INACTIVATION OF VIRUSES AND CELLS BY MUSTARD GAS

By ROGER M. HERRIOTT*

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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Recent studies have shown that the sulfur and nitrogen mustards are unique reagents of considerable fundamental and practical interest. Thus it has been reported that (*a*) these compounds induce mutations in a variety of organisms (1–9), (*b*) that the carboxyl groups of many enzymes and proteins combine with the agents resulting in a decrease in enzymatic activity (10), (*c*) that several different animal viruses are inactivated by the sulfur mustard and the inactive preparations have vaccine properties (11), and (*d*) that preliminary reports indicate some hope for these agents being of therapeutic value (12–14, 50).

In the present communication it will be shown that viruses in general are particularly susceptible to the action of mustard gas (hereafter referred to as H). Following exposure to this agent, six animal, one plant, and two bacteria viruses, and the pneumococcus-transforming principle were inactivated at rates higher than the rates for the most sensitive enzymes but of about the same order of magnitude as noted for yeast and a number of bacteria.

Theoretical Considerations.—It was shown earlier (10) that the method of treating proteins with H did not affect the qualitative nature of the reaction. It was noted, however, that the extent of the inactivation of enzymes following a single exposure to H (formerly referred to as the Dixon method and hereafter designated as the single method) could not be calculated from a derived equation. It did fit a simple reciprocal relationship obtained empirically. Results of the inactivation of viruses, on the other hand, fit the theoretical bimolecular equations for both methods; *i.e.*, single and stirring methods. The single method has some practical advantages over the stirring method when dealing with viruses, bacteria, etc. under sterile conditions and has therefore been used in most of the experiments in this paper. The derivation of the equation used in conjunction with the single method will now be given.

Research work carried out during the war led to the conclusion that the active principle of mustard gas is not the β-chloroethyl structure but a cyclic intermediate ethylene sulfonium ion formed when mustard reacts with water (15). Although for the sulfur mustard a cyclic intermediate product has never been isolated, it has been generally assumed that one exists for the reaction

*Present address, Department of Biochemistry, The Johns Hopkins School of Hygiene and Public Health, Baltimore.

properties of sulfur and nitrogen mustards are very similar and for the latter the intermediates have been clearly demonstrated (15, 16, 51).

The concentration of the active intermediate (hereafter referred to as H*) is proportional to the H concentration or

$$[H^*] = C\,[H]$$

With the symbols P = protein, n = number of mols of H, t = time in minutes, and a = the rate of hydrolysis of H, then the reaction of H with any protein be it virus or enzyme may be written in the general form

$$nH^* + P \rightarrow P\,(H^*)n$$

and

$$\frac{-dP}{dt} = K[P][H^*]^n$$

$$\frac{-dP}{[P]} = K\,[H^*]^n\,dt$$

In the case where the [H] is held constant throughout the experiment as in the stirring method, the integrated equation (see reference 10 for details) is

$$K = \frac{2.3}{[H^*]t} \log \frac{P_0}{P}$$

but since only H can be measured and not H*, the equation becomes

$$KC = K' = \frac{2.3}{[H]t} \log \frac{P_0}{P} \tag{1}$$

In the single method the [H] decreases logarithmically during the reaction or at any time t the $[H] = [H_0]e^{-at}$ and at time t, $[H^*] = C[H_0]e^{-at}$. Therefore the differential for the single method becomes

$$\frac{-dP}{dt} = K[P](C[H_0]e^{-at})^n$$

and

$$\frac{-dP}{[P]} = KC^n[H_0]^n e^{-atn}\,dt$$

upon integrating the following is obtained

$$-\ln P = \frac{-K(C[H_0]e^{-at})^n}{an} + B$$

where B is the integration constant. When $t = 0$ and $P = P_0$ then

$$B = \frac{K(C[H_0])^n}{an} - \ln P_0$$

Substituting this for B and rearranging:

$$\ln \frac{P_0}{P} = \frac{K(C[H_0])^n}{an}(1 - e^{-atn})$$

In both the previous and present experiments it was found that the inactivation rate did not vary as a higher power of the mustard concentration as predicted by the formula immediately above. Instead the results agreed with the assumption that $n = 1$ in spite of the fact that n, the number of H residues per mol protein, was actually greater than 1 as shown by direct analysis. A discussion of this apparent anomaly was given in the preceding paper (10). Since both the previous and present experiments clearly indicate that as far as the kinetics of the reaction with proteins is concerned $n = 1$, then the above equation simplifies to

$$\ln \frac{P_0}{P} = \frac{KC[H_0]}{a}(1 - e^{-at})$$

As t increases e^{-at} approaches zero. Therefore, if all samples are analyzed when t is relatively large (*i.e.*, after most of the H has reacted) the equation simplifies still further to

$$KC = K' = \frac{a}{[H_0]} \ln \frac{P_0}{P} \quad \text{or} \quad = \frac{2.3a}{[H_0]} \log \frac{P_0}{P} \tag{2}$$

It follows from Equation 2 that if measurements of virus activity are made after reaction with H is complete, the fraction of virus left will decrease logarithmically with increasing initial mustard concentration. It may also be predicted from Equation 2 that the rate or K' will be independent of the concentration of protein; *i.e.*, the same per cent inactivation will occur for a given H concentration regardless of the concentration of protein. These predictions are qualitatively confirmed by the results shown in Figs. 1–3.

