Rubella virus serology: detection of residual lipoprotein inhibitors of haemagglutination using sensitive indicator arboviruses

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SUMMARY Immune and non-immune rubella sera were examined in the haemagglutination inhibition (HAI) test to evaluate the removal of lipoprotein non-specific inhibitors (NSI) of haemagglutination after kaolin and manganous chloride/heparin treatments. The sera were titrated fresh or after lipoprotein deterioration brought about by ageing the samples at 4°C for various periods of time and by freezing at -20°C with subsequent thawing. Deterioration was seen as altered electrophoretic mobility while the lipoproteins in treated sera were detected by indicator arboviruses whose haemagglutination is known to be strongly inhibited by the native macromolecules. After both treatments, notably manganous chloride/heparin, residual NSI activity was detected in deteriorated samples, particularly with group B arboviruses such as Japanese encephalitis and west Nile viruses but generally less so or not at all with the group A arboviruses employed. Absolutely fresh sera are considered highly desirable for rubella virus HAI assay, and it is suggested that the efficiency of lipoprotein NSI removal regardless of treatment protocol could be monitored in parallel HAI tests using carefully chosen indicator arboviruses. This could be done in conjunction with density gradient centrifugation of doubtful sera should ultracentrifuging facilities be available. The suitability of the monitoring procedure would be dependent to some extent on whether certain arboviruses are known to be endemic in a particular area.

The haemagglutination inhibition (HAI) test has found ready application diagnostically for detecting antibody arising as a consequence of rubella virus infection. There occur in serum non-antibody substances or non-specific inhibitors (NSI) which also inhibit haemagglutination the removal of which is essential for determining specific antibody levels. Rubella virus NSI activity has recently been shown to reside in the lipoprotein fraction, notably low density (LDL) and very low density lipoproteins (VLDL), and only weakly or not at all with high density lipoprotein (HDL) (Shortridge and Ho, 1974).

Kaolin and manganous chloride/heparin have been frequently used to remove lipoprotein NSIs although they do suffer the disadvantage that they may remove some antibody, particularly IgM (Monath *et al.*, 1970; Schmidt *et al.*, 1971). However,

some recent studies on kaolin-treated serum suggest that lipoprotein, particularly LDL, may not be completely removed and the ensuing inhibitory activity may be confused as specific antibody (Haukenes, 1973).

My interest in this topic is twofold. First, I have noticed that over a period of months the apparent specific antibody titre for rubella virus continued to rise although the same methods and reagents were always employed for NSI removal. This phenomenon was more noticeable if the sera had been previously freeze-thawed, suggesting that the conditions under which the sera were stored or held were important for the efficiency of NSI removal. Secondly, an earlier independent study on the susceptibility of two representative togaviruses to inhibition of haemagglutination by lipoproteins (Shortridge and Ho, 1974), since extended to cover 23 different arboviruses from five antigenic groups, has shown that not only are all haemagglutinating togaviruses

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inhibited by the lipoprotein subfractions but they differ widely in the extent to which they are inhibited (Shortridge and Ho, 1976). For example, rubella virus which is least inhibited and some arboviruses such as Ntaya, a group B arbovirus, differ by as much as 50-fold in the extent of their inhibition of haemagglutination.

Thus it is an obvious possibility that very sensitive arboviruses might find application as indicators for monitoring the efficiency of procedures used to remove lipoprotein NSIs from sera during rubella antibody assay. With the introduction of rubella vaccination programmes and the awareness of rubella virus as a causative agent of congenital abnormalities, the services of the diagnostic virus laboratory are becoming increasingly important for detecting rubella virus antibody. Against the background of the above comments low or even moderate levels of antibody may be of questionable significance. This communication reports findings on the effect of ageing of serum on the efficiency of lipoprotein NSI removal and shows how various arboviruses may be used to detect lipoproteins that might otherwise be interpreted as specific rubella virus antibody.

