Inactivation of Vaccinia Virus by Ascorbic Acid

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SUMMARY

Ascorbic acid undergoing auto-oxidation inactivated vaccinia virus. Copper ion was shown to have a catalytic effect on the inactivation. Neither unoxidized ascorbic acid nor its oxidation product, dehydroascorbic acid, were inhibitory. When ascorbic acid was oxidized at high pH in the absence of copper ion no inactivation took place. Similarly, enzymic oxidation of ascorbic acid in the absence of copper was without effect on the virus. Catalase prevented inactivation but not the oxidation of ascorbic acid. Glutathione prevented both inactivation and the oxidation of ascorbic acid. Inhibition experiments with ascorbic acid under anaerobic conditions were inconclusive. The mechanism of ascorbic acid inactivation is discussed in the light of these data and that of other authors with different viruses.

INTRODUCTION

During experiments on the stability of vaccinia virus in the presence of various reducing agents, it was found that ascorbic acid was strongly inhibitory. Kligler & Bernkopf (1937) noted this phenomenon, and Jungeblut (1935) reported that ascorbic acid inactivated poliovirus. Similar results have been reported for herpes virus (Holden & Resnick, 1936; Holden & Molloy, 1937), bacteriophages (Lominski, 1936) and tobacco mosaic virus (Lojkin, 1936). Lojkin made a systematic study of the inhibitory effects of ascorbic acid on tobacco mosaic virus infectivity; our results with vaccinia virus are in many respects like hers.

METHODS

Virus. The Lister Institute vaccine strain of vaccinia virus was used throughout the experiments. It was prepared from sheep dermal pulp in 0.004 M-McIlvaine buffer (pH 7.2). The virus was partially purified by extraction with 'Arcton 113' (trifluorotrichloroethane, I.C.I.) followed by one cycle of high-speed centrifugation. It was stored in small volumes at -70° . Such preparations produced cytopathic changes in monkey kidney tissue cultures in dilutions of 10^{-6} to 10^{-9} .

Virus titrations. Suitable dilutions of samples were inoculated in 0.1 ml. volumes into roller tube cultures of 2nd cycle monkey kidney cells; four tubes were used per dilution. Cytopathic changes were evident in 48 hr with high concentrations of virus, but the tubes were examined daily until the seventh day after inoculation, when no further changes were recorded. Fifty % end-points (TCD₅₀) were calculated by the method of Reed & Muench (1938).

Buffers were prepared from McIlvaine's 0.2 M-disodium phosphate + 0.1 M-citric

acid solutions, which were mixed and diluted to give the desired pH value and molarity. Unless otherwise stated, all experiments were done in buffer at pH 6.9.

Enzymes. Catalase was a commercial beef-liver preparation (Light and Co.). It was used in different experiments in final concentrations of 10 and 4.0 mg./ml.

Ascorbic acid oxidase (ascorbate oxidase) was prepared from fresh cabbage by freezing whole cabbage-heart at -70° and then expressing the juice in a press. The juice was dialysed overnight against distilled water, and an equal volume of cold acetone was added to the dialysis residue. The resulting precipitate was dissolved in distilled water of one-tenth of the original volume of juice. This preparation was stored at -70° ; at pH 6.8 and 37° , 0.2 ml. of this solution in a total volume of 2.0 ml. completely oxidized 180 μ g. ascorbic acid in 2 hr.

Copper solution was 0.1 M-CuSO₄ in distilled water. It was added to ascorbic acid + virus mixtures to a final copper concentration of 5 μ g./ml.

Ascorbic acid (British Drug Houses Ltd.) was prepared in solutions of various strengths in pH 6.8 buffer immediately before use. The concentration of ascorbic acid was estimated by titration against standard B.D.H. dichlorophenolindophenol.

Cystein hydrochloride, glutathione and thioglycollic (mercaptoacetic) acid were dissolved in distilled water and diluted in buffer of the desired pH value.

RESULTS

In preliminary experiments samples of virus containing 5×10^8 TCD 50/ml. were exposed to cysteine, glutathione, thioglycollic acid or ascorbic acid at 100 μ g./ml. for 18 hr at 37° and pH 7.4. Whereas the three thiol-containing reducing substances were without effect, ascorbic acid decreased the virus titre to < 10 TCD 50/ml. This inactivation was not abolished by dialysis of the virus + ascorbic acid mixture against water. Tissue culture cells, pretreated for 2 days at 37° with ascorbic acid 200 μ g./ml. had no altered susceptibility to virus. The inactivating effect of ascorbic acid on vaccinia virus was also shown by titration of samples on the chorioallantoic membranes of chick embryos.

Effect of copper. The autoxidation of ascorbic acid is a well-known phenomenon and is catalysed by copper ions (Barron, Demilio & Klemperer, 1936). Preliminary experiments showed that Cu²⁺ 5 μ g./ml. was itself without effect on the virus, but it increased both the rate and degree of inactivation by ascorbic acid in ascorbic acid + vaccinia virus mixtures. The Cu²⁺ also increased the rate of oxidation of ascorbic acid in the mixture (Fig. 1).

