0.15 M glutamate, 0.2 ml of a solution of 0.012 M $MgCl_2$, and 0.004 M $MnCl_2$, 0.2 ml of 0.11 M potassium phosphate, pH 7.5, 0.8 ml 0.145 M KCl, and 1 ml rickettsial suspension. The center well contained 0.1 ml 10 per cent KOH.

Cotton rat infectivity. Rats were injected intra-

TABLE 1

Effect of ultraviolet irradiation on infectivity, toxicity, and hemolytic activity of epidemic typhus rickettsiae (Rickettsia prowazeki)

STRAIN	DURATION OF IRRADIATION	INFECTIVITY FOR COTTON RATS		INFECTIVITY FOR CHICK EMBRYOS	TOXICITY FOR MICE		HEMOLYTIC ACTIVITY	
	min	ID_{50}*	*per cent*†	*per cent*	LD_{50}‡	*per cent*	*HE*§	*per cent*
Madrid E	0			100	67	100	122	100
	1			0.01	45	67	90	74
Madrid E	0			100	144	100	660	100
	1			0.005	45	31	530	80
	3			< 0.001	30	21	360	54
Madrid E	0‖	$10^{7.2}$	100	100	50	100	370	100
	0.5‖	$10^{3.6}$	0.025		27	54		
	1‖	$10^{3.4}$	0.016	0.001	25	50	220	60
Breinl	0	$10^{9.4}$	100		50	100	56	100
	1	$10^{4.7}$	0.002		72	144	62	112
	4.5	$10^{3.6}$	0.0002		27	36	36	64
Breinl	0	$10^{9.2}$	100		33	100	26	100
	1	$10^{4.2}$	0.001		27	82	25	96
	4.5	$10^{3.2}$	0.0001		10	30	12	46

* The ID_{50} represents the denominator of the dilution required to immunize 50 per cent of the cotton rats.

† Per cent of initial activity remaining after irradiation.

‡ LD_{50} represents the denominator of the dilution required to kill 50 per cent of the mice.

§ HE is the hemolytic end point, the denominator of the dilution required to give sufficient hemolysis to produce an optical density reading 0.3 in the method described by Snyder *et al.* (1954).

‖ In this experiment rickettsiae were frozen after irradiation and stored at −70 C for about one week before the assays were carried out.

abdominally with 0.25 ml of serial 1.5 log dilutions of rickettsial suspensions, using 6 rats per dilution. Three weeks later the rats were challenged intracardially with approximately 3 to 6 times the certainly fatal doses of the corresponding strain. The ID_{50}, or dilution required to immunize 50 per cent of the rats, was calculated by the method of Reed and Muench from the number of rats surviving 7 days after challenge (Morgan, Stevens, and Snyder, 1947).

Infectivity for chick embryos. Serial tenfold dilutions of the rickettsial suspensions were prepared. Six day embryonated eggs were inoculated by the yolk sac route with 0.2 ml quantities of the 10^3, 10^5, and 10^7 dilutions of the control suspensions and with the same quantity of the 10^2 and 10^5 dilutions of the irradiated suspensions. Twelve eggs were used for each dilution. The eggs were incubated at 35 C and were candled daily for 14 days; the average time of death of eggs dying after the third post inoculation day was calculated for each group. The time of death of different dilutions of the control suspension was plotted against the logarithm of the dilution injected. From this curve the dilution of the control suspension required to give the same time of death as that of the irradiated suspensions was estimated. The reduction in infectivity on irradiation was taken as the ratio between the dilution of the irradiated suspension used and that of the control suspension required to produce the same time of death. For example, in experiment I, figure 1, eggs inoculated with the 10^2 dilution of the suspension of rickettsiae that had been irradiated had an average time of death of 8.9 days. This time of death corresponds to that expected for the $10^{6.3}$ dilution of the control eggs, as read from curve I. Therefore, the relative infectivity after irradiation is $10^2/10^{6.3}$ or $10^{4.3}$. The results with the 10^5 dilution of the irradiated organisms which killed only 3 out of 12 eggs in 14 days indicate only that this dilution contains fewer viable rickettsiae than a 10^7 dilution of the control suspension. In our experience with this method, differences in results up to one log unit may be attributable to chance.

