*EFFECT OF PERIODATE OXIDATION ON HEMAGGLUTINATING AND
ANTIBODY-PRODUCING CAPACITIES OF CERTAIN
ENTEROVIRUSES AND REOVIRUSES**

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Experiments in this laboratory have been concerned with the nature of the functional units of the capsids of reoviruses and enteroviruses. Hemagglutinating capacity of reoviruses types 1, 2, and 3 and of coxsackie virus group B, type 5, was destroyed after incubations with dilute potassium periodate.¹ Moreover, the infectivity of reovirus type 2 was also tested after similar periodate treatment, and was found to be markedly reduced. Hemagglutination (HA) by reoviruses and a number of enteroviruses was inhibited by digestions with β -glucosidase and incubations with certain simple sugars.^{2, 3} The patterns and quantities of sugars necessary for these inhibition reactions were distinct for each virus. Smaller aldoses and aldehydes also inhibited these HA reactions but their polyhydroxyl counterparts did not. Radioactive studies with carbon-14-labeled sugars, aldoses, and aldehydes indicated that compounds which avidly inhibited these HA reactions attached to erythrocytes, but similar nonactive compounds did not. These active sugars, aldoses, and aldehydes did not attach to purified preparations of the viruses.⁴ These data have suggested to us that the capsids of reoviruses and enteroviruses may contain previously unsuspected glycoproteins, and that virus-erythrocyte union may involve a free carbonyl group on oligosaccharide side chains of the viruses.

Here we report that periodate oxidation simultaneously affects HA by a number of enteroviruses and reoviruses, reduces their infectivity, and alters these viruses in such a manner as to inhibit markedly their abilities to produce homotypic antibodies.

Materials and Methods.—Viruses: Suspensions of rhesus kidney cells were obtained from Parke-Davis and Co. through the courtesy of Dr. W. Richtsel, and grown in 32-oz bottles with Eagle's medium and 10% calf serum. These cultures were inoculated with stocks of echoviruses types 3 (NIH-1), 7 (NIH-1), 11 (1867), 12 (NIH-1), and 19 (Burke); coxsackie viruses group B, types 1 (201468) and 5 (201482); and poliovirus type 1 (Brunhilde). When cytopathic effects were complete, cells which remained attached to glass were removed with a rubber policeman, and the virus-cell suspension was transferred to a 250-ml centrifuge bottle. It was then rapidly frozen and thawed three times in acetone and dry ice to lyse cell membranes and release intracellular virus.

Reoviruses types 1 (Lang), 2 (988), and 3 (Abney) were produced in L-60 cells by methods previously described.⁵ L-cell debris containing these viruses was treated with 100 μ g/ml of sodium deoxycholate for 2 hr (4°C) to release reoviruses which are attached to lipids of the sediment.

Concentration and purification of viruses: Supernates from coxsackie, echo-, and polioviruses were treated 2 hr with deoxyribonuclease (50 μ g/ml), ribonuclease 50 μ g/ml, and trypsin (2%) at 4°C, and the sediment was separated by centrifugation for 10 min at 1200 *g* (4°C). These final supernatant fluids were centrifuged in the Spinco model L ultracentrifuge at 27,000 rpm (19°F) for 5 hr. Pellets containing these viruses were taken up in 2–3 ml of Eagle's medium. These concentrated suspensions of coxsackie and echoviruses were further purified by ultracentrifugation in 15–65% sucrose gradients for 14 hr in the SW-25 swinging bucket rotor at 23,000 rpm (–8°C). Fractions were collected in 50-drop aliquots by puncturing the bottom of the cellulose

tubes. HA titers of coxsackie virus group B, type 1, and the echoviruses were done to locate the fractions of each gradient containing these viruses.⁴ Tubes containing the highest HIA titers were pooled, and sucrose was removed by dialysis against Earle's saline (BSS).

The concentrated suspension of poliovirus type 1 was purified by column chromatography with Sephadex G-200 in BSS. The eluate was collected in 1-ml samples, and virus recognized as the first peak in optical density (260 m μ).

