THE FORMATION OF BACTERIAL VIRUSES IN BACTERIA RENDERED NON-VIABLE BY MUSTARD GAS

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It has been found that *E. coli* B and *Staphylococcus muscae* after treatment with mustard gas [bis(β-chloroethyl)sulfide] can produce phage but apparently can no longer multiply. Most of the experiments were carried out on the *coli* system so that this will be discussed first and in more detail. Table I shows that at a mustard concentration of 0.8×10^{-3} M the phage formation is still 50 per cent or more of the untreated control but the number of organisms which can multiply in a veal-peptone infusion has decreased from 1×10^8 per ml. to about 1×10^3. When the concentration of mustard gas was $1.25 - 2.0 \times 10^{-3}$ M there were no viable cells and the phage formed from these cells was still 5 to 20 per cent of the untreated control cells. In an occasional experiment after treatment with 1.25×10^{-3} molar mustard the undiluted suspensions showed a few (less than 10) viable cells.

Since the test for viability is the crux of the present problem, an explanation of the tests used is important. Both colony count and culture in liquid media were used in testing for viability. Colony counts were made 24 to 72 hours after plating out on veal-peptone-agar plates; the cells being (*a*) spread on the surface and (*b*) mixed with 42°C. veal-peptone–0.7 per cent agar, and then layered onto the plate (2). In the liquid culture serial tenfold dilutions of the organisms were made in rich media and shaken at 37°C. The different media tested were (1) veal infusion–peptone broth; (2) (1) + 0.3 per cent yeast extract; (3) (1) in which normal *E. coli* had been grown from 1×10^6 cells per ml. to 1×10^8 per ml. and then the cells removed by a Berkefeld N filter; (4) difco-brain-heart infusion; and (5) a "complete" media in which were most if not all the vitamins and growth-promoting agents in addition to amino acids, yeast extract, and peptone.[1] Dilutions of untreated cells calculated to contain only

[1] The "complete" medium kindly supplied by Dr. A. C. Braun and R. J. Mandle was made up of the following materials plus water to a liter of solution: 3 gm. of yeast extract, 5 gm. of peptone, 5 gm. of glucose, 0.025 μg of biotin, 0.01 mg. folic acid, 5 mg. of *p*-aminobenzoic acid, 5 mg. of inositol, and 1 mg. each of thiamin, pantothenic acid, pyridoxine, niacin, riboflavin, and choline. Besides the above, 10 mg. of each of the following amino acids was added to the liter of solution: *dl*-lysine, *dl*-methionine, *dl*-threonine, *d*-arginine, valine, *dl*-histidine, *dl*-phenylalanine, *dl*-leucine, and tryptophane.

2 cells per tube became turpid in 24 hours in all the various liquid media. In a number of test runs using cells treated with varying amounts of mustard, all the various media showed the same number of viable cells for any given treatment. Therefore, only one medium, the veal infusion–peptone broth, was used in the majority of the experiments.

The viable cells present after treatment with 0.8×10^{-3} M mustard are not responsible for the phage formation in this suspension for the following reasons:

TABLE I

Tube No.			I	II	III		IV	
H, final molar concentration			0 (Saline)	0 (1.25×10^{-3}M hydrolyzed mustard)	0.8×10^{-3}M		1.25×10^{-3}M	
Cells per ml			1×10^8	1×10^8	1×10^8		1×10^8	
Viability tests	Plates	Total cells spread	50	50	1×10^6	1×10^4	1×10^7	
		Colonies found	10–60	10–60	6	0	0	
	Liquid	Total cells in 5 ml. veal infusion	2	2	5×10^5	5×10^4	5×10^7	5×10^6
		Turbidity after 4 days.	+	+	+	−	−	−
Phage formation		Total cell concentration per ml.	1×10^7	1×10^7	1×10^7	1×10^4	1×10^7	
		Viable cell concentration per ml.	1×10^7	1×10^7	60	0	0	
		T_1 phage concentration per ml. in suspension at start	2×10^7	2×10^7	2×10^7	2×10^4	2×10^7	
		Phage per ml. (final)	$4 \times 10^{9*}$	2.1×10^9	2×10^9	1.8×10^8	4×10^8	
		Phage per cell (final)	400	210	200	180	40	
		Phage per viable cell	400	210	3.3×10^7	∞	∞	

* The difference between this value and the corresponding value of Tube No. II is probably accidental since other experiments showed no such difference.

