Although it is known that mice so treated show an enhanced susceptibility to many pharmacological agents, histamine, serotonin, Proteose-Peptone, etc., the mechanism involved in probably specific; for other agents, compound 48/80, epinephrine, Bacto-Peptone, etc., are without effect.

Summary. Short ragweed pollen extract is shown to be a poor antigen for mice even with recourse to the pertussis-technic. A subsequent challenge dose demonstrates a striking secondary response. Extracts fail to display any lethal effects in mice previously treated with *B. pertussis*.

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Precipitating Antibody in Normal Human Urine.* (26748)

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Agglutinating antibody has been demonstrated in human urine against several organisms(1-5). Significant amounts of globulins have been found in normal human urine, and some of them were shown to be immunologically and electrophoretically similar to those of the serum(6-10). The present paper reports the demonstration of precipitating antibodies in normal urine.

Materials and methods. Patients. Two groups of patients with normal blood urea nitrogen and urine that was free of protein by the sulfosalicylic acid test were chosen for these studies. In the first group, 4 patients who gave a negative Schick test but whose serum failed to react with diphtheria toxoid were injected with 1.0 ml of purified diphtheria toxoid‡ subcutaneously; specimens of serum and 24-hour collections of urine were obtained at the end of the second and third weeks after the vaccination. The second group consisted of 11 patients convalescing from pneumococcal pneumonia; serums from these patients were tested for precipitins using as antigen the supernatant of a 5-day culture of the pneumococcus isolated from their own sputum or blood. Urine was collected over a period of 48 hours from each of those patients whose serum gave a precipitate in the ring test.

Urine. The urines were collected in clean containers without preservative. Each specimen was filtered through Whatman #12 filter paper and the filtrate was dialysed against running tap water at 4°C for 24-48 hours. Comparisons were first made of the efficacy of several concentration procedures which included: evaporation under negative pressure, pervaporation at 4°C and at room temperature, ultrafiltration(11), ammonium sulfate precipitation(10), and lyophilization. The following method was chosen as the most efficient for the purpose at hand: Immediately following dialysis the urines were concentrated at room temperature by pervapora-

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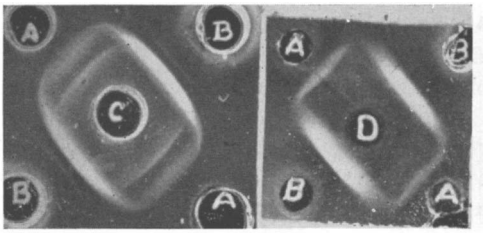
‡ 50 Lf units/ml; obtained from Div. of Biological Labs., Mass. Dept. Public Health.

tion. By using Visking tubing (inflated diameter 20/32 in., wall thickness 0.0008 in.) and large portable fans in a walled-off enclosure, a 24-hour output of urine could be reduced to approximately 50 ml within 8 hours. The concentrated urine was then lyophilized and the resulting dry powder was suspended in 2 to 4 ml of barbital buffer, pH 8.6. The preparation was kept at 4°C overnight, centrifuged, and the clear supernate was used. By this technic, a 24-hour sample of urine could be processed completely within 48 to 72 hours.

Immunological technics. Double diffusion in Agar gel. A modification of the Ouchterlony technic described by Grasset *et al.*(12) was employed. Glass slides, 25 × 75 mm, were overlaid with 4 ml of 2% agar (Difco). The wells were formed with Pasteur pipettes into which agar plugs were drawn up with a rubber bulb attached to the free end; these wells could hold from 0.01 to 0.03 ml of the substance to be tested. After filling the wells, the slides were placed in Petri dishes, the bottoms of which were covered with wet filter paper. The dishes were covered and sealed with vaseline to maintain a high humidity and were kept at room temperature. Depending on the concentrations of the constituents being tested, precipitin lines appeared between 4 and 12 hours. When urine was used as an antibody preparation, up to 48 or 72 hours were necessary for complete development of the precipitin lines and it was often necessary to refill that well several times during the first day.