After incubation with sodium deoxycholate, reoviruses were concentrated and further purified by ultracentrifugation, first in a #30 angle rotor (5 hr, -8°C) and then on sucrose gradients (5 hr, -8°C) as outlined for coxsackie and echoviruses.

Hemagglutination: HA tests were performed in 13 $\times$ 100-mm glass test tubes with virus suspensions diluted in BSS (23°C). Erythrocytes obtained from umbilical cords at the time of delivery were used with coxsackie viruses group B, types 1 and 5.

Infectivity titrations: Logarithmic dilutions in Eagle's medium were done, and 0.1 ml of each virus dilution was inoculated into 2-3 rhesus kidney tube cultures. Tubes were incubated on stationary racks (37°C) for 1 week, and cytopathic effects observed daily. End points were estimated by the method of Reed-Muench.⁶

Periodate oxidations: Concentrated and purified preparations of viruses were divided into equal portions, and treated in parallel with either 0.04 *M* potassium metaperiodate (KIO_4) in BSS (pH 5.6), BSS (pH 7.4), 0.04 *M* potassium chloride (KCl) in BSS (pH 7.2), or 1% formaldehyde (HCHO) in BSS (pH 7.2). All such incubations were done at 4°C in the dark. Times of incubations varied from 10 to 48 hr. Periodate oxidations were performed: (1) in 20-ml screw-capped tubes under constant agitation with a magnetic stirrer, or (2) by dialysis of the viruses with 10-20 times their volumes of 0.04 *M* of potassium metaperiodate in BSS. With the latter method the dialyzing fluid was exchanged at 6 hr, and again at 24 hr if the incubation was continued beyond this period.

Serology: Hemagglutinating-inhibiting antibody (HIA) was assayed in rabbit sera which had been absorbed with kaolin.⁷

Immunoinactivation tests were performed to measure neutralizing antibody (NA) by a modification of the method of Gard.⁸ This procedure was used because of its increased sensitivity in the detection of small amounts of type-specific antibody. Serum titers were calculated by the method of Reed and Muench.⁶

Procedures with rabbits: Young male rabbits weighing 3-5 lb were obtained. Serum was collected by arterial puncture from the ear, and tested for HIA and NA to the several viruses to be studied. Only rabbits with HIA and NA titers of less than 20 and 5, respectively, were subsequently used. Such rabbits were inoculated intramuscularly with periodate-, BSS-, or formaldehyde-treated preparations of concentrated and purified viruses. In most experiments sera were obtained 14 days later, at which time rabbits were reinoculated. All animals were sacrificed by exsanguination 28 days after the initial virus injections.

Results.—Concentration and purification of viruses: Representative data from preparative procedures are shown in Figure 1.

Effect of periodate on erythrocytes: Potassium periodate at a concentration of 0.04 *M* caused immediate hemolysis, but 0.02 *M* caused no red-cell destruction within 4 hr, and with 0.005 *M* none was observed at 8 hr. In the experiments reported here, erythrocytes were never in contact with concentrations of periodate greater than 0.002 *M*, and the time of incubation was never greater than 1 hr. These conditions do not alter receptors on erythrocytes necessary for HA of enteroviruses or reoviruses.^{1, 9, 10} The pH of the periodate, 5.6, also has no effect on HA.¹¹

Effect of periodate on hemagglutinating capacity of enteroviruses and reoviruses: Purified and concentrated preparations of echoviruses types 3, 7, 11, 12, and 19; coxsackie virus group B, type 1; and reoviruses types 1, 2, and 3 were diluted with BSS to a concentration of 160 HA/0.1 ml. A 2-ml vol of each of these viruses was incubated with similar volumes of 0.04 *M* KIO_4 in BSS, BSS, and in some cases with 0.04 *M* KCl in BSS in screw-capped tubes on a magnetic stirrer at 4°C .

FIG. 1.—Fractions A (5–9) and B (12–16) were used in continuing experiments. (A) Column chromatography of poliovirus type 1, on Sephadex G-200 in BSS. (B) Sucrose gradient ultracentrifugation of echovirus type 12.

