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THE EFFECT OF IODINATION ON THE HAEMOLYTIC PROPERTY AND THE FATTY ACIDS OF NEWCASTLE DISEASE VIRUS

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Summary

Treatment of Newcastle disease virus with iodine inhibits haemolysis and infectivity, but has no effect on haemagglutination. This is shown to be concurrent with the incorporation of iodine in the hydrocarbon chain of fatty acyl residues of the viral membrane lipid. It is concluded that iodine incorporation, by reducing membrane fluidity, is responsible for these biological phenomena.

Introduction

Newcastle disease virus is a fowl pathogen belonging to the paramyxovirus genus of the family Paramyxoviridae. Myxoviruses also include influenza A, B and C. The common characteristics of the family include maturation by budding from the host protoplasmic membrane and the presence of an ether-sensitive envelope. On the outside of this envelope are the projections (spikes) which are responsible for the attachment manifest in the haemagglutinating property and neuraminidase activity. The spikes are attached to the other constituent of the envelope, the viral (basal) membrane. The virus membrane consists of converted host cell membrane with intact glycolipid antigenic properties but total absence of host membrane proteins. In addition to the haemagglutinating and neuraminidase properties, paramyxoviruses also produce haemolysis and cell fusion. The haemolytic property is unique amongst the viruses. Although it has been shown that the protein spike is involved in haemolysis, cell fusion and infectivity [1], there is a solid body of evidence that the agent for all these properties is the virus membrane [2,3]. The virus membrane has fusogenic properties and is responsible for the entry of the virus into

host cells. Following fusion and integration into erythrocytes, the viral membrane breaks and leaks haemoglobin and also, by bridging with membranes of other cells, leads to erythrocyte fusion. In a recent paper [4] it was shown that iodination of Newcastle disease virus under defined conditions could lead to selective inhibition of haemolysis and loss of infectivity with no effect on haemagglutination. In this paper we present the result of investigations into the effect of iodination on the fatty acids of Newcastle disease virus.

Methods

Newcastle disease virus (Texas strain) was prepared as previously described [2] and further purified by isopycnic separation on a sucrose gradient using zonal ultracentrifugation [5]. The yield of virus was assessed by titration of haemagglutinating capacity. This was done in the cups of standard perspex serology trays using 0.25 ml vols. and 0.5% washed chick erythrocytes. The haemolytic activity of the virus was determined as previously described [6]. Iodination of Newcastle disease virus was carried out at 4 °C and 37 °C. In each case an iodine-free control was similarly processed. 1 vol. of freshly prepared Lugol's iodine (1% (w/v) I_2 in 2% (w/v) aqueous KI) was added to approx. $5 \cdot 10^4$ haemagglutinating units of virus in 5 ml water, together with 1 vol. of phosphate buffer (26.5 mM KH_2PO_4 plus 145.8 mM Na_2HPO_4/l, pH 7.5). These were incubated at 37 °C for 15 min or 4 °C for 30 min as appropriate. After incubation the reaction was stopped by the addition of an excess of sodium thiosulphate (5 ml 0.2 M $Na_2S_2O_3 \cdot 5\,H_2O$). The control virus was similarly treated except for the replacement of Lugol's iodine with an equivalent volume of distilled water. The virus was then pelleted by high-speed centrifugation (145 000 × *g* for 60 min at 4 °C), washed twice with distilled water and finally resuspended in 10 ml distilled water. An aliquot of each iodinated, twice washed virus was examined for haemolytic and haemagglutinating capacities as described in order that the biological effects of iodine treatment could be assessed.

Lipids were extracted by the method of Bligh and Dyer [7] incorporating 0.05% butylated hydroxytoluene as antioxidant to all solvents. Following extraction, qualitative separation of major lipid classes was performed by column chromatography on silicic acid [8]. A 1.5 × 10 cm column of heat-activated silicic acid was prepared in $CHCl_3$. The sample of mixed lipids was applied in 0.5 ml and neutral lipids eluted with 200 ml $CHCl_3$. Subsequently, glycolipids were eluted with 700 ml acetone and finally phospholipids eluted with 200 ml CH_3OH. Since it was anticipated that the glycolipid fraction may contain residual phospholipid, this fraction was reduced in volume under nitrogen and run again through the column, the resultant CH_3OH fraction being pooled with that collected earlier. Each fraction was reduced to dryness under nitrogen and heated to 100 °C for 15 min in 5 ml of methanolic NaOH (1.25 M NaOH in 50% (v/v) aqueous CH_3OH). The saponified material, containing free fatty acids and alkali-stable glycolipid was cooled and 5 ml saturated aqueous NaCl added, the pH being adjusted to approx. pH 2 with phosphoric acid. Lipids were extracted three times with 4 vols. $CHCl_3$/hexane (1 : 4) and reduced to dryness under nitrogen. To the dry residue was added