1. There are too few of them. A single viable cell would have to produce about 10,000,000 phage particles to account for the observed result. Normal cells produce about 200 per cell.

2. Lysis and liberation of the phage occurs in an hour or two which does not allow time for much growth of the relatively few *viable* organisms.

3. The yield of phage is approximately independent of the inoculating phage concentration. This would not be expected if the viable cells were multiplying.

4. Diluting out the viable cells does not affect the phage yield per cell.

5. The capacity to form phage does not disappear at higher mustard concentrations where the number of viable cells is zero.

The addition of a few (0.1 and 1.0 per cent) normal cells to a broth suspension of mustard-treated *coli* did not revive any observable number of the latter for

the number of cells that grew up in 3 to 5 hours was the same as in the tubes containing no mustard-treated organisms.

E. coli B received from two different sources, Professor M. Delbrück and Professor A. D. Hershey, have given qualitatively similar results. Most of the

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Materials and Procedures for Table I

Preparation of Suspension C.—*E. coli* B were grown from a small innoculum in veal infusion for 10 to 12 hours, then diluted in veal infusion down to 1–3 $\times$ 10^7 cells per ml. and grown up to 2 $\times$ 10^8 cells per ml. as judged by Klett colorimeter readings. These cells in the log phase of growth were then centrifuged and resuspended in same volume of 0.85 per cent saline – M/100 pH 8 phosphate buffer.

H.—0.01 ml. mustard gas (M.P. 14.2°C.) in 32 ml. saline in a 125 ml. glass-stoppered bottle and shaken hard for 15 seconds. The concentration of mustard was checked by the bromine titration (1).

Tube I.—2 ml. of C + 2 ml. of 0.85 per cent saline.

Tube II.—2 ml. of C left at 27°C. for an hour followed by addition of 2 ml. of H which had stood an hour at 37°C.

Tube III.—2 ml. of C + 0.72 ml. saline and 1.28 ml. H.

Tube IV.—2 ml. of C + 2 ml. H.

After all the tubes had stood an hour at 27°C., samples were diluted 1/10 in ice cold veal infusion.

Counts of Cells.—0.5 ml. of indicated cell concentrations in veal infusion was spread on a previously poured veal infusion–agar plate and then incubated at 37°C. Colonies were counted after 24 and 72 hours. Seldom was there any change after 24 hours.

Viability in Liquid Media.—5 ml. of the dilutions in veal infusion containing the indicated cell concentration were shaken in 20 mm. $\times$ 170 mm. tubes at 37°C. for as long as 4 days. Turbid suspensions which exhibited a swirling sheen on shaking were considered positive. Clear tubes indicated no viable organisms.

Phage Formation.—To 5 ml. of the indicated concentration of organisms was added 0.1 ml. of filtered T_1 phage which was 50 times the desired final concentration. The tube was shaken at 37°C. for 1 to 3 hours and then the phage concentration determined by plaque count. A phage control to which no cells were added was analyzed with each experiment.

Plaque counts were obtained after mixing a dilution of the phage with 3 $\times$ 10^7 per ml. log phase *E. coli* B in 0.7 per cent agar-veal infusion and layering 1 ml. of this mixture on a previously poured veal-agar plate (2). Plaques were counted after 12 to 24 hours' incubation at 37°C.

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experiments have been performed with subcultures of the sample from Professor Delbrück. Besides the T_1 phage used for most of this work, T_4r and T_6 phage have also been formed from non-viable mustard-treated *coli*.

The phage-forming capacity of mustard-treated *coli* cells decreases with time. Even in veal broth the phage formation may be virtually zero in an hour or two at 37°C. It is more stable at low temperatures.

In some instances non-viable mustard-treated *coli* can swell or elongate when placed in veal infusion medium. This was observed microscopically and also by turbidity measurements. Table II shows how the capacity for swelling and phage formation varied with increasing mustard gas concentrations. In experiments not shown in Table II the turbidity increase varied several-fold with different concentrations of mustard with no measurable effect on the phage formed per cell. After treatment with 1.5×10^{-3} M mustard there was no change in turbidity and the phage formed was 10 to 20 per cent of the yield with untreated cells. It appears from these various results that the phage formation does not parallel the capacity to swell.

