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The effects of iodine on the biological activities of myxoviruses

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SUMMARY

Lugol's solution destroys the biological activities of Newcastle disease virus (NDV) after 15 s incubation at 37 °C. The rates of inactivation are slower at lower temperatures and at acid pH. At 4 °C and pH 5·8, the functions associated with the virus membrane, (haemolysis (HL), cell fusion and infectivity) are inactivated within 32 min, while haemagglutination (HA) and neuraminidase (N) are resistant to inactivation for several hours. Adjustment of NDV, Sendai and influenza A virus allantoic harvests to pH 5·8 and subsequent treatment with undiluted Lugol's solution (pH 5·8) for 15 min has a minimal effect on HA but results in complete loss of infectivity. It is suggested that iodination could be a useful method for vaccine production with membrane-bound viruses. It is postulated that the separation and dissociation of the membrane-associated properties of paramyxoviruses from the glycoprotein functions is due to the higher affinity of iodine for the lipids. Iodine could react with the carbon–carbon double bond (C=C) of the unsaturated fatty acids. This could lead to a change in the physical properties of the lipids and membrane immobilization.

INTRODUCTION

Iodine has been used in medicine since it was shown by J. Lugol in 1827 that its solubility in water can be increased by the addition of potassium iodide. Free iodine in the form of Lugol's solution (1% I_2, 2% KI in distilled water), tincture of iodine (15% I_2 in 70% ethanol), or more recently as Betadine (10% polyvinylpyrrolidone-iodine in water or ethanol) is a powerful bactericidal and virucidal agent. In addition, the radioactive isotopes I^{125} and I^{131} are widely used in radiotherapy, tracer experiments and immunochemistry.

However, although there is a considerable literature on the bactericidal and virucidal effects of free iodine (Plotkin, 1972), little is known about the conditions and the mechanism of its activity. In this paper, we present the results of a study of the effects of free iodine on the biological properties of influenza, Sendai and Newcastle disease viruses.

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MATERIALS AND METHODS

Viruses

The Queensland strain of NDV, and Sendai virus, were grown, harvested and purified as described previously (Apostolov & Almeida, 1972; Apostolov & Sawa, 1976). The laboratory PR8 strain of influenza A was also grown in 10-day-old chick embryos. Freshly harvested allantoic fluid after 48 hour's incubation was used for iodination. The virus was also partially purified by differential centrifugation at 75000*g* for two hours.

Haemagglutination (HA)

The titre was measured using 0·5% chick erythrocytes in 0·25 ml volumes.

Haemolysis (HL)

This was assayed by standardized methods (Apostolov & Sawa, 1976). Briefly, 0·2 ml. of virus preparation in plastic conical tubes was incubated with 5 ml 10% human group O erythrocytes for 30 min in a 37 °C water bath. After centrifugation at 1500 rev./min for 10 min, the supernatant was assayed for haemoglobin in a spectrophotometer at 540 nm wavelength.

Neuraminidase assays

These were kindly performed by Dr D. J. Alexander, according to a method previously published (Alexander, Reeve & Allan, 1970).

Infectivity

NDV was assayed in embryonated eggs (Apostolov & Sawa, 1976). PR8 was titrated in 10-day-old embryonated eggs and also in commercially obtained monolayers of monkey kidney cells. The inoculated eggs were tested for HA after 48 h incubation. The tissue culture cells were tested for haemadsorption after 10 day's incubation at 37 °C.

Erythrocyte monolayers and erythrocyte fusion

Chicken erythrocytes as 2% (v/v) suspension in isotonic saline were mixed with an equal quantity of NDV virus (0·2 ml) and incubated at 4 °C for 30 min, then lightly centrifuged (5000 rev./min) (Terry & Ho-Terry, 1976). After incubation at 37 °C for 60 min, the pellet was shaken loose and 0·2 ml was added to 2 ml of saline and the mixture poured into poly-L-lysine treated plates (Bächi, Eichenberger & Hanri, 1978). The cells were allowed to attach to the plastic plates (5 cm Falcon Petri dishes) for 10 min and then examined by a phase contrast microscope. The presence of fused chick cells with two or more nuclei was recorded.

