

Influenza A Neuraminidase Antibody Assay with Sensitized Erythrocytes

J. L. HOLSTON, JR.,¹ AND W. R. DOWDLE

*Respiratory Virology Unit, Center for Disease Control, Health Services and Mental Health Administration,
U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333*

Received for publication 21 September 1972

Erythrocytes sensitized with purified neuraminidase (Hong Kong) antigens were used for assay of influenza A neuraminidase antibodies. The neuraminidase indirect hemagglutination test was equal to the neuraminidase hemagglutination-inhibition (enhancement) test and appeared to be better than the neuraminidase inhibition test for detection of fourfold or greater antibody rises in paired sera from influenza patients or vaccinees. It was better than both tests for detection of neuraminidase antibody. The neuraminidase indirect hemagglutination test is simple to perform and has the advantage of direct antigen-antibody assay.

The recognition that the surface of the influenza virus is composed of a second major antigen, the neuraminidase (N), in addition to the hemagglutinin (H) antigen, has stimulated efforts to develop practical methods for assaying neuraminidase antibodies. The neuraminidase inhibition test (NI), in which antibody titer is expressed in terms of inhibition of enzyme activity (14), has proved useful, but even with modifications (5) it is often impractical for small laboratories or for large-scale studies. The neuraminidase-hemagglutination-inhibition (N-HI) enhancement test (6), in which antibody titer is determined indirectly through interference with hemagglutination, is simple to perform, but its dependence upon four reactants (virus, antibody, anti-antibody, and erythrocytes) makes it a complex test system (W. R. Dowdle, D. Sarateanu, and C. B. Reimer, *J. Immunol.*, *in press*). In this report (submitted by J. L. Holston, Jr., in partial fulfillment of the requirements for the Doctor of Public Health degree in Laboratory Practice from the University of North Carolina, Chapel Hill, N.C.) we described a serological test which uses glutaraldehyde-fixed and tannic acid-treated erythrocytes sensitized with neuraminidase antigen. The test is simple and sensitive and has the advantage of direct antigen-antibody assay.

MATERIALS AND METHODS

Seed viruses. Influenza strains A/

NWS/33(H0N1), A/Hong Kong/8/68(H3N2), A/Aichi/2/68(H3N2), and B/Massachusetts/3/66 were from the reference virus collection of the World Health Organization (WHO) International Influenza Center for the Americas (IICA), Atlanta, Ga. Recombinant virus A/NWS/33(HO)-Hong Kong/8/68(N2) was prepared according to the method of Webster (13). Recombinant virus A/equine/Prague/1/56 (Heq1)-Hong Kong/16/68(N2) was furnished by E. D. Kilbourne, Mount Sinai School of Medicine, New York. Parent viruses and recombinant strains are named according to the system of nomenclature recommended by WHO (15).

Reference antisera. Chicken antisera prepared against A/NWS(H0N1) and the recombinant viruses A/Prague(heq1)-HK(N2), A/HK(H3)-NWS(N1), and A/NWS/33(HO)-Taiwan/1/64(N2) were obtained from the reference antisera collection of the IICA. Rabbit antisera to isolated type A ribonucleoprotein, isolated type A matrix protein, and purified Hong Kong neuraminidase (N2) were furnished by G. C. Schild, National Institute for Medical Research, London. Guinea pig antisera to type A RNP were obtained from the IICA reference serum collection.

Human sera. Human serum specimens were from a 1968-69 vaccine study conducted among inmates at the Georgia State Prison, Reidsville, Ga. "Vaccine" sera consisted of paired serum specimens, the first drawn 1 week before the administration of vaccine and the second 28 days later. Vaccinees received either 300 or 3,000 chick cell-agglutination (CCA) units of A/Japan/170/62(H2N2) or 300 or 3,000 CCA units of A/Aichi/2/68(H3N2). "Illness" sera consisted of paired sera from volunteer controls. Each subject had been naturally infected with Hong Kong influenza as shown by a fourfold or greater serum antibody rise in the hemagglutination-inhibition (HI) test. The details of this study are published elsewhere (9, 12).

