normal subjects, 60-90% of acid mucopolysaccharides are equally distributed between the 1.25 M and 1.5 M NaCl fraction. With increasing age, less acid mucopolysaccharides are distributed in these two fractions. 5. The present report suggests the presence of heparitin sulfate, heparin and chondroitin sulfate B as minor components of AMP in normal urine. 6. There was no evidence for the presence of keratosulfate in the normal urines examined. The possible presence of hyaluronic acid in normal urine is not excluded by the results of the present study.

The authors gratefully acknowledge suggestions and advice of Dr. C. R. Treadwell and the technical assistance of Mr. William Hitt.

1. Brante, G., Scand. J. Clin. Lab. Invest., 1952, v4, 43.
2. Kerby, G. P., J. Clin. Invest., 1954, v33, 1168.
3. Astrup, P., Acta Pharmacol., 1947, v3, 165.
4. Heremans, J. F., Vaerman, J. P., Heremans, M. Th., Nature, 1959, v183, 1606.
5. Dorfman, A., Lorincz, A. E., Proc. Nat. Acad. Sci., 1957, v43, 443.
6. Meyer, K., Grumbach, M. M., Linker, A., Hoffman, P., Proc. Soc. Exp. Biol. & Med., 1958, v97, 275.
7. King, J. S., Jr., Fielden, M. L., Boyce, W. H., Clin. Chim. Acta, 1962, v7, 316.
8. Di Ferrante, N., J. Lab. Clin. Med., 1963, v61, 633.
9. Linker, A., Terry, K. D., Proc. Soc. Exp. Biol. & Med., 1963, v113, 743.
10. Berenson, G. S., Dalferes, E. R., Biochim. Biophys. Acta, 1965, v101, 183.
11. Teller, W. M., Nature, 1967, v213, 1132.
12. Kelley, W. R., Poncet, I. B., Di Ferrante, N., ibid., 1963, v197, 1204.
13. Di Ferrante, N., Rich, C., Clin. Chim. Acta, 1956, v1, 519.
14. Kao, K.-Y. T., Hizer, C. A., Dawson, R. L., McGavack, T. H., Proc. Soc. Exp. Biol. & Med., 1965, v119, 193.
15. Schiller, S., Slover, G. A., Dorfman, A., J. Biol. Chem., 1961, v236, 983.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., ibid., 1951, v193, 265.
17. Dische, Z., ibid., 1947, v167, 189.
18. Dische, Z., Shettles, L. B., ibid., 1948, v175, 595.
19. Weimer, H. E., Moshin, J. R., Am. Rev. Tuberc., 1953, v68, 594.
20. Warren, L., J. Biol. Chem., 1959, v234, 1971.
21. Rondle, C. J. M., Morgan, W. T. J., Biochem. J., 1955, v61, 586.
22. Bailey, N. T. J., Statistical Methods in Biology, John Wiley & Sons, Inc., New York, 1959, p169, 172.
23. Matalon, R., Dorfman, A., Proc. Nat. Acad. Sci., 1966, v56, 1310.
24. Pedrini, V., Lennzi, L., Zambotti, V., Proc. Soc. Exp. Biol. & Med., 1962, v110, 847.
25. Di Ferrante, N., J. Clin. Invest., 1957, v36, 1516.
26. Craddock, J. G., Kerby, G. P., J. Lab. Clin. Med., 1955, v46, 193.
27. Azerad, E., Morard, J. C., Ghata, J., Path. Biol. Semaine Hop., 1962, v10, 217.
28. Rich, C., Laird Myers, W. P., J. Lab. Clin. Med., 1959, v54, 223.
29. Kambikubo, K., Acta Pediatrics, 1965, v69, 904.
30. Asboe-Hansen, G., Clausen, J., J. Invest. Derm., 1964, v42, 81.
31. Christiaens, L., Fontaine, G., Cuvelier, R., Pediatrics, 1965, v20, 84.
32. Utermann, D., Klempien, D., Maass, E. J., Klin. Wschr., 1965, v43, 117.