Material and methods

SERA

Laboratory diagnosis of rubella virus infection in Hong Kong is requested infrequently. Sera for this study were obtained from two sources. The first comprised samples submitted to the Department of Pathology, University of Hong Kong for routine biochemical examination and were derived from donors resident in the central urban area of Hong Kong. Although Japanese encephalitis (JE) occurs in Hong Kong, serological studies indicate that infection appears to be confined to the rural districts rather than to the highly populated urban area (Shortridge, unpublished data). The serum samples used are unlikely to contain arbovirus antibody although the possibility cannot be completely excluded. On receipt these fresh sera were divided into two portions, one held at 4°C and the other at -20°C before assay three days later. Sera from the second source were generously provided by Dr P. Higgins, Virus Reference Laboratory, Central Public Health Laboratory, London and comprised portions of samples taken from women in southern England which were sent to the laboratory for serological evaluation of suspected rubella virus infection. Although it was not ascertained whether these women had travelled to arbovirus endemic areas, the possibility that their sera might contain arbovirus antibody would seem remote.

VIRUS ANTIGENS

Commercial rubella antigen was purchased from Flow Laboratories Inc, Rockville, USA and diluted as required in HSAG buffer, pH 6.2 (0.6% HEPES, 0.8% NaCl, 0.01% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1% bovine albumin Fraction V, 0.003% gelatin) as described by Liebhaver (1970). Lyophilised acetone/sucrose or acetone/ether antigen extracts of the arboviruses listed in Tables 1 and 2 were generously provided by Drs B. Johnson (London School of Hygiene and Tropical Medicine, England), R. Shope (Yale Arbovirus Research Unit, USA), and A. Oya (National Institute of Health, Japan). These antigens were reconstituted with either distilled water or borate saline, pH 9.0, containing 0.4% bovine serum albumin (BSBA) and then diluted as required in BSBA.

RED CELLS

Trypsinised human group O erythrocytes were used in preference to gander cells in rubella and arbovirus titrations as they are usually more sensitive to agglutination by these viruses and are able to circumvent the need for adsorbing test sera for removing natural agglutinins (Shortridge and Hu, 1974; 1976). Red blood cells were prepared as a 0.5% (vol/vol) suspension in Tris-saline (0.15 M NaCl in 0.01 M tris (hydroxymethyl) aminomethane-hydrochloride, pH 7.5) and then treated with 1:250 trypsin (Difco Laboratories, Detroit, USA) at a concentration of 1 mg/ml for one hour as reported (Shortridge and Hu, 1974). The trypsinised red cells for rubella titrations were resuspended at a concentration of 0.5% (vol/vol) in HSAG and those required for arbovirus titrations in 0.2 M phosphate buffer at the optimal pH which ranged between 5.85 and 6.2 (Shortridge and Hu, 1976).

HAEMAGGLUTINATION INHIBITION (HAI) TESTS

These were performed as described by Clarke and Casals (1958) and Liebhaver (1970) for arboviruses and rubella virus, respectively, with volumes appropriately reduced to 100 μl final volume for micro-trays. Doubling dilutions of serum in 25 μl volumes were challenged with four agglutinating doses of virus in 25 μl . In each case 50 μl of red cell suspension was added and 50% end-points were read.

SERUM TREATMENT

Kaolin and manganous chloride/heparin (Mn/hep) were used to remove NSI from sera exactly as described by Plotkin (1969), resulting in 10- and 8-fold dilutions of the sera, respectively. Immune rubella sera from London were similarly treated with kaolin but to a 1:8 final dilution (Dr H. G. S.

Murray, Standards Laboratory, Central Public Health Laboratory, personal communication). When required, the treated samples were concentrated back to their original volume by the addition of 1 g Lyphogel^R (Hawksley and Sons Ltd, Lancing, Sussex) for each 5 ml liquid to be absorbed. Concentration was achieved without any apparent loss of inhibitory potency using JE virus for monitoring HAI activity.

LIPOPROTEINS

The serum lipoprotein pattern was evaluated by electrophoresis using precast agarose slides (Bio-Rad Laboratories, Richmond, Calif, USA) according to the manufacturer's instructions (Bio-Rad Laboratories Instruction Manual, Bio-Gram A Lipoprotein Profile BGA-03) and barbitone-acetate, pH 8.6, I = 0.05 as buffer. On completion of electrophoresis the slides were stained with Fat Red 7B.