Solutions

Stocks of Lugol's solution were prepared by dissolving 1% iodine and 2% KI by weight in water or phosphate buffers. Sodium thiosulphate was prepared as a 2% stock solution in saline.

Table 1. *The effect of undiluted Lugol's solution on the biological activities of Newcastle disease virus at* 4, 20 *and* 37 °*C*

Temp.	Time (s)	HA	N	HL	Inf.
4 °C	15	128	480	024	10^{-2}
	30	128	460	006	10^{-1}
	60	128	480	0	0
	120	128	440	0	0
	240	32	420	0	0
	480	16	194	0	0
	960	4	40	0	0
20 °C	15	128	400	0	0
	30	128	350	0	0
	60	16	70	0	0
	120	8	30	0	0
37 °C	15	0	0	0	0
Control		128	480	1·2	10^{-12}

Equal quantities (0·2 ml) of NDV in saline and Lugol's solution in phosphate buffer (pH 7·2) were mixed and incubated in water-baths. The reaction was stopped at various times by addition of an equal amount of 2% $Na_2S_2O_3$. The preparations were then stored at 4 °C until assayed. Haemagglutination titre (HA) is expressed as the reciprocal of the dilution, neuraminidase (N) as the OD value of change in substrate colour, haemolysis (HL) as the OD of haemoglobin and infectivity (Inf.) as the dilution factor of the ETD50.

RESULTS

The biological effects of iodine depend on the concentration, the time and temperature of exposure, and the pH. By manipulation of the experimental conditions of iodination, the biological properties of myxoviruses can be conveniently separated. The interaction of NDV with erythrocytes was used as a model, measuring HA and HL as the most convenient indicators of virus activity.

The effects of iodine in the classical prescription of Lugol's solution in distilled water are temperature-dependent. It was possible to separate the two sets of biological activity, HA and neuraminidase, from HL and infectivity, by changing the temperature of the reaction. It was found that the separation is better at lower temperatures (Table 1). The reaction of iodine with the virus, and the degree of separation between HA and HL is also pH dependent. Using Lugol's solution in the Sørensen's range of phosphate buffer and a reaction temperature of 4 °C, it was found that HA and HL could be even more effectively dissociated, especially in the acid pH range (Fig. 1). On the strength of this result, Lugol's solution buffered at pH 5·8 was selected for further studies.

The kinetics of the effects of Lugol's solution pH 5·8 at 4, 20 and 37 °C are shown in Fig. 2. As expected, the best dissociation of HA from HL was achieved at 4 °C. HA was not affected, even after several hours' treatment with Lugol's solution (pH 5·8) at 4 °C, but HL was destroyed in 32 min. The virus preparation with full HA but no HL, also lacked cell fusion properties when tested with chick erythrocytes (Terry & Ho-Terry, 1976).