Received February 14, 1967. P.S.E.B.M., 1967, v125.

A New Micro-Neutralization Test for Antibody Determination and Typing of Parainfluenza and Influenza Viruses. (32272)

HERTA WULFF, JOHANNA SOEKEN, JACK D. POLAND, AND TOM D. Y. CHIN
(Introduced by H. A. Wenner)
Virus Diseases Section, Ecological Investigations Program, National Communicable Disease Center, Public Health Service, U. S. Department of HEW, Kansas City, Kan.

The conventional tube method for determining the neutralizing antibody titer of sera for the parainfluenza viruses is relatively time consuming. Recently, Schmidt *et al*(1) reported a micromethod which utilizes the hemadsorption technique for end point determination. The results are read with a standard light microscope. This report describes a new

technique which is a modification of Schmidt's method in that reading of the hemagglutination pattern is done macroscopically. This method facilitates the technical procedures considerably because it is not necessary to establish an intact monolayer in the microtiter plate wells. Furthermore, reading of the plates macroscopically for hemagglutination is considerably simpler and faster than by microscopic examination for hemadsorption.

Materials and methods. Equipment and technique. The microtiter neutralization test was carried out in disposable microtiter plates. The new hemagglutination method was developed by using new style Cooke* microtiter plates† in which cells were grown in suspension but without the necessity of establishing a complete cell sheet in each well; reading was done by hemagglutination. For comparison, selected sera were also tested in old style plates‡ in which a complete cell sheet could be consistently established and reading was done by hemadsorption. The microtiter plates were prepared by immersing them in 70% alcohol for 1 hour; they were inverted and dried under ultraviolet light.

Dropping pipettes calibrated to deliver 0.025 ml and 0.05 ml were used for delivering reagents into the wells. Spiral wire loops calibrated to transfer 0.025 ml were used for diluting the sera. Handling of the equipment has been described in detail by Lennette(2).

To compare this new microtiter method to standard tube techniques, neutralization tests for parainfluenza virus antibodies were also conducted in primary or secondary Rhesus monkey kidney tube cultures; the tube tests were read by hemadsorption(3).

Monkey kidney cells. Secondary Rhesus monkey kidney cells were used. The cells were obtained by trypsinizing primary cultures of Rhesus monkey kidney cells; a suspension containing 150,000 cells per ml was prepared by diluting the cells with an appropriate volume of medium 199 containing 0.5% lactalbumin hydrolysate, 2% chicken serum, and 1.68 g per liter of $NaHCO_3$.

Serum dilution. All serum dilutions and virus antigen titrations were made in medium 199 to which 1.68 g per liter of $NaHCO_3$ were added. The sera were diluted 1:4 and inactivated at 56° C for 30 minutes.

Virus titration. Parainfluenza virus strains used for the micro-neutralization and tube tests were the HA-2 strain of type 1, the CA strain of type 2, and the HA-1 strain of type 3. For microtiter neutralizing antibody determination, the virus was diluted so that 0.025 ml contained 100 $TCID_{50}$. This virus concentration was further diluted in tubes in 10-fold steps to determine the exact virus concentration used in the test. Aliquots (0.05 ml per well) of these tube dilutions were placed in each of 3 plates to be tested at specified intervals in order to determine when the optimal virus titer was achieved for reading the test.

Antibody assay. An 0.025 ml volume of diluting medium was dropped into all wells of the microtiter plates except those in the first row. An 0.05 ml volume of the 1:4 dilution of each serum was dropped into 2 adjacent empty wells and diluted in 2-fold steps with 0.025 ml microtiter loops. An 0.025 ml volume of antigen containing 100 $TCID_{50}$ of virus was added to each well. The virus-serum mixture was incubated in a 5% CO_2 incubator at 37°C for 1 hour then 0.05 ml of the monkey kidney cell suspension was added to each well. The plates were sealed with a special adhesive tape§, and incubated at 37°C in a 5% CO_2 incubator for 5 to 7 days.