Experimental Procedure for Table II

E. coli B were prepared as described for the experiments in Table I, centrifuged, resuspended in saline – M/100 phosphate buffer pH 8 to a cell concentration of 2×10^8 cells per ml. in one instance and 4×10^8 in a second. These were then mixed with saline and mustard gas, dissolved in saline so the final concentration was that shown in Table II. After an hour at 27°C., each suspension was divided into two equal portions and centrifuged at 7,000 R.P.M. for 10 minutes at 10°C. and resuspended in veal infusion. One portion was shaken at 37°C. and the turbidity determined every 15 minutes in a Klett-Summerson colorimeter employing a 66 filter. The other suspension was inoculated with 2 T_1 phage particles per cell and then shaken at 37°C. till the suspensions had cleared which was usually an hour.

TABLE II

Swelling of and Phage Formation of Mustard Gas-Treated E. coli B

Mustard gas concentration ($\times 10^{-3}$ M)		0	0.5	1.0	1.5	0	0.8	1.15	Phage control
	Time of shaking in veal infusion at 37°C.	Klett turbidity readings (66 filter)							
Swelling	0	20	17	17	15	42	36	34	
	1 hr.	65	27	20	16	137	45	34	
	3 hrs.					345	93	38	
	3.5 hrs.	365	84	24	14				
	10 hrs.					530	144	34	
T_1 phage formation from 1×10^8 cells per ml.									
Plaque count at 1×10^9 dilution		80	82	48	11	51	26	12	2
Per cent of zero mustard			100	60	14		50	24	4

Respiration studies following treatment with varying concentrations of mustard have been made parallel to estimations of phage formation. Little or no change in oxygen uptake was observed even when the phage formation had

been reduced to 10 per cent or less of normal cells by 2×10^{-3} molar mustard. CO_2 liberation, however, showed a measurable depression at 0.8×10^{-3} molar mustard and was about 50 per cent of normal following treatment with 1.3×10^{-3} molar. The phage formed in these two instances was 70 and 20 per cent respectively of the controls.

It was found that *Staphylococcus muscae* are not as susceptible to the action of mustard gas as the *coli* organism. However, as may be seen in Table III, approximately 98 per cent of the organisms were rendered non-viable for veal infusion media by treatment with 1×10^{-3} molar mustard. This treatment did not depress the phage formation to any appreciable extent. 2×10^{-3} M mustard apparently obliterated the phage-forming mechanism.

Materials and Procedures for Table III

The materials and methods used in the experiments on *Staphylococcus muscae* were the same as for *coli* except that Locke's solution was used in place of saline – M/100 phosphate for the medium in which the mustard treatment was performed. For the general methods of growth of the organism and its phage see reference 3.

TABLE III

Tube No.		I	II	
Final molar concentration of mustard		None	1.0×10^{-3} M	
Cells per ml		1×10^8	1×10^8	
Viability test	Total cells in 5 ml. broth (4 tubes each)	2	500	50
	Turbidity after shaken 4 days at 37°C.	+	+	–
Phage determination	Cells per ml	1×10^8	1×10^8	10
	Initial phage count per ml.	2×10^8	2×10^8	50
	Final phage count per ml.	2.2×10^9	1.1×10^9	280
	Final phage count per cell	22	11	28

Over a dozen experiments have been performed on the *coli* system of which only one failed to show phage formation after mustard treatment. In eleven experiments on *Staphylococcus* four failed to show an increase in phage. Since the mustard-treated organisms are quite labile, this may have been responsible for the negative experiments.

DISCUSSION

A number of investigators (3) have presented evidence that phage can be formed by cells which are not actively dividing. Zinsser and Schoenbach (4)

reported a number of years ago that rickettsia multiplied in cells which were not viable but they failed to obtain growth of equine encephalomyelitis under similar circumstances. Anderson (5) concluded that after ultraviolet light treatment *E. coli* would not produce colonies but could form phage; however, no experiments have appeared to substantiate this conclusion.

There can be no doubt from the present results that mustard gas has altered the cells. It is not possible to prove beyond any doubt that the cells are strictly non-viable or dead and cannot be revived for this would require an infinite number of tests, but the media and nutrients used have been varied enough to indicate that they do not multiply when placed under conditions very favorable for phage and cell multiplication. It follows from our results that the capacity for cell division is not necessary for phage formation.

The nature of or the site of the mustard reaction in the cell is not known but one of us has found (6) that bacteria exhibit the same order of sensitivity to mustard as such nucleic acid and nucleoproteins as the pneumococcus-transforming principle and animal, plant, and bacterial viruses. Most enzymes are much less sensitive.

SUMMARY

E. coli B and *Staphylococcus muscae* rendered non-viable by aqueous solutions of mustard gas at pH 7.5 to 8 can still produce phage.

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