Immunoelectrophoresis. A modification of the method described by Williams and Grabar(13) was used. The Spinco Model R cell operated with a Heathkit variable voltage regulated power supply, Model PS-3 was employed. With this system, 5 large (50 × 75 mm) or 13 small (25 × 75 mm) slides could be run in parallel. The gradient across each slide was maintained at 40 volts, a 2 to 3 hour run producing satisfactory separation of the proteins. Large slides were overlaid with 9 ml of 2% agar prepared by adding equal volumes of barbital buffer (pH 8.6 and ionic strength 0.075) and 4% agar (Difco) in distilled water. Wells, similar to those



(a) (b)

FIG. 1. Double diffusion in agar of urine (A) and serum (B) with rabbit anti-human globulin (C) and diphtheria toxoid (D). Patient was immunized with diphtheria toxoid.

described above, were cut out of the agar for the substance to be electrophoresed. Following electrophoresis, longitudinal basins were cut out, with razor blades, parallel to the direction of electrical flow; these were filled and the slides placed in a humid atmosphere at room temperature. The precipitin pattern was usually established after 24 hours of diffusion and was completed in 2 or 3 days. Photographs of unstained slides were made with a modified dark field microscopy technic(14).§ In some instances it was found necessary to stain the precipitin lines for better photographic representation; in that event, the preparations were first washed in several changes of physiologic saline for 2 days. After staining with either Ponceau S or Nigrosin (Consolidated Laboratories, Inc., Chicago Heights, Ill.) the slides were dried at 37°C for permanent mounting.

The modification of Wadsworth and Hanson(15) was used to demonstrate the presence of precipitates not observed with the standard immunoelectrophoresis. This involved diffusion of the separated material from an additional basin to increase its concentration above the threshold for formation of a visible precipitate.

Results. Diphtheria. The specimens of concentrated urine from 3 of the 4 patients immunized with diphtheria toxoid reacted with that antigen. The serum of each of these 3 patients formed very clear and dense precipitates, whereas only a weak precipitin line resulted from the reaction of the fourth patient's serum and the toxoid. Fig. 1a il-

§ By Mr. Leo Goodman, Mallory Inst. of Pathology.

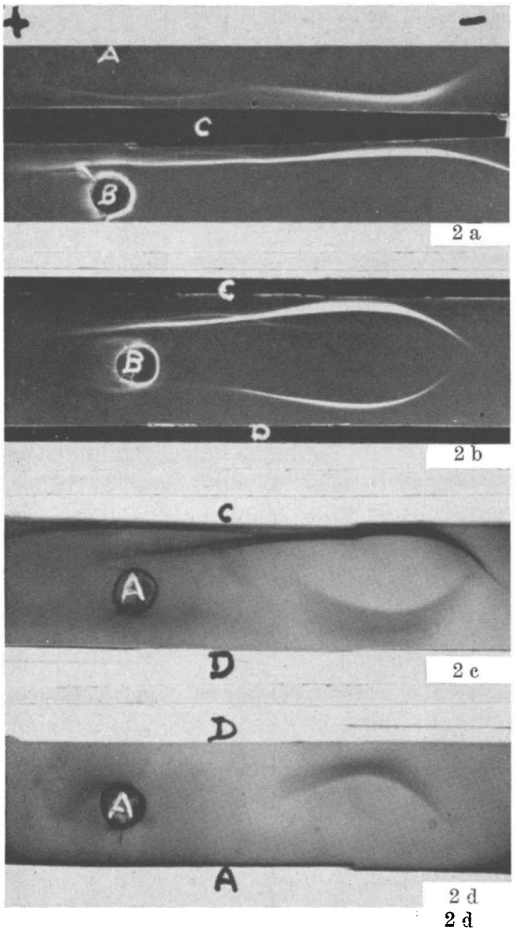


FIG. 2. Immunoelectrophoresis of serum and urine of same patient as in Fig. 1. A = urine; B = serum; C = rabbit anti-human globulin; D = diphtheria toxoid.

lustrates the precipitin lines obtained when a urine preparation and serum from one of these patients were reacted with an anti-human-γ-globulin.¶ The line closest to the urine well showed a reaction of complete identity with the major line formed by the patient's serum. These results are similar to those of Franklin(6) who reacted urine γ-globulin and serum 7S globulin in agar with an antiserum prepared against Cohn Fraction II; 2 or 3 lines were usually present in the case of the γ-globulins prepared from urine and the line closest to the antigen well showed a reaction of identity with that formed by the 7S γ-globulin. Fig. 1b shows the reaction of complete identity produced by the same serum and urine with diphtheria toxoid.