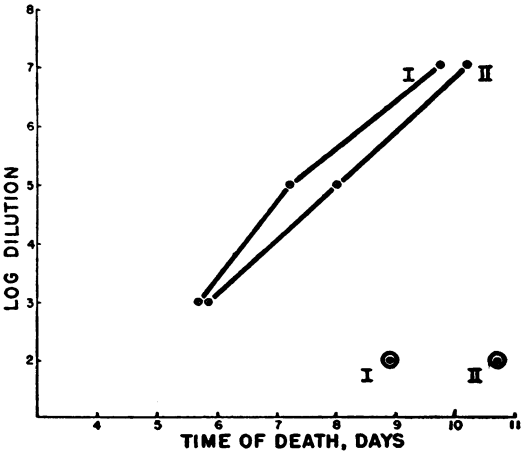

Figure 1. ⊙ = values obtained with control suspensions of rickettsiae in two different experiments, I and II. ⊗ = values obtained with portions of the same suspensions after irradiation for one minute with ultraviolet light.

In experiment I, nine out of twelve eggs and, in experiment II, eleven out of eleven eggs inoculated with the 10^{-5} dilution of the irradiated suspensions were alive 14 days after inoculation.

RESULTS

The changes in infectivity, toxicity, and hemolytic activity of both the Madrid E and Breinl strains after short periods of irradiation are shown in table 1. The change in infectivity for eggs is shown in more detail in figure 1. In the case of strain E, it can be seen that losses of the order of 10^4 to 10^5 in infectivity as estimated by egg or cotton rat titrations are accompanied by 20 to 70 per cent losses in toxicity for mice and 5 to 25 per cent losses in hemolytic activity. With the Breinl strain, after one minute irradiation there is a similar loss in infectivity and no significant change in toxicity or hemolytic activity; even after 4.5 minutes irradiation the

TABLE 2

Changes in toxicity, hemolytic activity, and rate of respiration of the Madrid E strain after prolonged irradiation

TIME OF IRRADIATION	TOXICITY FOR MICE	HEMOLYTIC ACTIVITY	RATE OF O_2 UPTAKE
min	LD_{50}	*HE*	*μL/ml/hr*
0	173	400	110
3	100	280	100
6	55	200	93
9	21	75	79
12	7	36	64

For the explanation of the figures for the toxic and hemolytic activities, see the footnote on table 1.

TABLE 3

Summary of experiments showing rate of loss of toxicity, hemolytic activity, and respiratory activity of Madrid E strain after irradiation with ultraviolet light

TIME OF IRRADIATION	TOXICITY			HEMOLYTIC ACTIVITY			RESPIRATORY ACTIVITY		
	No. of experiments	Per cent remaining		No. of experiments	Per cent remaining		No. of experiments	Per cent remaining	
		Mean	S.D.		Mean	S.D.		Mean	S.D.
min									
1	6	58	22	8	82	11	4	91	4.4
3	8	43	18	10	72	4	3	85	3
6	3	21	9	3	48	10	2	78	8
9	2	8	6	2	21	3	2	62	4

S.D. = standard deviation.

drop in the two latter end points is only about 50 per cent.

It seemed possible that more prolonged irradiation might lead to a separation of toxicity, hemolytic activity, and respiration. Results of measurements of these properties in one typical experiment with strain E are given in table 2. There is a certain amount of variability in the results obtained in separate experiments, especially in the toxicity for mice. This is shown in table 3 where the average recoveries after different times of irradiation found in all experiments are given together with the standard deviations of these values. In spite of the variations, it appears from the results of both individual and averaged experiments that the toxicity for mice is lost most rapidly, followed by hemolytic activity, and finally by respiration. However, the differences, while consistent, are not sufficiently great to make possible any complete separation of these three properties. It is also apparent that considerably greater doses of ultraviolet light than those which so drastically reduced the infectivity still leave intact considerable amounts of toxicity, hemolytic activity, and respiratory activity.

That the apparent loss in infectivity of the rickettsiae as compared to the slight losses found in the other properties on ultraviolet irradiation is not merely due to secondary inactivation taking place after cessation of irradiation, is indicated in one experiment in which control and irradiation suspensions of the Madrid E strain were incubated at 34 C for 5 hours after irradiation. Estimations of hemolytic activity and respiratory rate were made before and after incubation. As can be seen in table 4, the drop in activity of the irradiated suspensions was only slightly greater than that of the control suspensions, indicating that the major effect is immediate and that secondary effects, if they occur, do so too slowly to be significant.