¹Present address: State Department of Public Health, Montgomery, Ala. 36104.

HI test. Sera were assayed for HI antibodies according to the Center for Disease Control (CDC) standardized Microtiter procedure (4).

Neuraminidase assay. Neuraminidase activity of virus preparations was assayed as described by Webster and Pereira (14). Samples to be assayed were prepared in serial 0.5-log₁₀ dilutions. A 0.05-ml amount of each dilution was added to an equal volume of fetuin (24 mg/ml) substrate and buffer, pH 5.9, and incubated for 1 hr at 37 C. The optical density (OD) of the colored organic extract was read at 549 nm against a reagent blank (fetuin and saline) on a Beckman DU-2 spectrophotometer.

Neuraminidase inhibition test. Antineuraminidase serum titers were also determined according to the method described by Webster and Pereira (14). Virus concentrations with neuraminidase activity of OD 0.5 to 0.9 per 0.05 ml were mixed with equal volumes of serial 0.5-log₁₀ dilutions of antisera, and incubated at 37 C for 30 min. Fetuin (24 mg/ml) and buffer, pH 5.9, were then added, and the mixture was incubated for 1 hr at 37 C. The OD of the organic extract was read at 549 nm against a reagent blank (fetuin and saline). Antineuraminidase titers were calculated by plotting the corrected (from normal serum controls) OD readings against the dilution factor of antiserum. The NI titer of a serum was expressed as that dilution (per milliliter) which inhibited 50% of the enzyme activity.

N-HI test. The N-HI test for the assay of antineuraminidase was performed in Microtiter with the recombinant A/Prague(Heq1)-HK(N2). The test was similar to that described by Kendal et al. (6). Sera were serially diluted in duplicate in twofold increments in 0.025 ml of phosphate-buffered saline (PBS) (pH 7.2). Equal volumes of a virus suspension containing 4 hemagglutinin (HA) units were added to each row of diluted sera. After 30 min of incubation at room temperature, 0.025 ml of a 1:20 dilution of rabbit antihuman immunoglobulin G with an E value of 105 (10) was added to the first row and 0.25 ml of PBS to the second row of each duplicate titration. After 30 min of incubation at room temperature, 0.05 ml of 0.5% chicken erythrocytes was added to each row. Test patterns were read after 1 hr at room temperature.

Complement fixation test. The complement fixation (CF) test was performed by the Microtiter method (2).

Immunodiffusion test. The micro double-immunodiffusion (ID) test employed was similar to that previously described by Schild (11). Ten percent sodium dodecyl sulfate (SDS) (in water) was added to antigen wells to disrupt the test virus suspension and permit migration of structural components through the gel medium.

Neuraminidase indirect hemagglutination test. Neuraminidase indirect hemagglutination (NIHA) tests were performed with sheep erythrocytes stabilized by glutaraldehyde fixation as described by Bing et al. (1). Glutaraldehyde (50% biological grade) was diluted to 1% with a solution containing 1 part of Na₂HPO₄ (pH 8.2), 9 parts of 0.15 M NaCl, and 5 parts of distilled water. Before glutaraldehyde and

diluent were mixed, they were cooled in an ice bath for 10 min to avoid clouding. Sheep erythrocytes, washed twice in 0.15 M NaCl and packed for 10 min at 350 × g, were diluted to 2% with the cold 1% glutaraldehyde and mixed on a magnetic stirrer for 30 min at 4 C. The fixed cells were washed five times with 0.15 M NaCl and diluted to 1.5% with 0.1 M PBS, pH 7.2.

Tannic acid (reagent grade), diluted to 1:100,000 in PBS (pH 7.2), was mixed with equal volumes of the 1.5% glutaraldehyde-fixed cells and incubated at 37 C for 15 min. The tannic acid-treated cells were washed with PBS (pH 7.2) and resuspended to 1.5% with PBS (pH 5.5).