On the fifth day of incubation one of the 3 virus titration plates was tested by adding 0.025 ml of a 1% guinea pig erythrocyte suspension in 0.85% saline to each well. The plate was kept at 4°C for 1 to 1½ hours. Reading was done by hemagglutination pattern macroscopically. If the virus titer on day 5 was less than 30 $TCID_{50}$ a second virus titration plate was tested on day 6 and, if necessary, the third plate was tested on day 7. The test was read on the day one of the virus control plates showed between 30 and 300 $TCID_{50}$. The neutralization titer was recorded as the highest serum dilution which

* Use of trade names is for identification only, and does not constitute endorsement by USPHS or by U. S. Dept. of HEW.

† Cooke Engineering Co. No. 220-24A.

‡ Cooke Engineering Co. No. 220-24.

§ Linbro No. 35 PS or Cooke No. 220-30.

TABLE I. Comparison of Neutralizing Antibody Titers in Human Sera Determined by Hemadsorption in Tubes and by Hemagglutination in Microtiter Plates.

Categories	Parainfluenza virus Type 1 No.	%	Type 2 No.	%	Type 3 No.	%	Total tests No.	%
Same titer	8	66	15	71	5	42	28	62
1 dilution deviation (2-fold)	4	34	5	24	6	50	15	33
2 dilutions deviation (4-fold)	0	0	1	5	1	8	2	5
Total	12	100	21	100	12	100	45	100

showed complete hemagglutination inhibition. Cell controls and serum controls were also included in each test.

Typing of isolated hemadsorbing viruses. The new microtiter hemagglutination method was also used for typing of hemadsorbing agents recently recovered from children with respiratory disease. Parainfluenza and influenza hyperimmune sera, prepared in rabbits, were used for the virus typing. The typing sera were diluted to a concentration which neutralized 1,000 $TCID_{50}$ of virus.

Results. Neutralizing antibody determinations in children for parainfluenza virus types 1, 2, and 3. Sera of children 2 to 12 years of age were tested for parainfluenza antibodies with the conventional tube method and the newly established microtiter method. Fifteen of the children had recently experienced a parainfluenza virus infection, as indicated by recovery of virus or 4-fold antibody increase.

Table I shows the comparison of the neutralizing antibody titers determined by hemadsorption in tubes and by hemagglutination in microtiter plates. For comparative testing, from 12 to 21 known positive or negative sera were selected for each parainfluenza virus type. Table I shows that of the 45 comparative titrations, 95% had either an identical titer or a 2-fold difference in titer; 5% resulted in a 4-fold deviation in titer from that determined in tubes. Where deviation was observed, neither method was consistently higher than the other. The 8 negative sera (titer $<1:4$) as determined in tubes were also negative by the new method.

Neutralizing antibody titers for parainfluenza virus type 2 were determined on 69 sera in microtiter plates by the hemadsorption method and by the new hemagglutination method. These 69 included 26 pairs of acute and convalescent sera. Four-fold rises in antibody titer were demonstrated in 17 serum pairs, all of which were detected by both methods. Table II reveals that 94% of the sera tested had the same titer (within one 2-fold dilution) by both methods, and about two-thirds had no deviation between the methods. Six per cent of the sera showed a 4-fold deviation in antibody titer between the 2 methods. There were 15 negative sera ($<1:4$) all of which were negative by both methods.

Identification of hemadsorbing virus isolates by the new micro-neutralization test. The hemadsorbing viruses which were typed by the new micro-neutralization technique had been previously typed by the conventional tube method. The second or third passage of each isolate in monkey kidney tissue culture was used as antigen. The infectivity titer of these isolates was at least 10^{-3} $TCID_{50}$.

Fig. 1 gives a sample of one of the typing tests. The virus control was diluted in 10-fold steps with 6 wells per dilution. In this case the $10^{-3.5}$ dilution was the virus concentration used for the test. For each of the parainfluenza and influenza hyperimmune sera, 4 test wells and 1 control well were used. The isolated virus strain shown in the figure was typed as a parainfluenza virus type 3.

TABLE II. Comparison of Neutralizing Antibody Titers for Parainfluenza Type 2 Virus Determined by Hemadsorption and by Hemagglutination in Microtiter Plates.