DENSITY GRADIENT CENTRIFUGATION

This was carried out in linear sucrose gradients (35%-7% w/v in Tris-saline buffer) and the gradients were centrifuged in 5 ml swinging bucket rotors at $200\,000 \times g$ for 7 hours at 4°C. Test sera were layered onto two gradients and a serum marker in the form of a rabbit antiserum prepared against A/Asia/57 (H2N2) influenza virus containing 7S IgG, and 12S and 18S non-specific inhibitors of influenza virus haemagglutination (Pepper, 1968) onto the third. Gradient fractions were titrated in HAI tests as indicated earlier and those of the marker serum were titrated against A/Asia/57 influenza virus as reported by Biddle and Shortridge (1967) with volumes appropriately reduced for microtrays. In the case of the rubella immune sera from London, rubella virus specific IgG and IgM were detected by centrifuging samples through 37.5%-12.5% sucrose density gradients in PBS and titrating the resulting fractions in the HAI test (Murray, personal communication).

Results

INHIBITORY BEHAVIOUR OF RANDOM FRESH SERA

Initial studies on random local sera employed rubella and JE viruses and showed that the latter was more sensitive to inhibition (by lipoprotein) in untreated sera (Table 1) in accord with previous observations using these two viruses (Shortridge and Ho, 1974). Treatment of the sera with kaolin and $MnCl_2$ /heparin to remove lipoprotein NSI caused a marked drop in HAI titre. In the case of rubella virus, HAI titres were generally higher after $MnCl_2$ /heparin treatment and this was readily

apparent when the sera had been stored at -20°C before titration. With some sera, eg. No. 199, there was a 10-fold difference in residual activity. Thus, although storage at +4°C and -20°C caused no significant alteration in the gross HAI titres of control untreated sera, subsequent treatment with $MnCl_2$ /heparin to remove NSI appeared less effective after storage at -20°C. In contrast, the effectiveness of kaolin treatment was not significantly altered.

Epidemiological considerations suggested that these sera are unlikely to contain arbovirus antibody as indicated in Material and methods. Titration of the treated sera with JE virus showed that a number had residual HAI activity, suggesting that the lipoprotein inhibitors had not been completely removed. The greater incidence and higher HAI titres observed with the $MnCl_2$ /heparin treated sera again suggested that kaolin was more effective in removing these NSIs.

Arboviruses vary considerably in their susceptibility to inhibition by lipoprotein (Shortridge and Ho, 1976). In order to investigate further the present findings, seven sera were assayed for HAI activity after the two treatments using representative arboviruses to hand. Group A arboviruses showed some variation in susceptibility to inhibition; aura virus was not inhibited by any of the treated sera whereas Sindbis and Semliki Forest viruses were strongly inhibited to titres comparable with those recorded for the four group B arboviruses used, viz, Ntaya, Wesselsbron, west Nile, and dengue type 1 viruses (Table 2). Inhibition was less apparent with sera stored at +4°C, and only two of these, viz, 199 and 116, lacked detectable inhibitory activity for all the arboviruses studied.

INHIBITORY ACTIVITY OF AGED RUBELLA SERA

As indicated above, the conditions under which sera are stored appear to influence the outcome of kaolin and $MnCl_2$ /heparin treatments. Here, rubella sera originally submitted for diagnostic evaluation in London were re-examined after two and seven months' storage at +4°C. The HAI titres at the time of collection and information on the presence of specific rubella virus antibody in the sera were kindly provided by Drs P. Higgins and H. G. S. Murray, respectively.