Optimal dilutions of antigen for sensitization were determined as follows. Test antigens were diluted in 0.5-ml volumes in a twofold series with PBS (pH 5.5). Equal volumes of tanned cells were added to each dilution, mixed well, and incubated at room temperature for 60 min. Control tanned cells (0.5 ml) were mixed with an equal volume of PBS (pH 5.5) and incubated for the same period. Both sensitized and control cells were centrifuged at 150 × g for 5 min and resuspended in 0.1 M glycine (in PBS, pH 7.2). After 15 min at room temperature, the cells were washed in 0.15 M NaCl and diluted to a 0.5% suspension with a 1:100 dilution of normal horse serum (NHS).

Sera to be tested were inactivated at 56 C for 30 min and adsorbed with 50% sheep erythrocytes (0.2 ml/1 ml of serum) at 4 C for 60 min. Serial twofold dilutions of the antisera were prepared in Microtiter "U" plates containing 0.05 ml of NHS (1:100). Equal volumes of sensitized cells were added to the test wells. The plates were incubated for 2 hr at room temperature, and the hemagglutination pattern was examined. The optimal antigen dilution was defined as the lowest antigen dilution which produced the highest specific antibody titer.

Isolation of neuraminidase. The procedures used were similar to those described by Laver (7). Recombinant virus A/NWS(H0)-HK(N2) was grown in 11-day embryonated eggs, and the allantoic fluid harvests were clarified by low-speed centrifugation (350 × g). The virus was pelleted by centrifugation at 100,000 × g for 2 hr and purified by centrifugation at 43,000 × g for 90 min on a 10 to 40% sucrose gradient. Final virus concentrates containing 10⁶ to 10⁷ HA units/ml were disrupted at room temperature by adding (dropwise) 10% SDS in water. Disruption was judged to be complete when the opalescence of the suspension cleared. This usually required 0.05 ml of 10% SDS per 0.1 ml of virus.

Electrophoresis of SDS-disrupted virus was performed on 5 by 36 cm cellulose acetate membranes in tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid-boric acid buffer, pH 8.0, containing 0.1% SDS. One-tenth milliliter of the SDS-disrupted virus was divided equally among four membranes and electrophoresed for 6 hr at 180 v. Several small (5 mm) horizontal strips were cut from the membrane for location and identification of protein bands. One or more strips were stained with procion brilliant blue dye (0.5% [w/v] in hydrochloric acid, 1% [w/v] in methanol). Other strips were placed

on agar gels alongside strips saturated with either anti-neuraminidase (N2), -hemagglutinin (H0), -type A nucleoprotein or, -type A matrix protein rabbit antisera. A precipitin line with anti-N2 corresponded to the band closest to the cathode. There was no serological evidence of contamination of this band with other viral structural proteins. The N2 band was located on the membrane by matching stained strips. The membrane was cut vertically and its contents were eluted overnight at 4 C in 0.75 ml of distilled water. SDS was precipitated by reducing the temperature to 0 C for 2 to 3 hr, and the precipitate was removed by centrifugation (350 × g) in the cold. The remaining SDS was precipitated by adding 1 drop of saturated KCl (in water). After 2 hr at zero C, the precipitate was removed and the supernatant was dialyzed for 24 hr against PBS (pH 7.2) at 4 C. This product is referred to as the N2 antigen.

Preparation of antisera. Antisera were prepared in female New Zealand White rabbits (6 to 8 lb.; ca. 2.7 to 3.6 kg) by intramuscular injections of antigen mixed with an equal volume of Freund's complete adjuvant. Rabbits were injected at 3-week intervals and bled 3 weeks after the third injection.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (8) with adsorption readings at 750 nm.