4 ml BCl_3-CH_3OH complex (Sigma Chemical Co.), and this was heated to 100°C for 5 min. Initial experimentation showed that this combination of alkaline hydrolysis and methanolysis liberated the major portion of fatty acyl residues as their methyl esters. The glycolipids of Newcastle disease virus, which could be expected to be most resistant to alkali treatment, have been shown to comprise solely of cerebrosides, although precise identification has not been accomplished [9]. Moscatelli [10] has studied the time relationship of cerebroside methanolysis with BF_3-CH_3OH. Whilst 60 min was suggested as optimum, 30-min treatment was found to give almost complete methanolysis. In our system, exposure of the viral glycolipid to BCl_3-CH_3OH for 5 min was found to give almost complete liberation of fatty acid methyl esters, whilst prolonged treatment (20—30 min) did not significantly improve recovery. Fatty acid methyl esters were recovered from the reaction mixture by adding 5 ml saturated aqueous NaCl and extracting four times with an equal volume of $CHCl_3$/hexane. The combined extracts were dried with anhydrous $MgSO_4$ and evaporated to 0.1 ml under nitrogen. The prepared methyl esters were separated by GLC. Analyses were performed using 1.5 m × 4 mm glass columns of 3% SE-30 on chromosorb W(HP) and 10% FFAP on acid-washed diatomite. For SE-30 a 20 min isothermal period at 140°C was followed by a programmed temperature increase at 2°C/min to 190°C, whilst for FFAP separation was performed isothermally at 145°C. In each case, the apparatus was a Pye Unicam 104 gas chromatogram with dual flame ionisation detectors. Nitrogen was used as carrier gas. Identification of fatty acids was achieved by retention time comparison and co-chromatography with authentic fatty acid methyl esters (Applied Science Labs. Inc. and Sigma Chemical Co.).

Subsequent investigations carried out in this laboratory [4] show the optimum conditions for lipid-iodine interaction. These conditions were found, by titration of haemagglutinin, to have least demonstrable effect on the viral proteins. These data were employed to assess the kinetics of iodine incorporation. To 1 volume of Newcastle disease virus was added 1 volume phosphate buffer (pH 5.8) and 1 volume Lugol's iodine. Incubation was in an ice bath. Aliquots were removed at various times for lipid analysis and assessment of residual haemolytic property by a standardised method [6].

Results

Percentage fatty acid composition of each lipid class was calculated, pre- and post-iodination of Newcastle disease virus. It was presumed that the significant reductions in unsaturated acids following iodination together with a corresponding rise in a related fully saturated component, was due to the incorporation of iodine in the fatty acid hydrocarbon chain. This assumption was supported by examination of pure fatty acid iodinated under conditions identical to those used for virus. Typically, the iodination of *cis,cis*-9,12-octadecedienoic acid ($C_{18:2}$) resulted in altered retention times. The majority of the iodinated product eluted co-chromatographically with octadecanoic acid ($C_{18:0}$). A small proportion of the total co-chromatographed with *cis*-9-octadecenoic acid ($C_{18:1}$). It was concluded that these retention time shifts correspond to iodine incorporation at the C = C double bonds of the

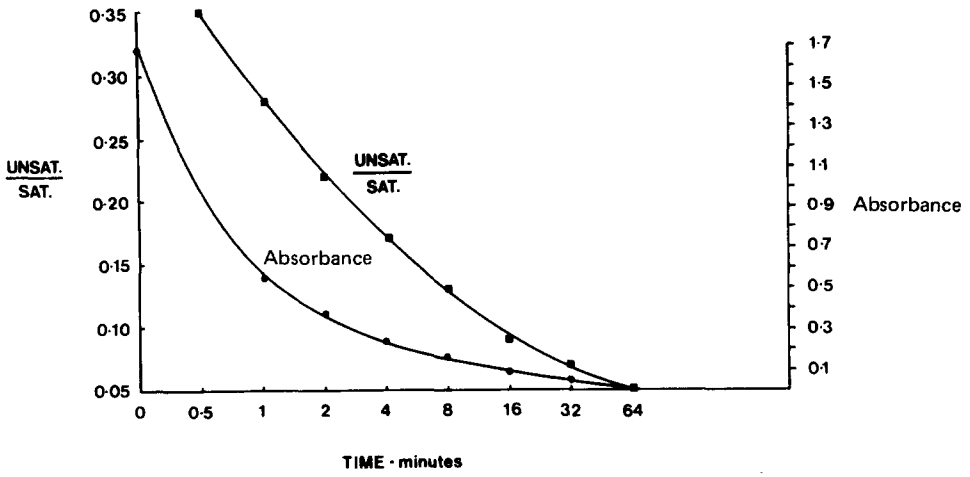

Fig. 1. The kinetics of iodination. Newcastle disease virus iodinated at pH 5.8 with ice bath incubation was assayed for haemolytic activity (expressed as absorbance against virus-free erythrocyte control) and for the loss, by iodine incorporation, of unsaturated fatty acids (unsaturated/saturated).

unsaturated fatty acid hydrocarbon chains. This indicates the products of the reaction to be 9,10,12,13-tetraiodooctadecanoic and 9,10-diiodooctadecanoic acids, respectively. This effect was not observed following iodine treatment of saturated and branched-chain fatty acids.