At intervals thereafter, HA titrations were done (Table 1 and Fig. 2). Hemagglutinating capacities of all of these viruses fell progressively with periodate oxidations, but titers were not significantly altered over these periods by an isomolar solution of KCl, or by Earle's saline. Total absence of HA by enteroviruses was noted within 12 hr, while this occurred somewhat more slowly with reoviruses. Once these hemagglutinins were destroyed, continued treatments with periodate or dialysis with BSS for up to 12 hr did not restore the hemagglutinating capacities of any of these viruses. These data clearly show that gentle periodate oxidation reacted with susceptible substrates on the viruses, and under these conditions did not affect the erythrocyte.

Effect of periodate on production of specific neutralizing and hemagglutinating-inhibiting antibodies in rabbits: Purified and concentrated preparations of echovirus type 12; coxsackie virus group B, type 1; poliovirus type 1; and reovirus type 2

FIG. 2.—Effect of periodate on hemagglutination. (A) Enteroviruses; (B) reoviruses.

TABLE 1
EFFECT OF PERIODATE ON HEMAGGLUTINATION TITERS (HA) OF ENTEROVIRUSES AND REOVIRUSES

Viruses	Reagent	Time of Incubation (Hr)													
		0	1	2	3	4	6	8	12	24	48	72	96	120	144
Coxsackie virus B, type 1	BSS*	80†	80	80					80						
	KCl	80	80	80					80						
	KIO ₄	80	<10	<2					<2						
Echoviruses, type 3	BSS	80	80	80	40	40	40	40							
	KIO ₄	80	20	8	4	4	<2	<2							
	BSS	80	80	80	40	40			40	40					
	KCl	80	40	40	40				40	40					
	KIO ₄	80	20	10	8	4	4	4	<2	<2					
	BSS	80	80	40											
	KIO ₄	80	10	<2	80	80	40	40	20						
	BSS	80	80	80	80	80	40	40	20						
	KCl	80	80	40	20	20	10	8	<2						
	KIO ₄	80	80	80	80	80	<2	<2							
	BSS	80	20	8	4										
	KIO ₄	80	20												
Reoviruses, type 1	BSS	80	80	80	80	80	80	80	80	80	80	40	20		
	KCl	80	80	80	80	80	80	80	80	80	80	20	20		
	KIO ₄	80	40	40	40	40	40	40	40	40	10	<2	<2		
	BSS	80	80	80	80	80	80	80	80	80	80				
	KCl	80	80	80	80	80	80	80	80	80	80				
	KIO ₄	80	80	80	80	80	80	80	80	80	<2				
	BSS	80	80	80	80	80	80	80	80	80	80	40	40	40	
	KIO ₄	80	20	8	4	<2									
	BSS	80	80	80	80	80	80	80	80	80	80	40	40	40	
	KCl	80	80	80	80	80	80	80	80	80	80	40	40	40	
	KIO ₄	80	80	80	80	80	80	80	80	80	80	40	40	40	
	BSS	80	80	80	80	80	80	80	80	80	80	40	40	40	
KIO ₄	80	80	40	40	40	40	40	40	40	8	2	<2	<2		
type 2	BSS	80	80	80	80	80	80	80	80	80	80	80	80	80	
	KCl	80	80	80	80	80	80	80	80	80	80	80	80	80	
	KIO ₄	80	80	80	80	80	80	80	80	80	80	80	80	80	
type 3	BSS	80	80	80	80	80	80	80	80	80	80	80	80	80	
	KCl	80	80	80	80	80	80	80	80	80	80	80	80	80	
	KIO ₄	80	80	80	80	80	80	80	80	80	80	80	80	80	

* BSS, Earle's saline; KCl, potassium chloride, 0.04 M; KIO₄, potassium periodate, 0.04 M. (Both KCl and KIO₄ solutions were prepared in BSS.)

† HA per 0.1 ml, expressed as reciprocal.