The K' in Equation 2 is a velocity constant even though time does not appear

Details of Experiments Shown in Fig. 1

The Newcastle virus used in these experiments had been twice centrifuged but the E. E. E. was diluted allantoic fluid from an infected chick embryo. The solvent was water and the pH was maintained at 7.5–8.5 by the addition of dilute NaOH or bicarbonate as needed. The temperature was 25°C.

Details of the several methods are to be found in the section on Experimental methods.

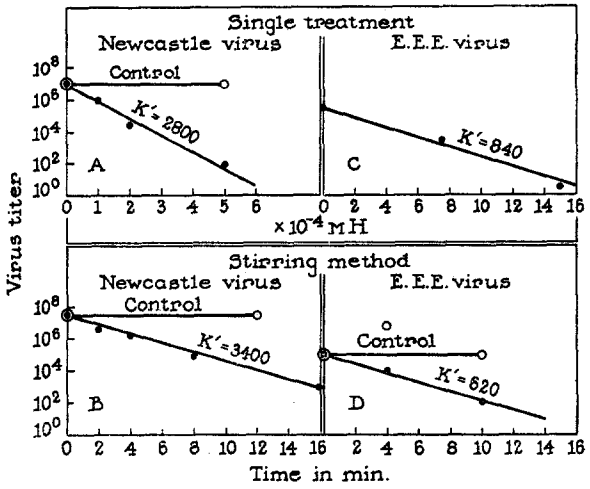

FIG. 1. Inactivation of Newcastle virus and equine encephalomyelitis virus by H.

Details of Experiments Shown in Fig. 2

The Type III transforming principle (TP) used in this experiment was kindly prepared and titrated by Dr. Rollin Hotchkiss and Dr. O. T. Avery following the current adaptation of the procedures previously described (33). To a series of 1 ml. aliquots of purified TP 54 containing 0.08 mg. nucleic acid in saline at pH 7.0–7.5 was added 1 ml. of saline solutions of H of various concentrations. Appropriate controls were included with hydrolyzed H and with saline alone. After standing 2 hours at 25°C. they were diluted and the activity of the residual TP determined.

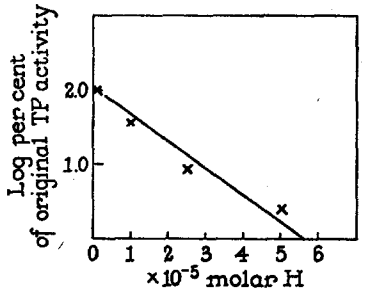

FIG. 2. Inactivation of purified Type III pneumococcus-transforming principle by varying concentrations of H.

Details of Experiments Shown in Fig. 3

The single method was used throughout this experiment. The pH was held between pH 7.5 and 8.5 with dilute NaOH or bicarbonate.

The experiment indicated in Fig. 3 by × was an allantoic fluid from an infected embryonated egg diluted one to five in water. In the experiment indicated by open circles ○, the virus had been twice ultracentrifuged and resuspended in water. For the experiment represented by filled circles ●, the virus had been once ultracentrifuged.

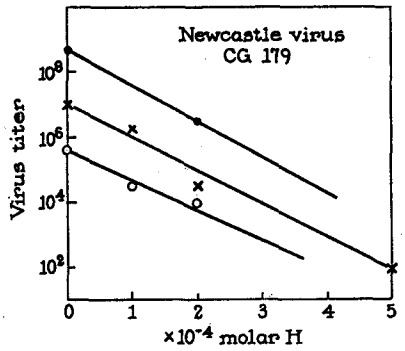

FIG. 3. Effect of the concentration of Newcastle virus on the rate of inactivation by H.

in the final integrated expression. The dimensions of the constant are: the fraction of P (protein or virus) reacting per mol of H per liter per minute.

EXPERIMENTAL RESULTS

Sensitivity of Various Biologically Active Materials to H

In Table I the sensitivity of various biologically active materials to H has been compared in two related ways. First, as the change in the logarithm of the biological activity per unit concentration of H and second, as the inactivation constants calculated with the aid of Equations 1 and 2. The first criterion is related to the second by the factor $2.3a$ for $\frac{\Delta \log P}{\Delta H_0} = \frac{K'}{2.3a}$ where a is the hydrolysis constant of H in the solvent used. Since the rate of reaction of H with any material including water is depressed by chloride ions, a comparison of rate constants in solvents of different chloride ion concentrations will involve this difficulty. Use of the direct measurement of the change in biological activity per unit H seems a preferable criterion of sensitivity to H for it is not dependent on the rate of reaction. This value also may vary with the chloride ion concentration as shown in Table I but the variation is specific for each material and independent of the effect on the rate of cyclization of H. It should be remembered in this connection that in the single method the reactions of H

Experimental Procedure for Table I

The solvent, temperature, and pH of the experiments analyzed and recorded in Table I are shown in the table. The pH was usually maintained with dilute bicarbonate or phosphate (*ca.* $^{M}/_{1000}$).

In general unpurified preparations of the viruses were examined but as will be discussed later no appreciable difference in the inactivation is observed upon purification.

Three to five samples at different mustard concentrations (in the single method) and at different time intervals (in the stirring method) were removed and the biological activity determined in the manner described in the section on Experimental methods. The end point of a titration was the estimated concentration or dilution of biological agent which, in the case of viruses, produced death in 50 per cent of the five susceptible organisms used for each test. With cells, viability on broth-agar plates incubated for 48 hours at 37°C. was the criterion of inactivation. Phage was determined by plaque counts.

TABLE I

Sensitivity of Certain Biologically Active Materials to Mustard Gas at pH 8.0 ± 0.5 and 25°C.