Categories	Number	Percent
Same titer	44	64
1 dilution deviation (2-fold)	21	30
2 dilutions deviation (4-fold)	4	6
Total	69	100

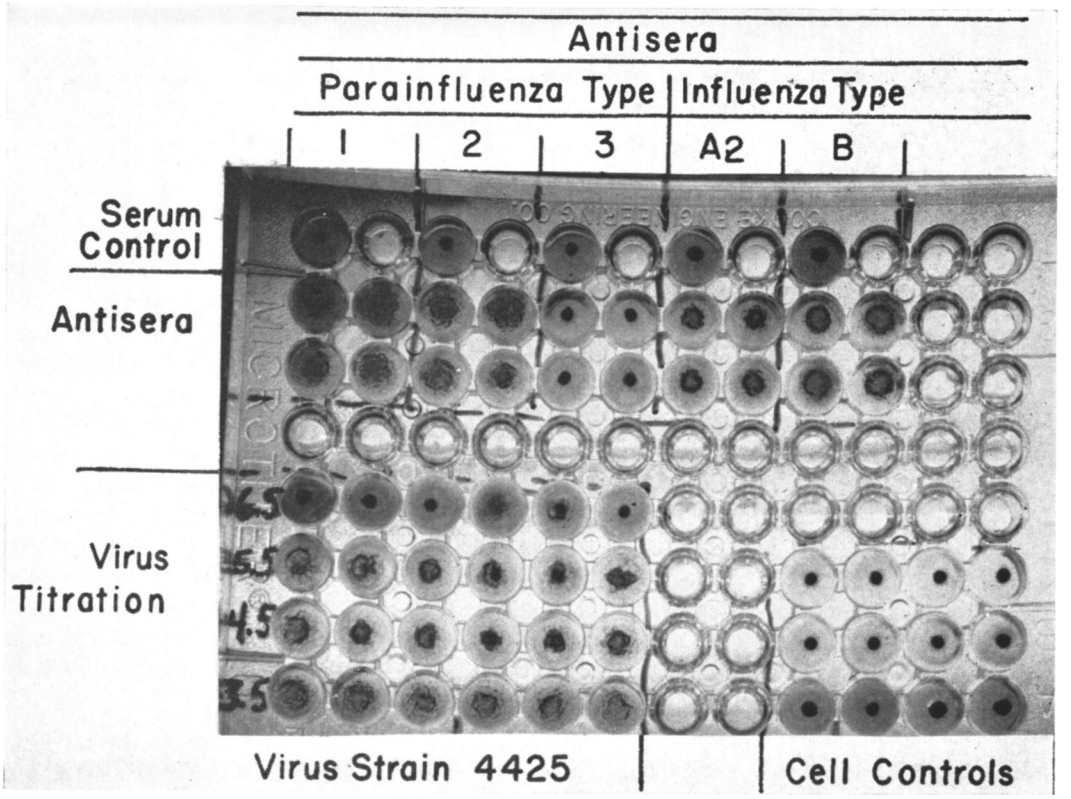

FIG. 1. Micro-neutralization test for typing of isolated hemadsorbing virus.

At the time of this report only a small number of isolates were typed by this method; these included one parainfluenza type 1, two parainfluenza type 3, one influenza type A2, and two influenza type B. For each of these isolates the micro-neutralization method confirmed the identifications made by the conventional tube method. Subsequently, using this method 38 additional strains of parainfluenza types 1 and 3 were identified. No type 2 or type 4 parainfluenza viruses had been encountered.

Discussion. The development of a simple micro-neutralization test for determination of antibody titers for parainfluenza viruses and for typing hemadsorbing isolates will be of great value, particularly to those laboratories participating in parainfluenza vaccine field trials. In our laboratory the microtiter method enables 4 employees to set up a test for 500 sera in about 6 hours. This is an equivalent of using more than 8,000 tubes. A 500 sera microtest can be read macroscopically in 1 hour. This method has an advantage over the recently published microtiter method(1) in which reading is done microscopically, since microscopic examination of the microtiter plates is relatively time consuming and cumbersome.