RESULTS

Antigenic purity of the isolated neuraminidase. Rabbit antiserum against the electrophoretically isolated neuraminidase antigen had an NI titer of 1:400,000 with the recombinant A/Prague(Heq1)-HK(N2). NI tests showed the antiserum to be free of antibodies to the parent A/NWS(H0) neuraminidase, and HI tests showed it to be free of antibodies to HA. HI antibodies to the H3 antigen were absent, although low-level inhibition of hemagglutination by recombinant viruses possessing the N2 component of the parent A/HK(H3N2) was observed. When the ID test was used, a single precipitin line was formed by N2 antisera against SDS-disrupted A/Aichi virus. This line had complete serological identity with that formed with a reference purified anti-N2 rabbit serum prepared in another laboratory (World Influenza Centre, London). Control antisera to the remaining three viral structural proteins produced precipitin lines of non-identity to those with the purified N2 antisera. The rabbit N2 antiserum was also negative for RNP antibodies by CF tests.

Preparation of N2-sensitized erythrocytes. Glutaraldehyde-fixed and tannic acid-treated sheep erythrocytes were sensitized with the electrophoretically isolated N2 antigens. The optimal dilution for sensitization was determined by titration of human sera (NI titer: 1,380) against each sensitized antigen

preparation. Glutaraldehyde-fixed erythrocytes treated with tannic acid dilutions ranging from 1:20,000 through 1:400,000 were equally sensitive for adsorption of N2 antigen. Optimal dilutions for most N2 preparations ranged from 1:4 to 1:6, and corresponded to an OD reading of approximately 0.15 in the standard neuraminidase test (1 hr of incubation with fetuin). N-IHA titers decreased sharply when erythrocytes were sensitized with N2 preparations of an OD less than 0.1.

The attachment of neuraminidase to the erythrocyte under these conditions was confirmed by washing sensitized erythrocytes three or more times and testing for neuraminidase activity. One milliliter of 0.5% sensitized erythrocytes yielded an average OD value of 0.2317 in the standard neuraminidase test.

Since purified N2 preparations in PBS rapidly lost enzyme activity, only freshly prepared (immediately after dialysis) N2 suspensions were used. The N2 antigen appeared to be stable after it became attached to the erythrocytes. Sensitized erythrocytes could be stored at 4 C for at least 4 months (last time evaluated) without loss of sensitivity to antibody or alteration in erythrocyte settling patterns.

Specificity of agglutination of N2-sensitized erythrocytes. The specificity of the N-IHA test was evaluated with selected recombinant and component specific antisera. Agglutination was observed only with sera containing antibodies to the N2 component (Table 1).

Human paired sera with fourfold or greater HI antibody rises to A/Japan/305/57, B/Massachusetts/3/66, and A/Hong Kong/8/68 also were examined for agglutination of N2 sensitized erythrocytes (Table 2). Paired sera collected in 1957 from persons experiencing first infections with Asian viruses (H2N2) showed no evidence of antibody to Hong Kong N2 by N-IHA. Pre- and postinfection sera collected in 1969-70 from persons infected with influenza B had stationary titers by the N-IHA test. All five paired sera from Hong Kong infections had fourfold or greater increases in N-IHA titers.

Further evaluation of the specificity of the N-IHA test was undertaken by adsorbing human sera with sensitized and nonsensitized erythrocytes. Five-tenths of a milliliter of packed erythrocytes was added to 1 ml of a predetermined dilution (1:20-1:30) of serum. The serum-erythrocyte mixture was agitated intermittently. Sera were adsorbed for 18 hr at 4 C. CF antibody titers to the ribonucleopro-

TABLE 1. Specificity of the neuraminidase indirect hemagglutination (N-IHA) test: reaction with influenza component-specific antisera

Antisera	N-IHA titer		Control test
	Sensitized cells	Non-sensitized cells	
Guinea pig-anti-type A ribonucleoprotein	<10	<10	64 ^a
Rabbit anti-type A ribonucleoprotein	<20	<20	+ ^b
Rabbit anti-type A matrix protein	<20	<20	+ ^b
Rabbit anti-A/NWS (HON1) ..	<10	<10	630 ^c
Rabbit anti-type A purified N2 (Hong Kong)	1,280	<10	1,700 ^c
Rabbit anti-NWS (HO)-Tw (N2)	2,560	<10	2,560 ^c

^a Complement fixation titer.