Material	Solvent	Δ log biological titer* / Δ 1 × 10^{-3} M H	K'	
			Single* method	Stirring method
Pneumococcus Type III transforming principle	Saline	30	2,800	
T_2r^+ phage of *E. coli* B	Water	20	5,600	
" " " " "	Saline	6.3	600	
Newcastle virus (CG179 of Beach)	Water	12	3,500	4,000
" " (Beaudette)	"			3,500
E. coli B (*d*)	Locke's or saline	20	1,500	
" " " "	Water	10	2,800	
Swine plague bacillus	Broth and saline	9		
Feline pneumonitis virus (Baker)	Broth	9		
Staphylococcus muscae phage	Locke's	7	500	
Rabbit papilloma virus (Shope)	Water	6	1,700	
E. coli B (*h*)	Saline	5	500	
Equine encephalomyelitis virus (Eastern)	Water	3.6	1,000	800
Staphylococcus muscae	Locke's	3.6	250	
Rabbit myxoma virus	Saline	2.7	250	
Fixed rabies "	Water	2.5	700	
Bakers' yeast	M/10 NaCl	2.0		
" "	Water	0.8	220	
Tobacco mosaic virus	"	1.4	400	
Chicken pepsin	"	0.5	150	300
Crystalline yeast hexokinase	1 per cent glucose	0.2	*ca.* 65	

* These values have been calculated on the assumption that the change in the logarithm of biological titer varies linearly with changes in H concentrations. This is approximately true for most cases (Figs. 1–3). See the text for certain gross exceptions.

are allowed to go to completion before biological analyses are determined. Both methods of evaluating the sensitivity of biological materials to H are shown in Table I for various viruses,[1] cells, and a few enzymes for comparison.

In most instances the single treatment method of applying H was used but a few viruses were tested by both methods. In the single method several different mustard concentrations were applied to several equal aliquots of a given virus preparation and the logarithm of the remaining virus titer was plotted against the initial mustard concentration. The slope of such a plot is $\frac{\Delta \log P}{\Delta H_0}$ and equals $\frac{K'}{2.3a}$. Since a, the hydrolysis constant of H, is about 0.12 for water at 25°C. (16–20), 0.04 for saline, and 0.03 for Locke's solution, the value of K' is readily obtained. When the value of K' or the slope is known only a simple reversal of this calculation is necessary to find the change in virus titer that would be brought about by a given H concentration or to determine the H concentration necessary to reduce the virus titer by a certain value.

In Table I the solvent has been noted in each instance and just enough bicarbonate or phosphate was added to absorb the acid liberated by H and keep the acidity at pH 7.5–8.5. The temperature was 25°C.

The values in Table I for rabies, myxoma, papilloma, and swine bacillus were determined in each instance from only two experimental points per experiment though the experiments were repeated on several preparations of each agent. The minimum amount of H necessary to completely inactivate the disease agent and the initial titer are the two points on a log titer-H concentration plot from which the slope was obtained and the constants calculated. The values obtained in this manner are minimal since the concentration of H used to inactivate was presumably in excess. However, since the H concentration was varied by dilutions of two, the constants are low by less than a factor of 2 if the drop in titer with H concentration is linear with these viruses.[2] This assumption may prove to be incorrect but for many of the viruses no gross deviation was observed (Figs. 1–3).

It may be seen from Table I that the sensitivity to H of chicken pepsin, the

[1] The writer takes this opportunity to express his appreciation to a number of his colleagues who have made possible the examination of some of the most interesting material. Thanks are due Dr. Rollin Hotchkiss and Dr. O. T. Avery for their careful preparation and assays of the pneumococcus-transforming principle. I am also grateful to Dr. Carl TenBroeck for his titrations of the rabies virus and swine plague bacillus; to Dr. Margaret H. D. Smith for her preparation and titrations of the rabbit myxoma virus; and to Dr. Saul Malkiel for a number of preparations and titrations of tobacco mosaic virus.

[2] In the preparation of mustard vaccines in which the last trace of virus activity must be eliminated it is necessary to use somewhat more (1.5 to 2 times the mustard concentration) than that calculated from the values listed in Table I.

most sensitive enzyme examined in this laboratory, is considerably below all viruses and cells.

The values given in Table I are in general dependable within a factor of 2 and in some instances, depending on the virus and the number of points plotted, the reproducibility is better.

The pneumococcus-transforming principle is the most sensitive material yet analyzed. Its relationship to the general problem is discussed later.

In several instances the number of viable cells as determined by colony count did not decrease logarithmically as the mustard concentration increased. This was particularly true of *E. coli*. The constants for these systems were calculated from the average slope obtained during only the first 90 per cent inactivation. For reasons that are not apparent samples of *E. coli* B from two different laboratories have shown a marked difference in their sensitivity to mustard gas. A concentration of 1×10^{-4} molar mustard, which renders non-viable 50 per cent of the organism from one strain, similarly affects 99 to 99.9 per cent of the second strain; both having been grown in the same medium to the same concentration.

Constants calculated for the H inactivation of yeast from the figures reported by Kinsey and Grant (22) and by du Vigneaud and Stevens (21) are in agreement with the value in Table I.

It is of considerable interest that the cells were inactivated faster than the most sensitive enzyme and at about the same rate as found for the viruses. This is very suggestive that the sensitive locus of viruses and cells may have something in common. This bears further investigation particularly in view of the unique property of mustard of inducing mutations in cells such as *E. coli* (4). The criterion of "inactivated" or non-viable cells was their failure to grow on veal infusion peptone–agar plates on which normal cells grew luxuriantly. This does not mean that they could not have produced colonies on different media or evidenced growth under different conditions, although some recent experiments by the writer (52) render this less likely.