Effect of concentration of ascorbic acid. Vaccinia virus has an optimum stability at pH 6.8–7.0 at 37° ; accordingly, quantitative experiments with ascorbic acid were performed in undiluted McIlvaine buffer at pH 6.9. Vaccinia virus with an initial titre of $10^{8.5}$ TCD 50/ml. was diluted 1/100 and mixed with different quantities of ascorbic acid. The mixtures were incubated at 37° and samples examined at various times for infectious virus. A control sample of virus alone was titrated after incubation for the maximum time. All the mixtures contained 5 μ g. Cu²⁺/ml. The concentration of ascorbic acid and the time of exposure influenced the inactivation of vaccinia virus; quantities as small as 50 μ g. ascorbic acid/ml. fully inactivated $10^{8.5}$ TCD 50 in 2 hr at 37° and amounts as little as 10 μ g. caused partial inactivation after 24 hr (Table 1).

Fig. 1. Vaccinia virus 10,000 TCD 50 that was exposed to ascorbic acid 100 $\mu\text{g./ml.}$ at 37° and pH 7.4. Curve (a) open circles virus titre without Cu^{2+} . Curve (b) open circles virus titre + Cu^{2+} 5 $\mu\text{g./ml.}$ Curve (c) closed circles % ascorbic acid oxidized in the presence of Cu^{2+} 5 $\mu\text{g./ml.}$ Curve (d) closed circles % ascorbic acid oxidized in the absence of Cu^{2+} .

Effect of dehydroascorbic acid. An inhibitory concentration of ascorbic acid (100 $\mu\text{g./ml.}$) was oxidized with an equivalent quantity of potassium permanganate. Although potassium permanganate inactivates vaccinia virus, the amount required to oxidize 100 $\mu\text{g.}$ ascorbic acid was calculated to be less than the inhibitory concentration. Only partial inactivation occurred when the sample of oxidized ascorbic

Table 1. *Effect of ascorbic acid on the infectivity of vaccinia virus suspensions*

Ascorbic acid (mg./ml.)	Time of exposure (hr)						Amount of ascorbic acid oxidized at time of inacti- vation of virus ($\mu\text{g.}$)
	0	0.25	0.5	1.0	2.0	24.0	
	Relative degree of virus inactivation						
1.0	—	+	+	+	+	+	80–190
0.5	—	—	$\pm$	+	+	+	140
0.25	—	—	+	+	+	+	70
0.125	—	—	+	+	+	+	65
0.0625	—	—	—	$\pm$	+	+	50
0.05	—	—	—	—	—	$\pm$	50
0.01	—	—	—	—	—	$\pm$	10

+ = complete inactivation; $\pm$ = partial inactivation; — = > 10 TCD 50 active virus still present.

acid was held with virus overnight at 37°. Ascorbic acid (125 µg./ml.) completely oxidized with ascorbic acid oxidase did not inactivate vaccinia virus after 2 hr at 37°. Controls of unoxidized ascorbic acid in this concentration inactivated completely. The infectivity of untreated virus under these conditions was unaltered.

Effect of ascorbic acid oxidase (ascorbate oxidase). The rapid oxidation of ascorbic acid can be catalysed without added Cu²⁺ by the enzyme hexoxidase. Virus and ascorbate oxidase preparation in pH 6.9 buffer were mixed to give a virus titre of 10⁶ TCD 50/ml. and a concentration of enzyme preparation of 20 % (v/v). To one portion of this mixture ascorbic acid was added to 125 µg./ml. The second portion was adjusted to the same volume by adding pH 6.9 buffer. Controls consisting of virus alone and virus + ascorbic acid + Cu²⁺ were set up at the same time. All mixtures were incubated at 37° for 2 hr. There was no inactivation of vaccinia virus when the ascorbic acid used had been completely oxidized by ascorbate oxidase in the absence of added Cu²⁺. The enzyme alone was without effect on the virus, which was completely inactivated in the presence of the same concentration of ascorbic acid when oxidation was catalysed by Cu²⁺. These results confirmed that the oxidation product of ascorbic acid (dehydroascorbic acid) did not inactivate and indicate that the copper catalysed oxidation of ascorbic acid (probably in the presence of oxygen) was necessary for inactivation, and that the enzyme-catalysed oxidation proceeds by different mechanism. These findings are like those of Lojkin (1936) with tobacco mosaic virus.

Effect of catalase. The addition of catalase to ascorbic acid + tobacco mosaic virus mixtures abolishes the inactivating effect of ascorbic acid (Lojkin, 1936). Similarly, the addition of beef-liver catalase 4 mg./ml. completely prevented the inactivation of vaccinia virus in a buffered mixture containing 10^{6.5} TCD 50 virus/ml. + ascorbic acid 125 µg./ml. + Cu²⁺ 5 µg./ml. held at 37° for 2 hr. Inactivation occurred when the catalase was omitted from a similar mixture or when the enzyme was inactivated by boiling before addition. The enzyme alone was without effect on the virus, and did not prevent the oxidation of ascorbic acid. The ascorbic acid in the mixture was about 90 % oxidized.