^b Precipitin line in immunodiffusion test.

^c Neuraminidase inhibition titer.

TABLE 2. Specificity of the neuraminidase indirect hemagglutination (N-IHA) test: reaction with paired sera from humans infected with A/Japan/305/57, B/Mass/3/66, or A/Hong Kong/8/68 influenza viruses

Human sera	Infecting virus	N-IHA titer
Group I ^a	A/Japan	
P-131		0/0 ^b
P-132		0/0
P-133		0/0
P-135		0/0
P-138		0/0
Group II ^a	B/Mass	
RU 7382		160/160
RU 7381		160/160
RU 7380		160/160
RU 7603		40/40
RU 7385		40/40
Group III ^a	A/Hong Kong	
56017		160/640
56105		320/5,120
57369		160/2,560
57485		160/640
57518		80/1,280

^a Paired sera from persons with greater than fourfold rises in hemagglutination-inhibition titer to: I, A(H2N2), winter 1957-58; II, influenza B, winter 1970-71; III, A(H3N2), winter 1968-69.

^b Zero means less than 10.

tein and HI titers to A/NWS were not altered by adsorption of sera with N2-sensitized erythrocytes. N-IHA and NI titers to the N2 antigen were reduced threefold or greater (Table 3).

Evaluation of the N-IHA test. Forty-one acute and convalescent sera from subjects confirmed by HI to have been infected with A/HK/68 were tested by N-IHA, and the results were compared with those obtained by NI and N-HI tests. Ninety-three percent of the serum pairs were shown by at least one of the three tests to have a fourfold or greater rise in neuraminidase antibody titer. Of the total antibody rises, the N-IHA test detected 83%, the N-HI 68%, and the NI 54% (Table 4).

Pre- and postvaccination sera from A/Japan/170/62 and A/Aichi/2/68 vaccinees (confirmed by HI to have been successfully vaccinated) were also tested by N-IHA, N-HI, and NI. Of the paired sera from the 25 A/Japan/62 vaccinees, 84% were found by at least one of the three tests to have a fourfold or greater rise in antibody titer. The N-IHA detected 76%, the N-HI 64%, and the NI 44% (Table 4). Of the paired sera from the 25 A/Aichi/68 vaccinees, 96% were found by at least one test to have a fourfold or greater rise in antibody titer. The N-IHA again detected 76%, the N-HI 88%, and the NI 64% (Table 4).

The percent agreement between the N-IHA and the N-HI tests was similar with all three study groups (Table 5). The percent agreement between the N-IHA and the NI tests was significantly better with the A/Aichi/68 vaccine group than with the illness or the A/Japan/170/62 vaccine group ($P > 0.05$).

The N-IHA, N-HI, and NI tests were also evaluated for detection of antibody in prevaccination and preillness sera (Table 6). One hundred percent of the total 91 sera were positive for antibody by N-IHA. The numbers positive by the N-HI and NI test were considerably less, 25 and 22%, respectively.

TABLE 3. Specificity of the neuraminidase indirect hemagglutination (N-IHA) test: adsorption of human sera with N2-sensitized erythrocytes

Human sera	Adsorption	Test			
		N-IHA (N2)	NI ^a A/Prague (Heq)-HK (N2)	CF ^b RNP	HI ^c A/NWS/33 (HON1)
57078	Tanned cells	320	631	20	<10
	Sensitized cells	20	≤ 224	20	<10
57364	Tanned cells	960	1113	60	<10
	Sensitized cells	30	≤ 355	60	<10

^a Neuraminidase inhibition.

^b Complement fixation, ribonucleoprotein.

^c Hemagglutination inhibition.