The work on cells shown in Table I and on a number of other organisms suggests that Gram-positive organisms are less susceptible to a given H concentration than are Gram-negative ones. More work is necessary to establish this but it is interesting in this connection that Henry and Stacey (23) have found that Gram-positive organisms have a coating of magnesium ribonucleate which is apparently absent in Gram-negative organisms.

Effect of the Method of Applying H on the Rate of Inactivation

Virtually the same inactivation constants are obtained by the stirring and single treatment methods of applying mustard to viruses as may be seen from Table I and Fig. 1. It should be noted in Fig. 1 that for the stirring method the abscissa is time whereas for the single method it is the initial H concentra-

tion. Only the constants K' are comparable. Details of the two methods are discussed later in this paper.

Effect of H Concentration on the Inactivation of Viruses

The results illustrated in Fig. 1 *A* and 1 *C* show how in the single treatment method the initial H concentration affects the extent of inactivation of two representative viruses. The straight line obtained by plotting the logarithm of the virus concentration remaining against the initial H concentration was expected if the derived Equation 2 correctly describes this system.

Fig. 2 shows the results of a similar experiment performed on highly purified Type III pneumococcus-transforming principle. This material exhibits even greater sensitivity to H than the viruses. Its relationship to the general problem is discussed later.

Chemical Nature of the Agent

It has been reported that in general the nitrogen mustards ethyl-bis(β-chloroethyl)amine (EBA), methyl-bis(β-chloroethyl) amine (MBA), and tris(β-chloroethyl)amine (TBA) have properties qualitatively similar to that of H (15). These three agents were tested on Newcastle virus and equine encephalomyelitis.

The nitrogen mustards as crystalline hydrochlorides were dissolved in cold water and then an aliquot added to a solution of virus buffered with bicarbonate just sufficiently to neutralize the acid liberated. Experiments showed that after a half hour at 25°C. there was not much change in the virus. This is in general agreement with the chemical studies of these agents (24).

It was found that whereas the $\frac{\Delta \text{ log biological activity}}{\Delta\ 10^{-3}\ \text{M agent}}$ for EBA and MBA was not very different from H, for TBA it was two to three times higher; *i.e.* it required $\frac{1}{2}$ to $\frac{1}{3}$ as much TBA on a molar basis to obtain approximately the same inactivation.

Concentration of Virus

As was discussed in the previous paper (10), it follows from Equation 2 that the *fraction* of virus inactivated is independent of the virus concentration. Thus the slope of a plot of the logarithm of the virus concentration against the initial H concentration will be independent of the virus concentration. That this was observed may be seen in Fig. 3 where various initial concentrations of Newcastle virus were treated with various concentrations of H. All the curves have approximately the same slopes in agreement with the prediction.

Temperature

Temperature changes are known to affect markedly the rate of hydrolysis of mustard (17, 18). In an experiment with Newcastle virus it was observed

that the same inactivation per unit H was obtained at 0°C. as at 25°C. but it required many hours at the lower temperature for complete reaction compared to 30 minutes at 25°C. For the reader's convenience the values of the unimolecular hydrolysis constant a for mustard in water at 0°C., 10°C., and 37°C. are about 0.004, 0.017, and 0.6 (17, 18).

pH

Whereas it had been found (10) with enzymes and other proteins that the rate of action was in some instances markedly affected by a change in pH of the medium, no such effect has been observed with the viruses of Newcastle disease and equine encephalomyelitis. The results in Table II carried out by the stirring method show that at pH 6 or pH 8 the inactivation constants of at least two viruses are the same.

Details of Experiments of Table II

This experiment was carried out by the stirring method, described later in this paper, at both pH and with both viruses. The pH of the reaction mixture was examined continually with the aid of a Beckmann glass electrode and the acid liberated during the reaction was neutralized by careful addition of M/100 NaOH.

Samples were removed at 0, 1, 2, and 4 minutes for the Newcastle virus and 0, 4, and 10 minutes for the E. E. E. and the remaining activity determined by titrating on embryonated chicken eggs. The H concentration was 1×10^{-3} mols in all experiments.

TABLE II

Effect of pH on the Inactivation of Newcastle and Equine Encephalomyelitis Virus by Mustard

pH	Velocity constant K'	
	Newcastle	E. E.
6	5000	690
8	5000	620

Salt Concentration

Occasionally the reaction was carried out in saline solution instead of water. With certain of the viruses and most cells the results suggested that the presence of salt increased the degree of inactivation by H, while with other viruses there was no effect or it was in the opposite direction. Thus, equine encephalomyelitis, baker's yeast, and *E. coli* were inactivated more per unit H in the presence of M/10 NaCl or 0.85 per cent NaCl than in its absence, whereas *E. coli* phage was more sensitive in the absence of salt.

The hydrolysis or reactivity of H is retarded by the presence of chloride ion but by extending the time of contact between H and virus the reaction goes to completion.

Effect of Impurities or Other Reactants on the Rate of Inactivation

No consistent change in rate of inactivation could be demonstrated as the viruses of Newcastle disease or equine encephalomyelitis were purified by differential centrifugation or as the concentration of contaminating proteins varied. The three curves shown in Fig. 3 were obtained with an unfractionated Newcastle virus allantoic fluid, a once ultracentrifuged, and a twice ultracentrifuged sample. There is no appreciable difference in the inactivation rates of these three preparations.