The effect of iodine on HL depends on the concentration of Lugol's solution as

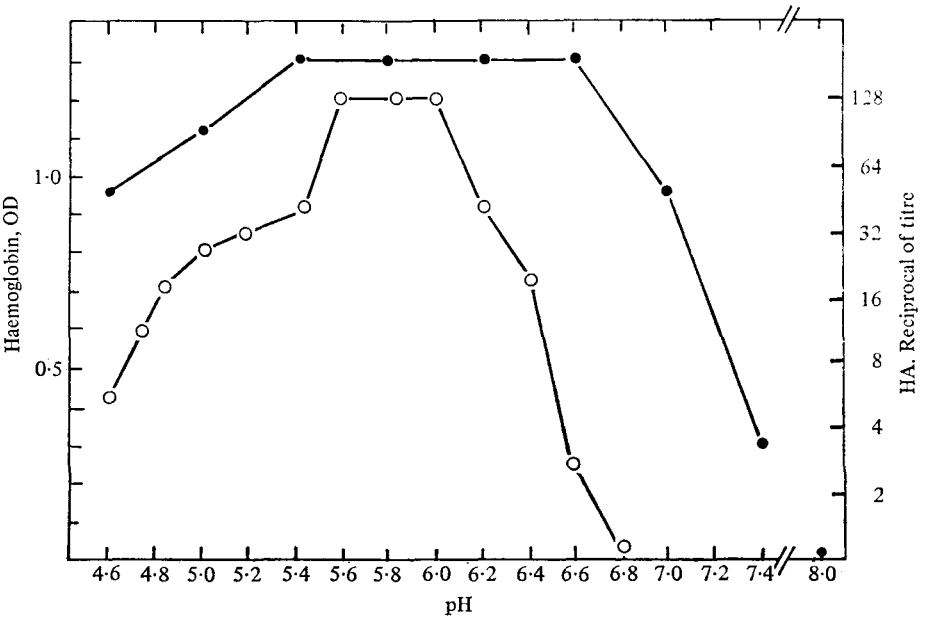

Fig. 1. The effect of pH iodination on HA and HL. The buffered Lugol's solution was prepared by mixing 9 parts of Lugol's solution with one part of 1/6 M Sørensen phosphate buffer. After addition of 0·2 ml of buffered solution to 0·2 ml of virus in saline, the mixture was incubated for five min at 4 °C and then the reaction was stopped by 0·2 ml of 2% $Na_2S_2O_3$. HA (●—), HL (○—).

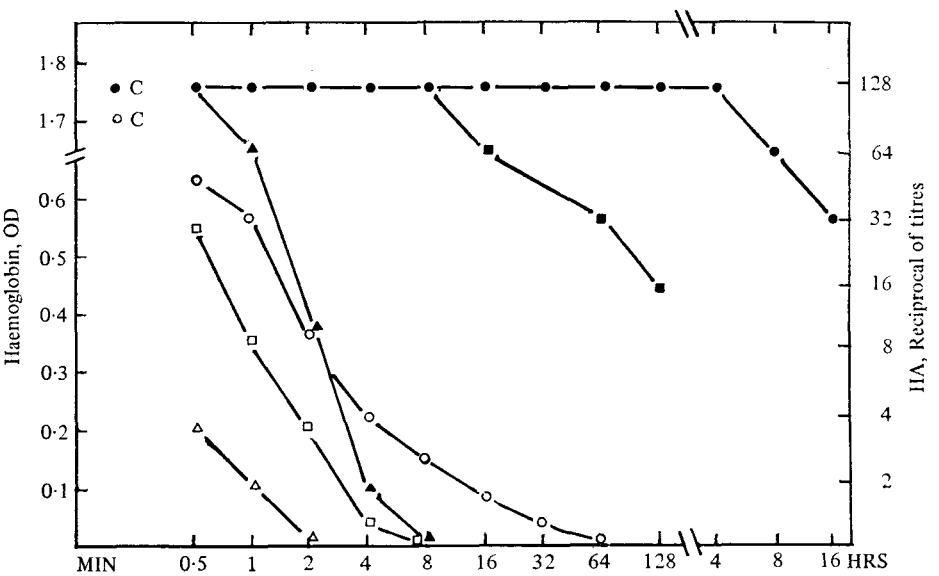

Fig. 2. Kinetics of iodination of NDV with Lugol's solution pH 5·8 at 4 °C, 20 °C, and 37 °C. Undiluted Lugol's solution in 1/6 M Sørensen's buffer, pH 5·8, was mixed with equal parts (0·2 ml) of NDV in saline. The mixture was incubated in water baths and the reaction stopped by sodium phosphate. HA titre, full symbols; OD, Hb value on open symbols. Temperature of incubation, 4° ●, 20° ■ and 37° ▲.

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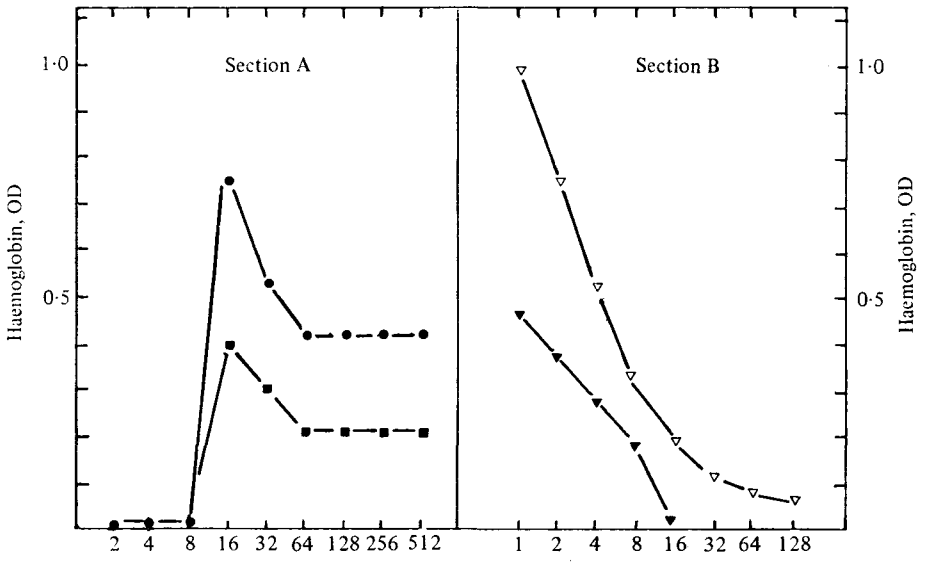