TABLE 2
EFFECT OF PERIODATE ON ANTIBODY-PRODUCING CAPACITY OF CERTAIN ENTEROVIRUSES
AND REOVIRUS TYPE 2

Rabbit no.	Virus	Reagent	Inoculations after Incubations			Antibody Response†				
			Time (hr)	Initial	Titers (log)	Final	14 Days HIA‡	28 Days HIA	NA‡	
	Enteroviruses:									
1	Poliovirus,	BSS	48	*	7.2	TCD ₅₀ †		>640		
2	type 1	BSS	48		7.2	TCD ₅₀		640		
3	(Brunhilde)	BSS	48		7.2	TCD ₅₀		>640		
4		KIO ₄	48		5.2	TCD ₅₀		10		
5		KIO ₄	48		5.2	TCD ₅₀		<5		
6		KIO ₄	48		5.2	TCD ₅₀		<5		
7	Coxsackie virus,	BSS	10, 24§	3.5 HA units	4	HA units	<20	80	160	
8	type B-1	BSS	10, 24	3.5	"	"	<20	20	113	
9	(201468)	BSS	10, 24	3.5	"	"	<20	160	640	
10		KIO ₄	10, 24	<2.3	"	<2.8	"	<20	40	<20
11		KIO ₄	10, 24	<2.3	"	<2.8	"	<20	40	<20
12		KIO ₄	10, 24	<2.3	"	<2.8	"	<20	40	40
13	Echovirus,	BSS	24	5.8	"	"		640	N.D.	N.D.
14	type 12	BSS	24	5.8	"	5.3	"	320	80	160
15	(NIH)	BSS	24	5.8	"	5.3	"	640	N.D.	N.D.
16		KIO ₄	24	<2.9	"	<2.9	"	80	N.D.	N.D.
17		KIO ₄	24	<2.9	"	<2.9	"	<20	20	<5
18	Reovirus,	BSS	48	4.9	"	"		1280	2560	320
19	type 2	BSS	48	4.9	"	5	"	2560	2560	56
20		BSS	48	4.9	"	5	"	1280	5120	320
21		KIO ₄	48	<2.4	"	<2.9	"	<20	40	<5
22		KIO ₄	48	<2.4	"	<2.9	"	<20	20	<5

N.D., test not performed because of apparently unrelated death of rabbit.

* Initial inocula of rabbits (nos. 1-6) were similar before BSS or KIO₄ incubations, but infectivity titrations were not successful because of overheating of incubator in laboratory.

† TCD₅₀, that amount of virus causing cytopathic effects in half of the inoculated tissue culture tubes.

‡ Antibody titers expressed as reciprocal; HIA, hemagglutinating inhibiting antibody; NA, neutralizing antibody as determined by the immunoinactivation procedure.

§ 10 = Incubation time of initial inoculum of coxsackie virus type B-1; 24 = incubation time of final inoculum 14 days later.

TABLE 3
COMPARISON OF FORMALDEHYDE, PERIODATE, AND EARLE'S SALINE ON
ANTIBODY-PRODUCING CAPACITY OF ECHOVIRUS TYPE 12

Rabbit no.	Reagent	Time (hr)	Inoculations after Incubations			Antibody Titer (14 days)	
			HA units	Titers (log)	TCD ₅₀	HIA	NA
23	BSS	48	4.8	8.7		20	>160
24	BSS	48	4.8	8.7		40	25
25	HCHO	24*	3.0	<2.2		160	32
26	HCHO	24	3.0	<2.2		20	>160
27	KIO ₄	48	<2.2	<2.2		<20	<5
28	KIO ₄	48	<2.2	<2.2		<20	<5

* Formaldehyde-treated virus was dialyzed for 24 hr with BSS before inoculation into rabbits.

were placed in equal initial amounts into dialyzing membranes for varying periods of contact with BSS or 0.04 M KIO₄. Incubations were continued until HA titers of periodate-treated viruses were <10 HA units/0.1 ml. These times of incubation varied from 10 hr for coxsackie virus group B type 1 to 48 hr for reovirus type 2. Poliovirus type 2 which does not cause HA, was dialyzed against KIO₄ for 48 hr (Table 2). The final quantities of these viruses after incubations that were inoculated into rabbits are recorded in Tables 2 and 3 and Figure 3.