TABLE 4

Stability of hemolysin activity and respiration of Madrid E strain after irradiation with ultraviolet light

TIME OF IRRADIATION	HEMOLYTIC ACTIVITY		RATE OF RESPIRATION	
	Initial	After 5 hr incubation	0–1 hr	4–5 hr
min				
0	660	555	117	107
1	530	420	108	88
3.5	360	260	95	72

The figures for hemolytic activity and respiration have the same explanation as in tables 1 and 2.

For measurement of stability of hemolytic activity the rickettsial suspensions were diluted 20-fold in a medium containing glutamate, diphosphopyridine nucleotide, and heated normal yolk sac as described by Bovarnick *et al.* (1953). They were tested at once and again after incubation for 5 hours at 34 C.

The oxygen uptake measurements were made as usual, except that readings were continued for 5 hours, the manometers being reset when necessary.

DISCUSSION

Cox and Wong (Cox, 1951) reported that the toxic and hemolytic properties of rickettsiae could be destroyed without change in LD_{50} titer for eggs, by aureomycin, and to a lesser extent by terramycin. Possibly their observations can

be explained in part by the facts that a large change in infectivity (20 to 30-fold) is required before statistically significant results are obtained in egg titrations, whereas the toxicity titration is very sensitive, showing 3-fold changes with reproducible results. In our irradiation studies reported above, the infectivity changes were determined both by animal and egg titrations and were far greater than the variations expected by chance with the methods used.

The striking loss in infectivity of the rickettsiae on irradiation with ultraviolet light without comparable loss in other properties is entirely analogous to results found with bacteria and viruses. Kelner (1953) has shown that *E. coli*, irradiated just sufficiently to reduce markedly the viability, appear also to have an undiminished rate of respiration and retain the ability to synthesize ribonucleic acid and can even increase in size. With most viruses there are fewer properties other than multiplication that can be followed. However, Luria and Delbrück (1942) showed that irradiated phage, which had lost its ability to multiply, could still adsorb onto the host cell. Also, Henle and Henle (1947) found with influenza viruses that infectivity was more sensitive to irradiation than toxicity, interference, or the ability to hemagglutinate and elute from red cells. With the influenza viruses, however, there was not only a marked difference between the sensitivity of infectivity and that of the other properties to irradiation, but the other properties also differed between themselves so markedly in sensitivity that, with properly chosen doses of ultraviolet radiation it was possible to effect a complete separation of each less sensitive property from the preceding more sensitive one. This has not been accomplished in the case of the rickettsiae; there are only relatively small differences in the rate of destruction of the toxicity, hemolytic activity, and respiration of these organisms.

The close association between the rates of loss of these three properties raised the question as to whether there is any intrinsic connection between them. The facts suggesting this are as follows: (a) Studies on factors affecting the rickettsial hemolytic activity have already suggested that hemolysis does not take place in the absence of respiration by the rickettsiae (Snyder *et al.*, 1954). (b) Studies of factors affecting the respiratory activity and the maintenance of toxicity and hemolytic activity at 34 C have shown that similar factors affect all three in a qualitatively similar way. All are increased by the presence of glutamate (Bovarnick *et al.*, 1950) and by diphosphopyridine nucleotide; all are depressed by a toxic factor obtained from autoclaved diphosphopyridine nucleotide (Bovarnick *et al.*, 1953). There are quantitative differences, however, in the rate of loss of these three properties. On irradiation with ultraviolet light, hemolytic activity is lost more rapidly than is respiration so that, as might be expected, some other function of the rickettsiae also must be involved in the hemolysis reaction. Also, after irradiation, toxicity for mice is lost more rapidly than either hemolysis or respiration. Toxicity also is lost more rapidly than hemolytic activity when suspensions of rickettsiae are kept at 34 C for several hours, which suggests that even larger numbers of rickettsial functions are required for this activity.

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SUMMARY

On irradiation of partially purified suspensions of epidemic typhus rickettsiae with ultraviolet light, it is possible to obtain a reduction in infectivity for chick embryos and cotton rats of 10^4 and 10^5-fold without a marked loss in toxicity for mice, hemolytic activity, or respiration.

More prolonged irradiation leads to a gradual loss of the latter properties, without a complete separation of any one. The most rapid loss is in toxicity; the least rapid, in respiratory activity.

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