Fig. 2 is a composite of the results obtained by immunoelectrophoresis with the same serum and urine. Fig. 2a shows the similar electrophoretic mobilities of the γ-globulin fractions of serum and urine, and Fig. 2b shows that the antibodies to diphtheria toxoid reside in the γ-globulin fraction of the serum. The precipitate formed by the interaction of urinary antibody and diphtheria toxoid is seen in Fig. 2c in the area corresponding to the electrophoresed γ-globulin of normal serum. Some difficulty was encountered in arranging the optimum proportions of diphtheria toxoid (antigen) and urine (antibody) to form a clear precipitate. The reinforcement technic(15) produced a more satisfactory precipitin line for photographic purposes as shown in Fig. 2d. When the longitudinal basins were arranged to form a rectangle around the separated urine and serum proteins, as suggested by Grant(9), the γ-globulins of serum and urine were found to join, showing, as with ordinary agar diffusion, that they are identical antigenically. Urine and serum collected from 3 Schick positive patients did not react with the diphtheria toxoid.

Pneumococcus. Of the 11 patients convalescent from pneumonia, the serum of 6 showed a visible precipitate by the ring test. By capsular swelling, the pneumococci from 2 of them were identified as type 3, in 2 others as type 4, in 1 as type 7, and in 1 the pneumococcus reacted with a pool of antisera of various types and was not specifically identified. The samples of serum from each of these 6 patients gave precipitin lines with the crude pneumococcal antigen preparation (culture supernates) in the micro-Ouchterlony technic. However, only the urine of the 2 patients convalescing from type 4 infection, and of one who recovered from type 3 pneumonia gave precipitin lines when reacted against the supernatant of a culture of their

¶ The antisera to human γ-globulin (Cohn Fraction II, provided by Protein Foundation, Jamaica Plain, Mass.) were prepared in rabbits by subcutaneous and intramuscular injections with Freund adjuvant and supplied by Dr. Ramzi Cotran, Mallory Inst. of Pathology.

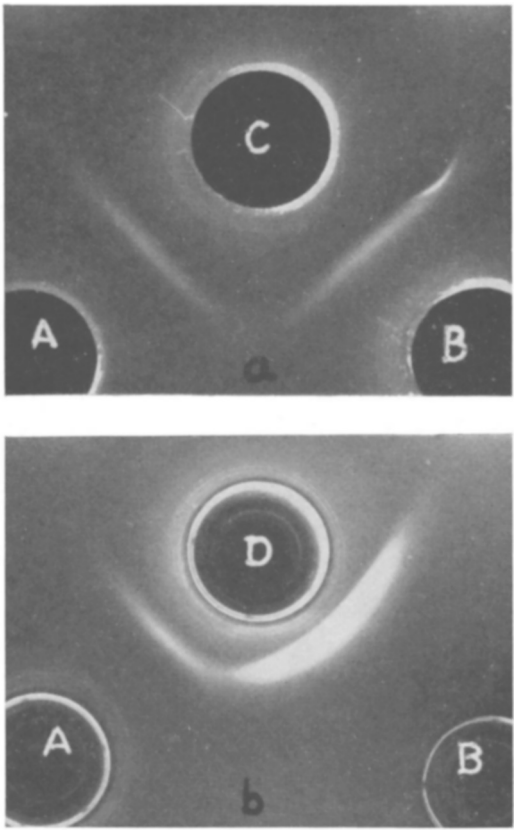


FIG. 3. Double diffusion in agar of urine (A) and serum (B) with culture supernate (C) of the pneumococcus isolated from the patient and with capsular polysaccharide (D) of the same type. The patient was convalescing from type 4 pneumococcal pneumonia.

own organism. An example is given in Fig. 3a.

The specimens of serum and urine which precipitated with their respective culture supernates also reacted with the corresponding purified type-specific pneumococcal polysaccharide.‖ This is illustrated in Fig. 3b. No reaction was discerned between any patient's serum or urine and any culture supernate or specific polysaccharide of a different pneumococcal type.