Effect of high pH values. Barron *et al.* (1936) showed that the autoxidation of ascorbic acid was increased in alkaline media in the absence of catalytic Cu²⁺. Vaccinia virus and ascorbic acid were mixed without added Cu²⁺ in pH 10.0 buffer to final concentrations of 10⁶ TCD 50 and 125 µg./ml. respectively. The mixture, and a virus control at the same pH value, were incubated for 2 hr at 37°. No inactivation of virus took place, although more than 40 % of the ascorbic acid was oxidized. Exposure to pH 10.0 for 2 hr at 37° had no effect on the virus alone.

Effect of glutathione. The inhibition of vaccinia virus by reduced glutathione reported by Kligler & Bernkopf (1937) was not confirmed. Not only were glutathione concentrations up to 1.0 mg./ml. without effect on the virus, but appeared to prevent the oxidation of ascorbic acid in vaccinia virus + ascorbic acid mixtures and consequently the inactivation of virus. Ascorbic acid was added to mixtures of virus and freshly prepared reduced glutathione. The final mixture, containing virus 10^{6.5} TCD 50/ml., glutathione 125 µg./ml., ascorbic acid 125 µg./ml., and Cu²⁺ 5 µg./ml. was held for 2 hr at 37°; suitable controls were included. Glutathione decreased the inactivation by ascorbic acid at 125 µg./ml., and abolished it at 1 mg./ml. Glutathione prevented the autoxidation of ascorbic acid, but did not reactivate virus when

added to virus + ascorbic acid mixtures in which inactivation of the virus had already occurred. The substitution of cysteine in the same concentration for glutathione had no effect on the inactivation by ascorbic acid. These experiments also indicate that ascorbic acid itself is not inhibitory when its autoxidation is prevented.

Effect of an atmosphere of nitrogen and anaerobiosis on ascorbic acid inactivation. Several attempts were made to prevent the autoxidation of ascorbic acid in vaccinia virus + ascorbic acid mixtures by gassing the system with oxygen-free nitrogen. In a typical experiment the buffer to be used as diluent was boiled to drive off dissolved air. Oxygen-free nitrogen (British Oxygen Company Limited) was bubbled through a mixture containing 10^6 TCD 50/ml. virus and $5 \mu\text{g. Cu}^{2+}$ /ml. for 30 min. before a freshly prepared solution of ascorbic acid was added to $125 \mu\text{g./ml.}$, the nitrogen bubbling was continued during the 2 hr incubation at 37° . The virus was completely inactivated, although only 15–20 % of the ascorbic acid was oxidized. A Thunberg tube was prepared containing similar concentrations of virus and Cu^{2+} . Ascorbic acid solution was placed in the side arm and the tap opened. This, together with a virus control, was placed in an anaerobic jar. After the usual procedure for producing anaerobic conditions within the jar, the ascorbic acid was tipped into the virus. Again, after incubation at 37° the virus was inactivated, although only about 11 % of the ascorbic acid was oxidized. The control virus retained its original infectivity. It appears that under these conditions even a small degree of autoxidation of ascorbic acid was sufficient to inactivate the vaccinia virus.

DISCUSSION

The observations reported here for the inactivation of vaccinia virus by ascorbic acid are similar to those of Lojkin with tobacco mosaic virus. The inactivation of other animal viruses by ascorbic acid has been investigated from different viewpoints. In at least one case the inactivation can be ascribed to a direct pH effect; in other cases no cognizance was taken of the ready autoxidation of ascorbic acid, the catalysis of this oxidation by minute amounts of cupric ion, or the effect of pH on the autoxidation process. Milas (1932), in a review of autoxidation, stated that autoxidations induce the oxidation of other substances that are relatively unaffected by free oxygen. The inactivation of tobacco mosaic virus by ascorbic acid has been explained not as a reduction, but an oxidation effected by an intermediate unstable peroxide formed during the autoxidation of ascorbic acid catalysed by Cu^+ . Although the identity of the peroxide was not established, the addition of catalase to ascorbic acid + tobacco mosaic virus mixtures abolished the inactivation (Lojkin, 1936). Catalase likewise prevented the inhibition of vaccinia virus by ascorbic acid, presumably by destroying peroxides, although it did not prevent the autoxidation of ascorbic acid. Peloux, Lofre, Cier & Colobert (1962) inactivated poliovirus with hydrogen peroxide and ascorbic acid and suggested that free hydroxyl radicals induced by iron and copper ions were responsible. Berneis (1963) showed that ascorbic acid degraded deoxyribonucleic acid, a degradation that could be abolished by catalase or peroxidase; he too concluded that free hydroxyl radicals rather than intermediate peroxides were involved. Free radicals drastically alter the properties of both synthetic and natural polymers. The inactivation of vaccinia virus might similarly be explained on this basis. Whether viral protein or nucleic acid is attacked remains to be established.

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