The results of NA and HIA tests 2 and 4 weeks after inoculations of rabbits indicate that in all cases higher antibody titers were produced by animals injected with BSS-treated viruses. Titers were most striking at 14 days, but were also obvious at 28 days. Thus, with saline-treated virus, high HIA titers to reovirus

FIG. 3.—Effect of periodate on antibody-producing capacities in rabbits of certain enteroviruses and reovirus type 2. Each symbol represents one rabbit. ∇ , BSS-treated poliovirus type 1; $\blacktriangledown$, KIO_4 -treated poliovirus type 1; $\triangle$, BSS-treated coxsackie virus B type 1; $\blacktriangle$, KIO_4 -treated coxsackie virus B type 1; $\square$, BSS-treated echovirus type 12; $\blacksquare$, KIO_4 -treated echovirus type 12; $\circ$, BSS-treated reovirus type 2; $\bullet$, KIO_4 -treated reovirus type 2.

type 2 were noted at 2 and 4 weeks; no HIA (<20) was recorded in rabbits inoculated with KIO_4 -treated virus at 2 weeks. At 4 weeks these latter rabbits showed low HIA titers of 20 and 40, while NA titers were <5 . The low titers of antibody in rabbits injected with KIO_4 -treated viruses indicate that most, but not all, of the specific antigenicity was destroyed by the periodate. Preliminary experiments showed that if rabbits had pre-existing homologous HIA or NA, periodate-treated virus would induce marked antibody responses. Thus, the immune responses to the second injection of periodate-treated virus (e.g., Table 2, see coxsackie virus B-1) may indicate that considerable unaltered virus remained even though the amount may not have been sufficient to cause HA. For instance, it is known that approximately 3,000–10,000 TCD_{50} of reovirus type 2 and 10,000 TCD_{50} of echovirus type 12 equal one HA unit of these viruses; likewise after periodate treatment of poliovirus type 1, although only 1 per cent of the initial virus infectivity remained. This represented 100,000 TCD_{50} .

All rabbit sera were tested by HIA tests to echoviruses types 3, 6, 7, 11, 12, and 19; coxsackie viruses types B-1 and B-5; and reoviruses types 1, 2, and 3 to determine if any cross-reactions occurred, or if periodate might change serologic specificities, possibly producing no homologous antibody, but inducing heterologous antibodies to related viruses. No such cross-reacting antibody was found to any of these former viruses in sera from rabbits inoculated with BSS- or KIO_4 -treated enteroviruses. However, rabbits inoculated with preparations of reovirus type 2, receiving BSS or KIO_4 treatments, developed the usual heterologous HIA to reoviruses types 1 and 3.

It still seemed possible that the difference in HIA and NA responses may have been due to asymptomatic multiplication of BSS-treated viruses, thus accounting for a larger antigenic mass. Formaldehyde was, therefore, used to produce antigenic, yet noninfective virus. Echovirus type 12 was produced, concentrated, and purified as outlined under *Methods*. Equal aliquots were dialyzed with Earle's saline and 0.04 M potassium metaperiodate for 48 hr. The third sample was dialyzed against 1 per cent HCHO for 24 hr, and with BSS for a second similar interval. Hemagglutination and infectivity titrations were done on each sample after 48 hr, and two rabbits were injected with each of the three virus suspensions.

After HCHO and KIO₄ dialysis, infectivity was destroyed. Although formaldehyde treatment decreased HA titers, these were at least five times those of similar samples incubated with periodate. However, after 14 days both HCHO- and BSS-treated viruses induced HIA and NA in rabbits, while again KIO₄-treated echovirus type 12 did not (Table 3). These data indicate the asymptomatic multiplication of virus was not necessary for antibody production in rabbits.