At collection the sera were treated with kaolin. However, if it is assumed that both treatments are of comparable ability in removing NSI from fresh sera (Table 1), ageing again would appear to alter the efficiency of NSI removal (Table 3). This is well illustrated with serum No. 22 which lacked specific rubella virus antibody yet showed a very significant rise in apparent HAI antibody titre after $MnCl_2$ /heparin treatment. On the other hand, both treat-

Table 1 Susceptibility of rubella and Japanese encephalitis (JE) viruses to inhibition of haemagglutination (HAI) after treatment to remove non-specific inhibitors in fresh unfrozen and freeze-thawed sera

Serum		HAI titres					
Sample	Storage (°C)	Rubella virus			JE virus		
		Untreated	Kaolin treated	Mn/hep treated	Untreated	Kaolin treated	Mn/hep treated
197	+ 4	240	80	96	1000	< 10	24
	-20	240	60	192	2600	< 10	64
184	+ 4	240	30	64	2600	40	128
	-20	320	20	128	1300	80	256
181	+ 4	120	15	32	2600	30	32
	-20	480	15	128	1900	60	192
195	+ 4	120	30	64	1900	40	64
	-20	480	40	192	3800	60	192
161	+ 4	160	30	16	1900	15	24
	-20	240	20	128	2600	10	8
194	+ 4	120	20	24	1000	20	24
	-20	160	30	64	1300	60	384
147	+ 4	60	10	32	480	< 10	24
	-20	120	10	48	480	10	64
192	+ 4	240	< 10	8	1900	< 10	16
	-20	240	30	96	1300	30	96
199	+ 4	60	< 10	12	5100	< 10	32
	-20	80	< 10	96	480	10	> 8
116	+ 4	240	80	128	1900	< 10	> 8
	-20	480	120	256	480	10	> 8

Mn/hep = MnCl₂/heparin

Table 2 Susceptibility of representative arboviruses to inhibition of haemagglutination after treatment to remove non-specific inhibitors in fresh unfrozen and freeze-thawed sera

Serum			HAI titres									
Sample	Storage (°C)	Treatment	Arbovirus group A					Arbovirus group B				
			AU	SB	Y62	SF	PX	NT	WB	WN	D1	
197	+ 4	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
		Mn/hep	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	< 10	< 10	NT	< 8
		Mn/hep	< 8	128	< 8	< 8	< 8	NT	< 8	192	NT	< 8
184	+ 4	Kaolin	< 10	< 10	< 10	< 10	10	120	60	80	120	120
		Mn/hep	< 8	12	< 8	128	24	384	384	128	384	384
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	60	60	NT	< 8
		Mn/hep	8	192	48	192	< 8	NT	128	512	NT	< 8
181	+ 4	Kaolin	< 10	< 10	< 10	20	< 10	60	30	60	60	60
		Mn/hep	< 8	12	< 8	128	16	128	128	48	128	128
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	60	60	NT	< 8
		Mn/hep	8	512	16	32	48	NT	16	384	NT	< 8
195	+ 4	Kaolin	< 10	< 10	< 10	15	< 10	60	30	60	60	60
		Mn/hep	< 8	12	< 8	128	16	128	128	32	128	128
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	60	60	NT	< 8
		Mn/hep	8	384	48	192	16	NT	16	512	NT	< 8
161	+ 4	Kaolin	< 10	< 10	< 10	< 10	< 10	15	< 10	20	15	15
		Mn/hep	< 8	12	< 8	64	16	96	96	12	96	96
	-20	Kaolin	< 10	10	< 10	< 10	< 10	NT	< 10	10	NT	< 8
		Mn/hep	< 8	384	< 8	128	32	NT	16	192	NT	< 8
199	+ 4	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	< 10	> 10	> 10	> 10
		Mn/hep	< 8	< 8	< 8	< 8	< 8	< 8	> 8	> 8	> 8	> 8
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	10	20	NT	< 8
		Mn/hep	< 8	64	< 8	< 8	< 8	NT	8	256	NT	< 8
116	+ 4	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	< 10	> 10	> 10	> 10
		Mn/hep	< 8	< 8	< 8	< 8	< 8	< 8	< 8	> 8	> 8	> 8
	-20	Kaolin	< 10	< 10	< 10	< 10	< 10	NT	< 10	10	NT	< 8
		Mn/hep	< 8	128	< 8	128	32	NT	< 8	384	NT	< 8