The above conclusion received support when it was found that the extent of inactivation of pepsin by H was not markedly altered by the presence of ten times its weight of egg albumin. This should not be taken to mean that no impurities affect the rate of inactivation for it is well known that thiosulfate and similar substances have a high affinity for mustard and markedly affect the reaction. All halides slow down the reaction of H.

Comparison of the Effectiveness of H, Iodine, and Permanganate as Inactivating Agents of Newcastle Virus

It was of some interest to determine how mustard compared with other chemicals such as iodine and permanganate as inactivaing agents. The results of an experiment are shown in Table III.

The amount of mustard combined is significantly less than the permanganate or iodine required to inactivate the virus. More mustard than is indicated in Table III was added to the virus solution to attain 90 per inactivation but from the nature of the reaction most of the H reacts with the solvent and only the amount indicated was bound to the protein.

Details of Experiments of Table III

A sample of twice ultracentrifuged Newcastle CG179 virus resuspended in water was mixed with sufficient reagent to make a final concentration in one tube of 1×10^{-4} M H, in another 1×10^{-5}M $KMnO_4$, and in a third 1×10^{-5}M iodine. The virus concentration was 0.03 mg. protein per ml. After an hour at 25°C. the samples containing permanganate and iodine had lost their color. Samples were removed for virus activity tests in the usual manner. The iodine experiment was also carried out on a centrifuge-purified preparation containing 0.6 mg. virus protein per ml.

The $KMnO_4$ and iodine reactions were assumed to be stoichiometric. One experiment to test this point indicated that this was qualitatively true for iodine. The reaction of H, however, is known to consist of two simulatneous competing reactions in which the reaction with protein is a small fraction of that reacting with the water. To determine the H bound to the virus it was necessary to use a solution of twice centrifuged Newcastle virus containing 5.6 mg. of protein per ml. and an initial 50 per cent end point titer of 10^{11}. After treatment with 1×10^{-4}M H for ½ hour at 25°C. which reduced the activity to 10 per cent of its original titer or to 10^{10}, this solution and an untreated virus control diluted to the same volume were angle centrifuged at 12,000 R.P.M. for an hour in a centrifuge of 9 cm. average radius. The uncombined H or its hydrolysis product in the supernatant was then titrated with bromine in acid solution using methyl red as the end point indicator (10). From this and the similar titrations of the appropriate controls, *i.e.* H solution plus no virus and virus supernatant when no H had been added, the value for the bound H was obtained. The value was very small and is the value shown in Table III. It should be emphasized that this is not an accurate determination for it was obtained as the difference between two close and large values. It does serve, however, as a qualitative upper limit of the amount of H combined.

TABLE III

Certain Chemical Agents on Newcastle Virus at pH 7–8 and 25°C.

Reagent	Reagent consumed or used up per milligram of purified Newcastle virus in inactivating (*ca.*) 90 per cent of the virus
	mM
Iodine	1×10^{-4}
$KMnO_4$	$>1 \times 10^{-4}$
H	$<0.03 \times 10^{-4}$

EXPERIMENTAL DETAILS

Materials and Biological Measurements

Equine Encephalomyelitis (Eastern Strain).—This virus was obtained from Dr. Carl TenBroeck of this laboratory. It was highly infectious for 10 day old chick embryos killing them in 24 to 48 hours. Allantoic fluids from embryos harvested after 23 hours' incubation at 37°C. usually contained 10^8 M.L.D. per ml. Titration of the virus followed the usual procedure (39). Serial tenfold dilutions of the virus were made in cold buffered (pH 7.4) saline. A drop (0.05 ml.) of the dilutions near the

expected end point was placed on the chorioallantoic membranes of 10 day old embryonated eggs after which they were incubated at 37°C. and examined daily with the aid of a candling lamp. All dead embryos were examined and pale uncongested ones were not counted as indicative of the presence of virus. Five embryos for each sample at each dilution were used. The virus titer was estimated as the concentration which would kill 50 per cent of the embryos. In our hands the results were reproducible to at least 0.5 logarithm units or about three times in the virus concentration.

Newcastle Disease of Chickens

Beaudette Strain.—This strain was obtained from Dr. F. B. Bang who in turn obtained it directly from Dr. Beaudette of the New Jersey Experiment Station, Rutger's Agricultural School. This strain kills embryos in 36 to 72 hours but in general does not kill adult birds. Allantoic fluid from embryos inoculated with 10^5 M.L.D. and subsequently incubated for 44 hours usually contained 10^8 or more M.L.D. per ml. Very few studies were made with this strain.

CG179 Strain of Beach

This strain which kills both embryos and adult birds was obtained from Dr. Carl TenBroeck who in turn obtained it from Dr. J. R. Beach in California. After inoculation with 10^5 to 10^6 M.L.D., allantoic fluids from eggs maintained at 37°C. for 38 hours usually contained 10^8 M.L.D. This strain kills embryos a little faster than the Beaudette strain. Practically all the studies on Newcastle virus in the present paper were made on this strain. Titration of the virus followed the same procedures described for equine encephalomyelitis except that the eggs were examined several days longer.

E. coli B

Most of our work was done on an organism kindly sent to W. H. Price by Professor M. Delbrück. Toward the end of our work we had reason to request a sample of *E. coli* B from Professor A. D. Hershey. We were considerably surprised to find that it exhibited greater resistance to the action of H than did the sample from Professor Delbrück. Both organisms are lysed by T_1, T_6, and T_7 phages in the same time. No other phages were available for test at this time.

We have indicated in Table I the organism from Delbrück as (*d*) and from Hershey as (*h*).