The data indicate that, for Newcastle disease virus, complete iodination was not attained using the conditions described (Table I). However, the partial iodination achieved was sufficient to impart the profound biological effects of loss of haemolysis and infectivity. Treatment with iodine at pH 7.5 resulted in approx. 50—75% reduction of fatty acyl residue unsaturation, the greater activity being at 37 °C.

TABLE I

THE SATURATION OF NEWCASTLE DISEASE VIRUS MEMBRANE ACYL RESIDUES BY LUGOL'S IODINE TREATMENT

	Neutral lipid	Phospholipid	Glycolipid
Untreated virus			
% saturated	74.3	66.1	67.3
% unsaturated	25.7	33.9	32.7
Following treatment with iodine at 4°C, pH 7.5			
% saturated	88.3	88.8	82.4
% unsaturated	11.7	11.2	17.6
Following treatment with iodine at 37°C, pH 7.5			
% saturated	93.8	90.3	95.5
% unsaturated	6.2	9.7	4.5

Discussion

Unsaturated fatty acids are found widely distributed in various membrane lipid complexes, imparting fluidity to that membrane [11]. The origin of viral lipids is known to be host cell membrane, although it has been shown that alteration of host lipid occurs on or before incorporation into the viral membrane [9]. In the pure state an increase in unsaturation of fatty acid is generally accompanied by an increase of fluidity and a relatively high chemical activity [12]. The former is perhaps best demonstrated by the melting point differences of various naturally occurring unsaturated fatty acids and the corresponding fully saturated form. $C_{18:2}$ and $C_{18:1}$ have melting points of −5 °C and 13 °C, respectively, whilst the corresponding fully saturated $C_{18:0}$ has a melting point of 69.6 °C [13–15].

The reactivity of unsaturated fatty acids with special regard to iodine has been much employed in the estimation of the degree of unsaturation (iodine number). Incorporation of iodine is primarily by addition to the double bonds of the fatty acid hydrocarbon chain yielding, under optimal conditions, a fully saturated iodoacid. The reactivity of the halogens is inversely proportional to their molecular weight. The rate of addition of iodine varies according to the relative positions of the C = C double bonds with respect to the carboxyl group and, in the case of polyunsaturated acids, to each other [16]. Therefore, the apparent absence of complete iodination of viral unsaturated fatty acids is not surprising for the mild iodination conditions employed.

Perusal of the literature shows no convincing data on the physical properties of iodo-fatty acids. However, for the bromo- and chloro-acids a significant rise in melting point occurs after halogenation towards, and often beyond, the melting point of the fully hydrogenated acid [14]. It is therefore highly probable that this effect would similarly occur with iodinated fatty acid.

The kinetics of the inhibition of haemolysis by iodine show a direct correlation with the incorporation of iodine in the unsaturated fatty acyl residues within the membrane lipids (Fig. 1). After 0.5 min treatment with iodine a large decrease in haemolytic activity occurred and a reduction in the concentration of unsaturated fatty acid (from 30.8% to 24.0%) occurred. Accounting for this reduction was the iodination of the predominant unsaturated fatty acids *cis*-9-hexadecenoic acid ($C_{16:1}$) and $C_{18:1}$. After increased exposure to iodine, further saturation of these compounds was noted and, by 2 min incubation, saturation of the other unsaturated acids detected in the virus membrane. $C_{18:2}$ and *cis*-9-icosenoic acid has occurred. That $C_{16:1}$ and $C_{18:1}$ were the first to become saturated by iodine presumably reflects their predominance as the major unsaturated components rather than increased reactivity with regard to iodine. From this data we suggest that the simple and rapid incorporation of iodine results in a reduction of membrane fluidity and hence fusibility brought about by the loss of highly fluidic unsaturated fatty acyl residues. This is supported by the reduction of viral membrane lipid-associated fusogenic properties following iodination with complete preservation of the glycoprotein-associated properties of the virus envelope (haemagglutination and neuraminidase) [4]. Indeed, that a reduction of the concentrations of $C_{16:1}$ and $C_{18:1}$ (from 9.8% and 9.2% to 6.2% and 5.9%, respectively) within 0.5 min

iodine exposure corresponds to a 42% reduction in haemolytic activity demonstrates the crucial role of these unsaturated fatty acyl residues in maintaining fluidity.

Chapman and Quinn [11] discuss the modulation of membrane fluidity by the catalytic hydrogenation of phospholipid fatty acyl residues. Their data show the importance of unsaturated fatty acids in membrane fluidity yet the system has not been successfully applied to intact biological membranes. Inhibition of haemolysis by the iodination of Newcastle disease virus represents a new biological system for the study of biological membrane fluidity. The degree of unsaturation of fatty acids imparting membrane fluidity can be easily and conveniently assessed by this method employing haemolysis as a rapid and convenient biological indicator system.

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