AU, aura; SB, Sindbis; Y62, Y62-33; SF, Semliki Forest; PX, Pixuna; NT, Ntaya; WB, Wesselsbron; WN, West Nile; D1, dengue type 1

Mn/hep = MnCl₂/heparin

NT, not tested

Table 3 Effect of ageing on the apparent antibody titre of rubella immune sera in the haemagglutination inhibition (HAI) test after treatment to remove non-specific inhibitor

Serum sample	Immunoglobulins present		Serum treatment	Rubella virus HAI titres at times ¹		
	IgG	IgM ²		Collection	2 mth	7 mth
18	Trace	Trace	Kaolin	< 8	< 10	15
			Mn/hep	NT	32	512
20	+	+	Kaolin	32	10	10
			Mn/hep	NT	32	32
22	-	-	Kaolin	< 8	< 10	< 10
			Mn/hep	NT	192	192
26	+	-	Kaolin	64	80	40
			Mn/hep	NT	384	384
27	+	-	Kaolin	256	240	160
			Mn/hep	NT	768	384
28	+	+	Kaolin	16	40	40
			Mn/hep	NT	512	512
29	+	+	Kaolin	1000	240	160
			Mn/hep	NT	512	256
51	+	+	Kaolin	128	80	20
			Mn/hep	NT	64	32

¹After collection the samples were frozen for varying periods of time before despatch to Hong Kong where they were held at +4°C and titrated at 2 and 7 months after receipt

²Rubella virus specific IgG and IgM detected by sucrose density gradient centrifugation (Murray, personal communication)

Mn/hep, MnCl₂/heparin

NT, not tested

Table 4 Susceptibility of rubella and Japanese encephalitis (JE) viruses to inhibition of haemagglutination after treatment to remove non-specific inhibitors in aged rubella immune sera

Serum sample ¹	HAI titres					
	Rubella virus			JE virus		
	Untreated	Kaolin treated	Mn/hep treated	Untreated	Kaolin treated	Mn/hep treated
1	640	10 (<8)	32	1000	10	64
2	480	10 (8)	32	2600	10	384
3	640	80 (16)	48	2600	320	192
4	1000	20 (32)	32	1900	40	256
5	1300	240 (64)	256	1900	15	128
6	640	60 (256)	128	1000	30	64

¹Aged sera titrated at 7 months, as indicated in Table 3

Mn/hep, MnCl₂/heparin

Figures in parentheses denote original antibody titres after kaolin treatment

ments caused a marked reduction in apparent antibody titre in two sera, viz, 29 and 51, suggesting that antibody as well as NSI was removed.

To extend these observations, six pools of rubella immune sera with HAI antibody titres < 8 to 256 at the time of collection were retitrated after seven months. The HAI titres for rubella virus obtained after one or other of the treatments were at variance with those originally recorded, and in all cases JE virus was inhibited, suggesting incomplete removal of NSI (Table 4). Some of the sera listed in Tables 3 and 4 were additionally examined for their ability to inhibit the HA of other arboviruses to hand (4 group A and 2 group B). All sera inhibited one or other of the viruses, this again being most evident with MnCl₂/heparin treated sera (Table 5). Aura and Y62-33

viruses (group A) were inhibited least and west Nile (group B) most.

LIPOPROTEINS IN AGED SERA

The findings to hand suggested that ageing rendered serum lipoproteins less susceptible to removal by kaolin and MnCl₂/heparin. It was therefore of interest to see what effect ageing had on the lipoproteins themselves. To this end, aged sera were selected at random and electrophoresed in parallel with a fresh serum sample followed by staining for lipoprotein. The fresh serum exhibited a type IV lipoprotein pattern (Beaumont *et al.*, 1970) with characteristic mobilities in the α , pre- β , and β positions (Fig. 1) corresponding to HDL, VLDL, and LDL, respectively, obtained by flotation centri-

Table 5 Susceptibility of representative arboviruses to inhibition of haemagglutination after treatment to remove non-specific inhibitors in aged rubella immune sera