Culture of this organism was in the usual broth media. Plating consisted of making the final dilution in 0.7 per cent agar in broth at 43°C. and 1 ml. was spread on warm previously poured agar-broth plates. Dilutions of the organism were made in saline buffered at pH 7.3.

Staphylococcus muscae Phage

W. H. Price kindly furnished this bacterial virus and host. He obtained them from Dr. R. E. Shope (41). This phage was diluted in Locke's solution, then into 0.7 per cent agar-broth with 1×10^8 actively growing *Staphylococcus muscae* cells per ml. One ml. of this suspension was spread on an agar-broth plate and incubated overnight at 37°C. after which the plaques were counted.

Staphylococcus muscae

The procedure for determining these cells was the same as that described above for *E. coli* B except that dilutions were made in Locke's solution.

Bakers' Yeast

This was fresh Fleischmann's bakers' yeast transferred and grown in a synthetic medium consisting of 2 per cent sucrose, 0.3 per cent $(NH_4)_2SO_4$, 0.2 per cent KH_2PO_4, 0.025 per cent $MgSO_4 \cdot 7H_2O$, 0.025 per cent $CaCl_2$, $2H_2O$, and 0.1 per cent extract. Colony counts of 2 per cent agar media plates are quite reproducible. Incubation temperature was 30°C.

Feline Pneumonitis (Baker)

This agent, a member of the psittacosis group (42), was obtained from Dr. J. A. Baker (43). The virus was handled as previously described in the above references. Suspensions and dilutions were made in veal infusion broth. Inoculations of 1 ml. were made into the yolk sac of 5 day embryonated eggs. A 10 per cent yolk sac suspension made from eggs in which the embryos were moribund contained at least 10^8 M.L.D. for chick embryos. It was shown by Hamre and Rake (42) that the time of death as well as the dilution end point could be used as a rough measure of the virus concentration in the inoculums.

Rabbit Papilloma Virus (Shope)

This virus was obtained through the kindness of Dr. R. E. Shope and consisted of glycerinated cottontail horny papillomas. The virus was prepared and titrated as previously described (44).

T_2r^+ Phage of E. coli B

This bacterial virus and host were originally obtained by W. H. Price from Professor M. Delbrück. Estimation of the phage was made by mixing 1 ml. of his buffered saline dilution of the phage containing about 10^3 M.L.D. per ml. with 9 ml. of an 0.7 per cent agar-broth mixture in which were also suspended about 3×10^7 actively multiplying *E. coli* B cells per ml. One ml. of this mixture was then spread on a broth-2 per cent agar plate and left at 37°C. overnight after which a count was made.

Crystalline Yeast Hexokinase

This purified protein was obtained from Dr. M. Kunitz and estimated as described by Kunitz and McDonald (45). In our previous paper (10) we indicated that the action of H on hexokinase was not examined at pH 7.5–8 because it was too unstable under these conditions. One per cent glucose stabilized the enzyme at this pH sufficiently to perform the experiment. As seen in Table I the rate of inactivation was still considerably lower than found for chicken pepsin or any of the viruses.

Chicken Pepsin

This enzyme was obtained as previously described (46). The activity was measured by its milk-clotting property (47).

Sulfur Mustard Gas

Most of the work reported herein was done with mustard prepared from thiodiglycol. It had a freezing point of 14.2 to 13.8°C., the change in melting point occurring over a long period. An experiment was performed on Newcastle virus with the above preparation and a highly purified preparation supplied by Dr. V. du Vigneaud (21) (M.P. 14.5°C.) and they showed the same degree of inactivation. Other experiments were performed in which *E. coli* B (*d*) and the T_2r^+ phage were treated with four different preparations of H the melting points of which varied from 12°C. to 14.5°C. All showed virtually equal effect on the biological agents. It may be concluded, therefore, that the impurities present in these preparations did not inhibit the biological test as was noted for yeast by du Vigneaud and Stevens (21) when certain of their impure preparations were used.

The Nitrogen Mustards

All three of these materials as crystalline hydrochloride salts were obtained from Edgewood Arsenal.

Aqueous Solutions of Mustard

Aqueous solutions of mustard were always made up fresh when they were to be used. A convenient method was to add 0.01 ml. of liquid H to 80 ml. of cold water or saline in a glass stoppered bottle and immediately shake hard for 15 to 30 seconds. Care must be taken to shake immediately after addition of the H to cold water for otherwise it may crystallize and the time required to dissolve the crystals has not been determined but it is probably considerably longer. Complete solution of the H results in a 1.0×10^{-3} molar solution. If the temperature is 10°C. or below, it will not hydrolyze perceptibly for several minutes. The concentration of H in water saturated at 5–10°C. is 4.5 to 5.0×10^{-3} molar. For other data in this connection one may find another paper (48) of interest.

If it is desirable the H can be first diluted in an organic solvent which is miscible with water such as alcohol or one of the "cellosolves." Such a solution permits a larger volume to be measured and mixed with water for the preparation of homogeneous solutions. Unless the aqueous solution is agitated as the organic solution of H is added, the H will precipitate locally and may not dissolve unless shaken hard.

General Methods

Determination of Mustard Concentration in Solution

Methods for differentiating free H from sulfonium salts and thiodiglycol are described elsewhere (10, 49). The basic titration is with bromine or hypochlorite in M/4 H_2SO_4 with methyl red as an indicator (10).

Methods of Exposing Materials to H

Single Method

This simple method consists of making an aqueous or saline solution of H and mixing in the desired proportions with the virus or biological material. Preparation of aqueous H solutions has been described in an earlier paragraph.