Discussion.—Periodate reacts quantitatively with α -glycols, α -hydroxyaldehydes, α -ketols, α -ketoaldehydes, and α -diketones to split carbon-carbon bonds. One mole of periodate (IO₄⁻) is reduced to iodate (IO₃⁻) for each C—C bond which is cleaved. Oxidation of 1 mole of a nonsubstituted hexose reduces 5 moles of IO₄⁻ and produces 5 moles of formic acid and 1 mole of formaldehyde. The rate of this periodic acid oxidation varies with the particular side chain on the C—C bond being split, the solvent, pH of the solution, temperature of the reaction, the concentration of the periodate, and exposure to light. Under the conditions of these experiments, that is, at 4°C, 0.04 M KIO₄, pH 5.6, and protected from light, gentle oxidation occurs without overoxidation or further degradation of the substrate by the IO₃⁻ produced.¹²⁻¹⁴ It seems unlikely, under the conditions described here, that amino acids such as serine or threonine with a hydroxyl group adjacent to an amino group were substrates for these oxidations.

Influenza viruses¹⁵⁻¹⁷ and several bacteria, including species of *Salmonella*,¹⁸ *Escherichia coli*,¹⁹ and *Streptococcus pyogenes*,²⁰ contain carbohydrates on their surfaces which are susceptible to periodate oxidations. As a result, agglutinations and other reactions requiring these antigens and their specific antibodies have been reduced or destroyed, suggesting that these moieties contain the antigenic-determinant groups. Periodate susceptibilities have also been suggested for reoviruses,^{1, 9, 21} polioviruses,²¹ and echoviruses.^{9, 22} In addition, more recently, Rueckert has analyzed the proteins of ME virus, a picornavirus of the Columbia SK group, and found small amounts of a hexoseamine-like material.²³

Here, gentle periodate oxidations have been performed on a number of enteroviruses and reoviruses. Following these reactions, the hemagglutinating, antibody-forming capacities, and, in a few instances, infectivity of these viruses have been studied. All of these functions have been concomitantly destroyed or markedly reduced.

The different rates at which periodate-inhibited HA (Table 1 and Fig. 1) may reflect the number of accessible polysaccharides or their configurations on virus capsids. These data suggest that substrates, in all probability carbohydrates, which are removed by periodate treatments, determine or are necessary for HA, infectivity, and serologic specificity of enteroviruses and reoviruses. Although it is possible that infectivity was lost on account of a reaction with the ribose of the ribonucleic acid cores of these viruses, oxidation at the surfaces of these viruses seems more likely. Moreover, it is well established that viral nucleic acid is not necessary for HA or antibody formation by enteroviruses, so that nucleic acid oxidations by periodate would not be a sufficient explanation. It seems likely that oligosaccharides on the capsids of these viruses were indeed the substrates utilized by periodate. It is of interest to note that these data would suggest that poliovirus which does not cause HA also contains glycoproteins on its capsid, for its infectivity and specific antigenicity were removed by periodate treatments. Indeed, these viruses which were altered in their antigenicity by periodate may have induced

other nonspecific antibodies, but other methods using these altered antigens in complement-fixation or precipitin procedures would have to be used to detect these.

Certainly the chemical demonstration of sugars from purified preparations of these viruses is necessary before the hypothesis offered can be fully accepted, but (1) inhibition of HA by these viruses by simple monosaccharides, (2) inhibition of HA by β -glucosidase, (3) attachment of inhibiting sugars to red cells, but not viruses, and (4) loss of HA and antigenicity after gentle periodate oxidations make it highly probable that there are oligosaccharides on the proteins of enteroviruses and reoviruses by which attachment to red blood cells and, perhaps, even tissue-susceptible cells occurs, and which further, as with blood group substances and some bacteria, determine serologic specificity.

Summary.—Gentle periodate oxidations of purified preparations of several echoviruses; reoviruses; coxsackie virus group B, type 1; and poliovirus type 1 were performed. These altered viruses lost their hemagglutinating capacity, were less or noninfective, and produced few or no homotypic-neutralizing or hemagglutinating-inhibiting antibodies. These experiments further support the concept that there are sugars on the capsids of these viruses which are important for cellular attachment to either erythrocytes or susceptible cells, and that these sugars are the antigenic determinants of these viruses. Definitive confirmation, however, requires biochemical analysis.

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