Serum ¹ sample	Treatment	HAI titres						
		Arbovirus group A					Arbovirus group B	
		AU ²	SB	Y62	SF	PX	WB	WN
1	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	15
	Mn/hep	< 8	16	< 8	16	16	16	192
2	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	15
	Mn/hep	< 8	32	< 8	16	16	16	1000
22	Kaolin	60	60	120	40	240	120	640
	Mn/hep	< 8	32	< 8	16	32	32	1000
23	Kaolin	40	60	60	30	120	60	640
	Mn/hep	< 8	128	< 8	128	32	32	1500
26	Kaolin	15	15	15	10	40	30	480
	Mn/hep	< 8	192	< 8	64	32	96	384
27	Kaolin	< 10	15	< 10	< 10	40	< 10	160
	Mn/hep	< 8	128	< 8	256	512	96	1000
28	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	15
	Mn/hep	< 8	8	< 8	16	< 8	8	384
29	Kaolin	< 10	< 10	< 10	< 10	< 10	< 10	10
	Mn/hep	< 8	< 8	< 8	< 8	< 8	< 8	64

¹Aged sera titrated at 7 months, as indicated in Table 3Mn/hep, MnCl₂/heparin²Viruses as in Table 2; NT and D1 were not available

Fig. 1 Lipoprotein electrophoretic patterns of fresh (C) and aged (numbered) sera. The aged sera had been held at 4°C for two months.

fugation. Ageing generally appeared to alter the mobilities of the α and pre- β lipoproteins, most of the staining being well in evidence in the β position.

BEHAVIOUR OF FRESH AND AGED SERA ON CENTRIFUGATION

Fresh non-immune or aged immune rubella sera were pooled and treated with kaolin and each serum pool was reconcentrated to its original volume (see Material and methods) before centrifugation. Control untreated pools were similarly diluted and reconcentrated. The inhibitory activity of fresh serum was confined to the top of the gradient indicative of lipoprotein and was greater for JE virus than for rubella virus (Fig. 2A). Fresh serum treated with kaolin was devoid of detectable inhibitory activity (Fig. 2B), indicating that the lipoproteins had been

effectively removed. In the case of the aged serum, inhibitory activity for rubella virus was observed in the macroglobulin (IgM) and IgG positions indicative of specific antibody and towards the top of the gradient in the lipoprotein position (Fig. 2C). This serum also contained inhibitory activity for JE virus which extended down the gradient further than did that in fresh serum, suggesting that the lipoproteins had undergone some form of denaturation on ageing. When the aged serum was treated with kaolin there appeared to be slight decreases in rubella IgM and IgG inhibitory titre levels but inhibitory activity still remained in the top part of the gradient, suggesting incomplete removal of lipoprotein which was confirmed using JE virus (Fig. 2D).

Discussion

The haemagglutination inhibition (HAI) test is still the method of choice for providing evidence of rubella infection. A major cause for concern with this test is the validity of low or even moderate levels of antibody in view of the fact that false positive reactions have been known to occur. This is especially important in evaluating the immune status of a pregnant woman who has been in contact with a case of rubella.

The chief cause for concern has been the reliability of established procedures such as kaolin and MnCl₂/heparin treatments of sera for removing non-specific inhibitors (NSI) of rubella virus haemagglutination. The recent recognition that

Fig. 2 Density gradient centrifugation of pooled fresh and aged (4°C for two months) sera, untreated or treated with kaolin before centrifugation. Gradient fractions were titrated in the HAI test for antibody and non-specific inhibitor using rubella (●—●) and Japanese encephalitis (★...★) viruses. The positions of IgM and IgG are indicated by arrows and were interpolated by centrifuging gradients in parallel containing markers of established sedimentation coefficient. Samples centrifuged: fresh, non-immune rubella sera untreated (A) and kaolin treated (B); aged, immune rubella sera untreated (C) and kaolin treated (D).

lipoproteins are responsible for NSI activity has opened the door to some basic studies which permit evaluation of removal methods. The findings reported here make use of the fact that arboviruses, which are classified with rubella virus as togaviruses, are susceptible to inhibition of haemagglutination by lipoproteins (Shortridge and Ho, 1974; 1976).