After mixing with the biological material the container was kept at 25°C. for at least 30 minutes if no salt was present but 60 minutes if 0.85 per cent saline was used.

Stirring Method

A variation in the previously described stirring method was used in the present experiment. In the earlier paper excess free H was stirred and a constant concentration of H resulted when an equilibrium was obtained between the rate of solution and the loss due to hydrolysis. In the present procedure the amount of H to give the desired concentration was first dissolved in the solvent (water or saline) in the usual way. The biological agent was then added and then the concentration of H maintained constant by permitting a "cellosolve"[3] solution of H to flow in very slowly through a fine capillary while the aqueous solution was stirred. By adjusting the initial volume of aqueous solution, the diameter and length of the capillary, and the concentration of H in the "cellosolve," any concentration in an aqueous solution can be maintained. When the concentration of "cellosolve" interferes with the reaction or the biological agents then this procedure will have to be modified. The amounts of "cellosolve" introduced in the present experiments were usually 0.05 to 0.10 ml. per minute and this was found to have little if any effect on the viruses studied.

In this method small samples are removed at intervals of time and diluted 10 to 100 times with cold buffered saline which virtually stops the inactivation by H. After dilution the activity of the sample was determined in the appropriate manner for the particular material.

The changes in pH were followed by means of external glass electrodes connected to a Beckmann pH meter. Corrections were made by permitting small quantities of dilute NaOH or bicarbonate to flow in through a capillary.

The apparatus was sterilized before use with phenol or 70 per cent alcohol followed by numerous washings and soaking with sterile water.

DISCUSSION

The obvious difference between viruses and enzymes that might account for the consistently greater susceptibility of the former to mustard is the nucleic acid present in viruses. In the past few years it has become well established that mustard gas combines with nucleic acid, nucleoproteins, and viruses (25–30, 53). In most of these instances cited the quantity of mustard used and the length of exposure were such that the treatment is considered more drastic than that described in the present work. In addition there was no indication of the rate of reaction in these earlier studies. Gilman and Phillips (15) have reported that du Vigneaud *et al.* and Bawden and Pirie found that mustard inactivates tobacco mosaic virus but a detailed account of this work has not yet appeared. More recently TenBroeck and Herriott (11) showed that the animal viruses equine encephalomyelitis, fixed rabies, and hog cholera are inactivated by small amounts of mustard but no consideration was given at that time to the rate of the reaction. Rose and Gellhorn (30) have recently found that influenza virus is inactivated by H as well as the nitrogen mustards.

[3] "Cellosolve" is a trade name for monoethyl ether of ethylene glycol.

The results of the present studies suggest that the viruses containing desoxyribose nucleic acid (DNA) are more rapidly inactivated than those containing only ribosenucleic acid (RNA). This may be seen in Table IV where certain chemical analyses are shown along with the relative sensitivity to mustard.

TABLE IV

Composition of Biologically Active Materials and Their Sensitivity to Mustard Gas

Material	Δ log titer / Δ 1 × 10^{-3} M H	Protein	Carbohydrate	Lipid	Nucleic acid		Reference No.
					Desoxyribose-	Ribose-	
			per cent	*per cent*	*per cent*	*per cent*	
Pneumococcus Type III transforming principle	30				*ca.* 100		33
T_2r^+ phage of *E. coli* B	20	51	13.6	2	30–40		34
E. coli B	20	67.7		9.1	2.4	21	34
Newcastle virus	12	67		27	1		35
Papilloma	6*	90		1.5	8.7		36
E.E.E.	3.6	49	3.5	48.5		4.4	37
Tobacco mosaic	1.4	94		0	0	6	38

* This is a minimum value and may be higher. See text p. 227.

Considerable interest was added to the above relationship when it was found that the purified pneumococcus-transforming principle (TP) was the most sensitive material yet examined. The present evidence indicates that the preparations of TP are relatively pure DNA in which the presence of protein has not been demonstrable.

The finding of Mirsky and Ris (31) that the isolated chromosomal material from lymphocytes is extremely rich in desoxyribose nucleohistone is interesting in view of the repeated observations on a variety of organisms that mutations may be induced with mustard gas (1–9).

Bodenstein and Kondritzer (32) have recently reported that the formation of DNA in amphibian tissue stops following exposure to dilute H which also arrests mitosis. In direct contrast the RNA formation proceeds apparently in a normal manner.

All of the above independent observations are suggestive but the work in progress employing mustard in which radioactive sulfur has been incorporated should provide a definite answer to the obvious question of the location of the bound mustard.

SUMMARY

The action of mustard gas on six animal, one plant, and two bacterial viruses; also on bacteria, yeast, and the pneumococcus-transforming principle has been studied. The viruses include Newcastle's disease of chickens, equine encephalo-

myelitis (Eastern strain), feline pneumonitis (Baker), rabbit papilloma (Shope), fixed rabies, rabbit myxoma, tobacco mosaic, T_2r^+ phage of *E. coli* B, and a *Staphylococcus muscae* phage. The cells include bakers' yeast, *E. coli* B, *Staphylococcus muscae*, and swine plague bacillus.

The rates of inactivation of the viruses and cells were of the same order of magnitude and faster than those of enzymes.

Of the viruses examined those containing desoxyribose nucleic acid were inactivated faster than those containing ribosenucleic acid. Preparations of the pneumococcus-transforming principle which were largely desoxyribose nucleic acid have shown the greatest sensitivity to mustard gas of all systems examined.

An expression was derived describing the inactivation rate when mustard gas decreases during the experiment.