Fig. 3. Dose–response relationships of Lugol's solution and NDV. Section A. Two-fold dilutions of Lugol's solution pH 5·8 were mixed with constant virus in saline in 0·2 ml amounts. After 60 min the reaction was stopped with 0·2 ml of 2 %$Na_2S_2O_3$. HL was first assayed in 0·85 % saline suspension of erythrocyte (■- - -) and the erythrocyte pellet was resuspended in 0·7 % saline and reincubated for 10 minutes at 37 °C. The additional haemolysis was added on to the original HL (●—). Abscissa reciprocals of Lugol's dilutions. Section B. Two-fold dilutions of the virus were reacted with constant 1/4 dilutions of Lugol's solution pH 5·8. HL was assayed in erythrocytes suspended in 0·7 % saline. Virus control (△—). Treated virus (▲—).

well as the concentration of the virus. Varying the concentration of iodine, in the presence of constant virus at the optimal time of exposure, does not result in a dose–response curve, but in an abrypt change from complete inhibition to enhanced HL activity (Fig. 3, Section A). Another important observation illustrated in the same figure is that additional double HL can be obtained when the reacted erythrocytes are suspended in 0·85 % saline and re-suspended in the hypotonic 0·7 % saline. Similar titration effects of iodine on HL were obtained when the virus concentration was changed in the presence of a constant concentration of iodine (Fig. 3, Section B).

Influenza A (PR8) is also inactivated after treatment with Lugol's solution. The degree of preservation of HA depends on the suspending medium. When the pH of the normally alkaline allantoic fluid is acidified with a few drops of M-NaH_2PO_4 to pH 5·8, there is only a small loss of HA after iodine treatment, but the infectivity is completely destroyed (Table 2). In a separate experiment with purified PR8 it was found that there was no change in HA titre after 3 hours' treatment with undiluted Lugol's solution pH 5·8 at 4 °C. From Table 2 it can be seen that similar results are obtained with Sendai virus, except that the HA of Sendai is more resistant to iodination. All treated preparations of PR8 and Sendai lose their infectivity. In addition, like NDV, treated Sendai lost HL.

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Table 2. *Iodine treatment of myxoviruses in allantoic fluid*

	Allantoic fluid		Allantoic fluid pH 5·8		Purified virus	
	C	T	C	T	C	T
Sendai	512	32	512	128	512	512
PR8	512	256	512	256	512	512

Equal quantities (0·2 ml) of virus and undiluted Lugol's solution pH 5·8 were reacted at 4 °C for 15 min. The allantoic harvests were stored at 4 °C. The pH was adjusted to 5·8 by addition of 1 to 3 drops of M-NaH_2PO_4 to 10 ml of fluid. The HA titre is expressed as the reciprocal of the dilution. C = Control. T = iodine-treated virus. The infectivity of the treated preparation was nil.

The effect of iodination of erythrocytes on their capacity to be lysed by haemolytic NDV virus was studied by the use of erythrocytes in monolayers. Iodination at 4 °C and pH 5·8 under the same conditions as for the virus did not noticeably affect the morphology of the erythrocytes in the monolayer. However, haemolytic virus failed to lyse them. They retain the capacity to adsorb and elute the virus because after adsorbing the virus in the cold, fresh erythrocytes could be adsorbed onto the monolayer. Incubation at 37 °C led to detachment of the extra layer of erythrocytes (elution).