Lipoproteins are unstable substances undergoing denaturation in serum stored either frozen or unfrozen, due to enzymic activity and oxidation of unsaturated fatty acids (Hatch and Lees, 1968). The resulting changes in lipoprotein structure may

account for the reduced effectiveness of kaolin, and MnCl_2 /heparin in particular, in removing lipoproteins, and this was more noticeable when the sera had been frozen and subsequently thawed. Although the molecular mechanisms of deterioration are not clearly understood it seems likely that the ensuing conformational changes in lipoprotein structure resulted in either more efficient union with the virus haemagglutinin or exposure of previously occluded receptor groups. This was best observed with some of the group B arboviruses, particularly JE and west Nile viruses, which suggests that they could find application as indicator viruses for evaluating the efficiency of lipoprotein removal. On the other hand, some arboviruses such as *aura* which are highly sensitive to native lipoprotein (Shortridge and Ho, 1976) were insensitive to inhibition by lipoproteins in deteriorating sera. Evidence that ageing caused structural changes in the lipoproteins was seen in the altered electrophoretic mobilities of sera stored for two months at 4°C in the laboratory refrigerator. Unless precautionary measures are taken, α -lipoprotein (HDL) spontaneously loses its lipid and the partially delipidated forms exhibit slower electrophoretic mobilities (Levy and Frederickson, 1965); this could explain in part the observations made here that the bulk of the lipoprotein staining activity was in the β -position.

Both kaolin and MnCl_2 /heparin treatments appeared to be of comparable ability in removing lipoproteins from fresh sera. Obviously it is better if rubella virus antibody assays are performed on absolutely fresh sera. If this cannot be done the sera may be stored for a few days at 4°C, preferably with the addition of a chelating agent such as EDTA to reduce enzymic activity and to slow down oxidative degradation (Hatch and Lees, 1968). Storage at 4°C for longer than a week seems inadvisable, making freezing the only alternative. This procedure is probably satisfactory for samples with normal or slightly raised lipoprotein levels; grossly hyperlipoproteinaemic samples undergo immediate change (Hatch and Lees, 1968). In this study MnCl_2 /heparin was notably ineffective in removing lipoprotein inhibitors from freeze-thawed samples. Sera may be classified into five types on the basis of their lipoprotein content and distribution according to the original Frederickson scheme (Beaumont *et al.*, 1970). The significance of serum type with respect to lipoprotein removal from deteriorated sera is at present being investigated.

It is of interest to note that the comments made about the efficiency of lipoprotein removal from sera for rubella virus antibody assay apply as much, and even more so, to arbovirus serology as these viruses are more sensitive to inhibition of haemagglutina-

tion. It is a matter of conjecture whether some of the low-titre cross-reactions seen among arboviruses in the HAI test might be due to residual lipoprotein.

While the conditions chosen for deterioration might be considered a little excessive, many laboratories do in fact hold sera for long periods at 4°C while others store samples frozen at -20°C for re-use on many occasions; this applies particularly to reference sera. The use of an indicator arbovirus could therefore be helpful for monitoring the efficiency of lipoprotein removal. Obviously such an approach would be restricted to those areas where arboviruses are not endemic. However, this need not be a problem; careful selection of a haemagglutinating arbovirus from one of the many arbovirus groups could resolve this apparent difficulty. Today many diagnostic laboratories have an ultracentrifuge; density gradient studies of doubtful sera using rubella virus and a suitable indicator arbovirus could readily detect residual lipoproteins at the top of the gradient, as was shown in this study.

Newer techniques such as the enzyme-linked immunoabsorbent assay (Voller and Bidwell, 1975) and single radial haemolysis (Grillner and Strannegård, 1976), have recently been applied to assay for rubella virus antibody with a view to circumventing problems caused by lipoproteins. In a pilot study in this laboratory an affinity chromatography procedure to remove lipoproteins from serum using dodecylamine attached to agarose (Ho and Shortridge, 1976) has given encouraging results. The merits of these procedures need to be evaluated in long-term studies.

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