BIBLIOGRAPHY

1. Auerbach, C., and Robson, J. M., *Nature*, 1944, **154,** 81; 1946, **157,** 302.
2. Auerbach, C., Robson, J. M., and Carr, J. G., *Science*, 1947, **105,** 243.
3. Koller, P. C., in press.
4. Bonner, D., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1946, **11,** 14.
5. Tatum, E., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1946, **11,** 278.
6. Horowitz, N. H., Houlahan, M. B., Hungat, M. G., and Wright, B., *Science*, 1946, **104,** 233.
7. Stahman, M. A., and Stauffer, J. F., *Science*, 1947, **106,** 35.
8. McElroy, W. D., Cushing, J. E., and Miller, H., *J. Cell. and Comp. Physiol.*, 1947, **30,** 331.
9. Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, 1947, **54,** 767.
10. Herriott, R. M., Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1946, **30,** 185.
11. TenBroeck, C., and Herriott, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62,** 271.
12. Gilman, A., *Fed. Proc.*, 1946, **5,** 285.
13. Rhoads, C. P., *J. Am. Med. Assn.*, 1946, **131,** 656.
14. Osborne, E. D., Jordon, J. W., Hoak, F. C., and Pschierer, F. J., *J. Am. Med. Assn.*, 1947, **135,** 1123.
15. Gilman, and Philips, F. S., *Science*, 1946, **103,** 409.
16. Bartlett, P. D., Davis, J. W., Ross, S. D., and Swain, C. G., *J. Am. Chem. Soc.*, 1947, **69,** 2971, 2977.
17. Grant, W. M., and Kinsey, V. E., *J. Biol. Chem.*, 1946, **165,** 495.
18. Hopkins, E. F., *J. Pharmacol. and Exp. Therap.*, 1919, **12,** 393.
19. Herriott, R. M., *J. Gen. Physiol.*, 1947, **30,** 457.
20. Price, C. C., and Wakefield, L. B., *J. Org. Chem.*, 1947, **12,** 232.
21. Du Vigneaud, V., and Stevens, C. M., *J. Am. Chem. Soc.*, 1947, **69,** 1808.

22. Kinsey, V. E., and Grant, W. M., *J. Cell. and Comp. Physiol.*, 1947, **29,** 66.
23. Henry, H., and Stacey, M., *Nature*, 1943, **151,** 671.
24. Golumbic, C., Fruton, J. S., Stahmann, M. A., Stein, W. H., Moore, S., and Bergmann, M., *J. Org. Chem.*, 1946, **11,** 518, 580.
25. Berenblum, I., and Schoental, R., *Nature*, 1947, **159,** 727.
26. Banks, T. E., Boursnell, J. C., Francis, G. E., and Wormall, A., *Biochem. J.*, 1946, **40,** 734, 778.
27. Chanutin, A., and Gjessing, E., *Cancer Research*, 1946, **6,** 599.
28. Gjessing, E., and Chanutin, A., *Cancer Research*, 1946, **6,** 593.
29. Young, E. G., and Campbell, R. B., *Canad. J. Research*, section B, 1947, **25,** 37.
30. Rose, H. M., and Gellhorn, A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65,** 83.
31. Mirsky, A. E., and Ris, H., *J. Gen. Physiol.*, 1947, **31,** 7.
32. Bodenstein, D., and Kondritzer, A. A., *J. Exp. Zool.*, 1948, **17,** 109.
33. McCarty, M., and Avery, O. T., *J. Exp. Med.*, 1946, **83,** 89.
34. Taylor, A. R., *J. Biol. Chem.*, 1946, **165,** 271.
35. Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, **55,** 69.
36. Taylor, A. R., Beard, D., Sharp, D. G., and Beard, J. W., *J. Infect. Dis.*, 1942, **71,** 110.
37. Taylor, A. R., Sharp, D. G., Beard, D., and Beard, J. W., *J. Infect. Dis.*, 1943, **72,** 31.
38. Loring, H. S., *J. Biol. Chem.*, 1939, **130,** 251.
39. Bang, F. B., *J. Exp. Med.*, 1943, **77,** 337.
40. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
41. Shope, R. E., *J. Exp. Med.*, 1927, **45,** 1037.
42. Hamre, D., and Rake, G., *J. Infect. Dis.*, 1944, **74,** 206.
43. Baker, J. A., *J. Exp. Med.*, 1944, **79,** 159.
44. Shope, R. E., *J. Exp. Med.*, 1933, **58,** 607.
45. Kunitz, M., and McDonald, M., *J. Gen. Physiol.*, 1946, **29,** 393.
46. Herriott, R. M., Bartz, Q. R., and Northrop, J. H., *J. Gen. Physiol.*, 1938, **21,** 575.
47. Herriott, R. M., *J. Gen. Physiol.*, 1938, **21,** 501.
48. Herriott, R. M., *J. Gen. Physiol.*, 1947, **30,** 449; 1947, **30,** 457.
49. Grant, W. M., and Kinsey, V. E., *J. Biol. Chem.*, 1946, **165,** 495.
50. Johnson, E. P., *Science*, 1948, **107,** 40.
51. Cohen, B., Van Artsdalen, E. R., and Harris, J., *J. Am. Chem. Soc.*, 1948, **70,** 281.
52. Herriott, R. M., and Price, W. H., *J. Gen. Physiol.*, 1948, **32,** 63.
53. Elmore, D. T., Gulland, J. M., Jordan, D. O., and Taylor, H. F. W., *Biochem. J.*, 1948, **42,** 308.