TABLE 4. Comparison of neuraminidase indirect hemagglutination (N-IHA), neuraminidase hemagglutination inhibition (N-HI), and neuraminidase inhibition (NI) tests for detection of fourfold or greater rises in neuraminidase antibody titers in paired sera^a

Test	Illness		Vaccinees			
	No.	%	A/Japan		A/Aichi	
			No.	%	No.	%
Any (at least one)	38	93	21	84	24	96
N-IHA	34	83	19	76	19	76
N-HI	28	68	16	64	22	88
NI	22	54	11	44	16	64
All 3	19	46	9	36	15	60
Only N-IHA	9	22	4	16	1	4
N-IHA and N-HI	6	15	5	20	2	8
N-HI	1	2	1	4	5	20
N-HI and NI	2	5	1	4	0	0
N-IHA and NI	0	0	1	4	1	4
NI	1	2	0	0	0	0
None	3	7	4	16	1	4

^a Subjects naturally infected with A/Hong Kong/8/68(H3N2) and from volunteers immunized with A/Japan/170/62(H2N2) and A/Aichi/2/68(H3N2) vaccines.

TABLE 5. Percent agreement between the neuraminidase indirect hemagglutination (N-IHA) and the neuraminidase hemagglutination-inhibition (N-HI) tests and the N-IHA and the neuraminidase inhibition (NI) tests for detection of fourfold or greater rises in neuraminidase antibody titers in paired sera^a

Tests	Percent agreement between tests (no. agreed/no. tested)		
	Illness	A/Japan	A/Aichi
N-IHA vs. N-HI	(29/41) 71%	(18/25) 72%	(18/25) 72%
N-IHA vs. NI	(23/41) 56%	(15/25) 60%	(22/25) 88%

^a Subjects naturally infected with A/Hong Kong/8/68(H3N2) and from volunteers immunized with A/Japan/170/62(H2N2) and A/Aichi/2/68(H3N2) vaccines.

DISCUSSION

All evidence suggests that erythrocytes sensitized with isolated N2 antigen were agglutinated only by neuraminidase antibody. Hemagglutination did not occur in the presence of high-titered antibody to the recombinant hemagglutinin, type A ribonucleoprotein,

TABLE 6. Positive^a preillness and preimmunization neuraminidase antibody titers by neuraminidase indirect hemagglutination (N-IHA), neuraminidase hemagglutination-inhibition (N-HI), and neuraminidase inhibition (NI) tests

Test	Total sera	
	No.	%
Any (at least 1)	91	100
N-IHA	91	100
N-HI	23	25
NI	20	22
All 3	16	18

^a Titers of at least 10 by N-IHA and N-HI and at least 60 by HI were considered positive.

or matrix protein antigens. In addition, adsorption of human influenza convalescent sera with N2-sensitized erythrocytes removed only neuraminidase antibody.

The N-IHA test was at least equal to the N-HI test and seemed to be better than the NI test for detecting fourfold or greater antibody rises in paired sera from infected patients or vaccinees. It was clearly better than both tests in its ability to detect N2 antibody. N-IHA titers were positive in all 1968 prevaccine and pre-epidemic sera. Evidence that these titers were not artifactual is suggested by the absence of N-IHA titers in sera collected in 1957, the time of first appearance of the N2 antigen. The high incidence of N2 antibodies in 1968 was not unexpected. Influenza A strains which circulated in the years immediately preceding the Hong Kong influenza epidemic possessed N2 antigens which were closely related to those of the Hong Kong virus (3). Whether the greater number of titers detected by N-IHA test indicates it is less strain specific than the NI test remains to be determined. It should be noted, however, that an improved NI test which has greater sensitivity than the present NI test is now available (World Health Organization Committee, Bull. W.H.O., *in press*).

The N-IHA test is simple. It requires only sensitized erythrocytes and can be performed in any laboratory. The stability (at least 4 months at 4 C and presumably longer at -70 C) of the glutaraldehyde-fixed and sensitized erythrocytes makes possible the use of the same reagents for multiple tests. Furthermore, the N-IHA test should permit, in theory, direct assay of antibody reactive with antigens associated with the neuraminidase structure.