The end points of the hemagglutination micro-neutralization tests are usually clear cut with either complete hemagglutination in a well or a clear button of guinea pig erythrocytes. In our experience, all sera which were positive or negative by previously described methods gave the same results as by the new technique; also, identical results in terms of 4-fold or greater rises in antibody titers were obtained with both methods.

The only difficulty we encountered was the occurrence of spontaneous agglutination of guinea pig erythrocytes in a few human sera in 1:4 and occasionally in 1:8 dilutions. Spontaneous agglutination could usually be distinguished from specific agglutination by the characteristics of the borderline. Virus

specific agglutination produced a much more diffuse pattern, whereas nonspecific agglutination usually had a more definitive but rough margin and appeared in the serum controls to the same extent. Whenever possible sera were aliquoted and stored at —20°C. The antibody titers to several antigens were determined in a single test; therefore, the serum samples were not frozen and thawed more than twice prior to the determinations herein described. The sera were not kept longer than 1 day at 4°C before testing. This method of handling sera could generally prevent the occurrence of spontaneous agglutination. When spontaneous agglutination did occur, it could usually be eliminated by a second inactivation of the sera for 10 minutes at 56°C.

Summary. A new microtiter method has been developed for determination of neutralizing antibody titers for parainfluenza viruses and for typing hemadsorbing viruses. The microtiter plates are read macroscopically by hemagglutination pattern. The new method has advantages over both the conventional tube technique and a previously described micro-neutralization test. The results obtained with the new method are comparable with those of the previously described methods.

1. Schmidt, N. J., Lennette, E. H., Hanahoe, M. F., Proc. Soc. Exp. Biol. & Med., 1966, v122, 1062.

2. Lennette, E. H., in Diagnostic Procedures for Viral and Rickettsial Diseases, Am. Publ. Health Assn., Inc., New York, 1964, 3rd ed.

3. Chanock, R. M., and Johnson, K. M., ibid.

Received February 14, 1967. P.S.E.B.M., 1967, v125.

Partial Characterization of Urine Protein with Insulin-Like Activity.* (32273)

LILIA B. GUEVERA,† JOSEPH C. MEEK, AND RORERT E. BOLINGER
University of Kansas Medical Center, Kansas City, Kan.

Previous work in this laboratory(1) confirmed the observation of Cahill, Pawan and Chalmers(2) that benzoic acid precipitates of human urine contain a factor capable of augmenting $C^{14}O_2$ production from C-1 labeled glucose by the rat epidymal fat pad. This activity was found to be increased in the urine from obese persons over that from nonobese controls and to increase during prolonged fasting(1). It was designated urinary insulin-like activity (UILA) because of the resemblance to plasma ILA.

The present paper describes a partial purification of the biological activity of an extract from urine of an obese female who excreted large amounts of UILA.

Patient material. The patient was a 27-year-old female with childhood onset of obesity. At time of examination she weighed 300 lb and was otherwise normal. Glucose tolerance was normal and no proteinuria was present. Twenty-four-hour urine collections were taken during feeding and fasting. Levels of UILA previously assayed(1) were elevated during fasting. A 24-hour sample of urine taken during fasting was submitted to the following studies.

Methods. Preparation of urine extracts. Benzoic acid precipitates of urine and the alkaline extracts were prepared for assay by the method described by Bolinger *et al*(1).

Column chromatography. An aliquot of the alkaline extract was diluted with buffer pH 7.6 (0.01 M PO_4, 0.15 M NaCl), placed in Visking dialysis bags (18/32) and dialyzed for 24 hours against the same buffer at 4°C. The bags were then transferred to 0.1 M acetate buffer at pH 4 and dialysis carried out for another 24 hours.

The samples were redialyzed against 0.05 M PO_4 buffer pH 7.6 for 24 hours, removed from the bags and lyophilized. One hundred

* Supported by USPHS Grants A-504 and FR-67.
† Trainee in Metabolism, USPHS Grant AM-5051.