Iodinated virus with no HL activity but with preserved HA and elution properties, did not appear to be changed morphologically when examined in the electron microscope by negative staining. Moreover, when treated with fresh adult human plasma, typical complement lesions were found in the virus membrane (Apostolov & Sawa, 1976).

DISCUSSION

The envelope of paramyxoviruses consists of a biological membrane to which projections (spikes) are attached on the outside. The projections consist of two polypeptides, one of which carries the HA and the neuraminidase activities (Tozawa, Watanabe & Ishida, 1973), and the other is believed to be involved in the fusogenic properties of the virus (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). The fusogenic properties of paramyxoviruses include infectivity, HL and cell fusion.

Evidence has accumulated in recent years supporting the concept that the fusing agent is the membrane of the virus envelope (Apostolov & Almeida, 1972; Hosaka, 1975, Homma *et al.* 1976). Agents that destroy the integrity of the envelope (lipid solvents, sonication), destroy the fusogenic properties of the virus but do not affect the functions of the polypeptides (Apostolov & Waterson, 1975).

The results presented in this paper show that the fusogenic properties of NDV can be effectively separated from the HA and neuraminidase functions by iodination. However, the morphology of the virion is intact also, and the antigenic properties of the virus membrane are preserved (Apostolov & Sawa, 1976). The experimental conditions described in the Materials and Methods section, and in the Results, can be simply and conveniently utilized for production of a virus

preparation with full HA and neuraminidase activity, but with complete inactivation of infectivity and HL.

HL is the most convenient indicator for the fusogenic capacity of a virus preparation. HL depends on the differential leakiness of the virus membrane after integration into the erythrocyte membrane (Apostolov & Damjanovic, 1973; Homma *et al.* 1976). Several methods for enhancement of the HL capacity of Sendai and NDV have been demonstrated (Apostolov & Damjanovic, 1973; Apostolov & Waterson, 1975). A new method described in this paper is the use of hypotonic solutions of saline as medium for HL testing (Fig. 3). The enhancement of HL obtained by this method is probably due to the differential sensitivity of the integrated virus membrane to osmotic pressure.

The selective inactivation of the fusogenic properties of the virus as measured by the HL is of considerable theoretical interest for membrane research. It is well known that iodine has a high affinity for the carbon–carbon double bond of the unsaturated fatty acids. Indeed, iodine number, i.e. the number of grams of iodine incorporated in fats after boiling is a convenient measure for unsaturated fatty acids in fats. However, more important biologically are the physical effects of saturation – the rise in the melting points of the constituent lipids (Harwood, 1962). It is therefore reasonable to postulate that iodination destroys the fusogenic properties of the virus by a change in the mobility of the lipid bilayer resulting from a change in the melting points, i.e. freezing, of the lipids. This concept is supported by the recent finding that catalytic hydrogenation of the unsaturated fatty acids leads to reduction in membrane fluidity in studies of artificial membrane systems (Chapman & Quinn, 1976). The support for this concept by chemical studies will be published elsewhere (Blenkharn & Apostolov, 1980).

In a morphological study of haemolysis by negative staining, it was postulated that the virus membrane and the cell membrane inter-react before integration of the virus membrane into the cell membrane (Apostolov & Almeida, 1972). Highly haemolytic virus does not lead to HL with iodinated erythrocytes, although the virus is effectively adsorbed and eluted from the same cells. It is likely that the iodination produces the same changes in the lipids of the erythrocyte membrane as those postulated for the virus membrane.

The influenza virus does not enter the cells by fusion, and does not produce HL (Bächi, 1970). The same conditions of iodination with selectively inactivated HL and infectivity of NDV also inactivate the infectivity of influenza without any effect on HA or elution. It is possible that the incorporation and uncoating of influenza involves an internal fusion step which could be inhibited by the iodination of the lipids of the virus membrane.

From a practical point of view, the inactivation of the infectivity of NDV and influenza by iodination of the membranes of their envelopes should be an advance on the present methods for production of inactivated vaccines. The chemical change is in the host-derived material, and in addition, these preparations could be more immunogenic. The saturation of the lipids could enhance their adjuvant effect. It is to be expected that iodination under the optimal conditions described could be used for infectivity inactivation of all enveloped and membrane-bound

viruses. This simple procedure should find use where safety is important in investigations in which virus infectivity is undesirable.

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