The one disadvantage of the test is the specialized equipment required for the initial virus preparation and isolation of the N2 anti-

gen. Even so, the test proved to be reasonably economical. A 0.1-ml amount of the purified recombinant (10^6 HA units) yielded sufficient N2 antigen to sensitize enough glutaraldehyde-fixed erythrocytes to test 400 sera through a full range of dilutions.

Our experience with the N-IHA test suggests that it should be a useful addition to the present methodology for the assay of N antibody.

ACKNOWLEDGMENTS

We gratefully acknowledge the advice and encouragement given by Marion T. Coleman and the able assistance of Wilma Yarbrough, Judy Galphin, Avis Sisson, and Ralph Pike.

This research was supported by research grant CC-00606 from the Center for Disease Control, Atlanta, Ga.

ADDENDUM IN PROOF

After our work was completed, a single-radial-diffusion test for assay of influenza neuraminidase antibodies was described by other workers (16).

LITERATURE CITED

- Bing, D. H., J. G. M. Weyand, and A. B. Stavitsky. 1967. Hemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. *Proc. Soc. Exp. Biol. Med.* 124:1166-1170.
- Casey, H. L. 1965. Adaptation of LBCF method to microtechnique. *In* Public Health Service Monograph no. 74. U.S. Govt. Printing Office, Washington, D. C.
- Coleman, M. T., W. R. Dowdle, H. G. Pereira, G. C. Schild, and W. K. Chang. 1968. The Hong Kong/68 influenza A2 variant. *Lancet* 2:1384-1386.
- Hierholzer, J. C., M. T. Suggs, and E. C. Hall. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. *Appl. Microbiol.* 18:824-833.
- Kendal, A. P., and C. R. Madeley. 1969. A comparative study of influenza virus neuraminidases, using automated techniques. *Biochem. Biophys. Acta* 185:163-177.
- Kendal, A. P., E. Minuse, and F. M. Davenport. 1972. An improved procedure for measuring neuraminidase antibodies by hemagglutination-inhibition. *Z. Naturforschung* 27:241-245.
- Laver, W. G. 1964. Structural studies on the protein subunits from three strains of influenza virus. *J. Mol. Biol.* 9:109-124.
- Lowry, O. H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mostow, S. R., S. C. Schoenbaum, W. R. Dowdle, M. T. Coleman, and H. S. Kaye. 1969. Studies with inactivated influenza vaccines purified by zonal centrifugation. I. Adverse reactions and serologic responses. *Bull. W.H.O.* 41:525-530.
- Reimer, C. B., D. J. Phillips, S. E. Maddison, and S. L. Shore. 1970. Comparative evaluation of commercial precipitating antisera against human IgM and IgG. *Lab. Clin. Med.* 76:949-960.
- Schild, G. C. 1972. Evidence for a new type-specific structural antigen of the influenza virus particle. *J. Gen. Virol.* 15:99-103.
- Schoenbaum, S. C., S. R. Mostow, W. R. Dowdle, M. T. Coleman, and H. S. Kaye. 1969. Studies with inactivated influenza vaccines purified by zonal centrifugation. II. Efficacy. *Bull. W.H.O.* 41:531-535.
- Webster, R. G. 1970. Antigenic hybrids of influenza A viruses with surface antigens to order. *Virology* 42:633-642.
- Webster, R. G., and H. G. Pereira. 1968. A common surface antigen in influenza viruses of human and avian sources. *J. Gen. Virol.* 3:201-208.
- World Health Organization Committee. 1971. A revised system of nomenclature for influenza viruses. *Bull. W.H.O.* 45:119-124.
- Schild, G. C., M. Henry-Aymard, and H. G. Pereira. 1972. A quantitative, single-radial-diffusion test for immunological studies with influenza virus. *J. Gen. Virol.* 16:231-236.