Discussion. In this study, although precipitating antibody could be demonstrated in the serum of each patient, it was not demonstrable in the urine of some of them by the technics used. Since total daily output of urinary protein varies widely(8), concentration of larger volumes from the other patients might have produced positive results. If the antibody found in urine is derived from the serum, a threshold level may be necessary in the serum for it to be excreted or to become demonstrable in the urine(16). The results of the present study are only qualitative and no attempt at quantitation was made.

Several studies of urinary antibody to specific bacteria have been reported. Burrows and Havens(1) demonstrated agglutinating antibodies to cholera vibrio and to typhoid bacilli in the urine of normal volunteers immunized with the corresponding vaccines. The cholera antibodies seemed to appear and disappear in the urine independently of the levels in the serum, peak titers occurring in the former 16 to 19 days and in the latter 26 to 42 days after inoculation. Unconcentrated urine was used in those studies, but the agglutinins to *V. cholerae* were also demonstrated in globulin isolated from the urine.

Naylor and Caldwell(4), working in Egypt with patients who had urinary shistosomiasis and also were urinary carriers of Salmonella, presented data which they interpreted as indicating that the urinary antibody was derived in part from serum but was also formed locally within the urinary tract. Their patients, like those of Archer, *et al.* in another study(5), had gross proteinuria, so that the results cannot be compared with those of the present study which was done in patients with normal urine; in the urine of such patients they failed to demonstrate flagellar agglutinins after TAB vaccination.

Agglutinating antibody to Leptospira has been found in the urine of patients during acute and convalescent stages of infection with this organism(2,3). The urines of a few of the patients' studies by Stuart(2) were negative for protein when examined by the sulfosalicylic acid test, and the agglutination was demonstrated in voided specimens without prior concentration. Stuart considered urinary antibody to Leptospira to be of considerable diagnostic value because of its re-

‖ The type specific polysaccharides were supplied in 1935 by Dr. Rachel Brown, Bureau of Laboratories, New York State Dept. of Health; the type specific antisera were provided by Lederle Laboratories.

markably greater species specificity when compared with serum of the same patients.

The normal daily urinary excretion of protein varies widely in different individuals and may be as high as 150 mg in 24 hours(6,8,10, 17,18). Rigas and Heller(8) found the mean normal excretion in 24 hours of protein to be 39.0 mg in 17 normal subjects. The average for albumin and globulin was 14.8 mg and 25.8 mg respectively, with an average A/G ratio of 0.51, the reverse of that found in normal serum. In the present study the total protein in the 24-hour urinary output of the 11 patients studied averaged 43.5 mg (range 23-60 mg) by the Biuret method(19).

Electrophoretic analysis of the proteins present in normal urine revealed mobilities corresponding to those of the 5 electrophoretically distinguishable components of normal serum(8). Certain of the physicochemical and immunochemical properties of the biocolloids (of normal urine) have been characterized by Webb, Rose, and Sehon(7) and by Franklin(6). They demonstrated urinary proteins which were antigenically closely related to serum γ-globulins but which differed from them in being smaller in size. Molecular weights of these globulins ranged from 10,600(7) to 35,000(6). Franklin reported a sedimentation constant of approximately 1.6 S for the major constituent of the γ-globulin fraction of normal human urine and demonstrated, by isotopic studies, that this constituent is derived primarily from the serum γ-globulin fraction. An additional component corresponding to the 7S γ-globulin of serum was also demonstrated but never exceeded 10% of total urinary protein. This more rapidly sedimenting component increased in absolute and relative concentration and constituted more than 75% of the total γ-globulin fraction in 5 patients whom they studied; these patients who had pyelonephritis, glomerulonephritis, or nephrosclerosis excreted 5 to 10 g of urinary protein per day.

Isotopic studies(6,18) add support to the hypothesis that urinary γ-globulins are derived from the corresponding serum fractions. However, Franklin was not able to demonstrate the presence of the low molecular weight globulins in the sera of patients with renal shutdown in whom an accumulation of these proteins might be expected. It is not clear whether these small globulins are products of breakdown or stages in the synthesis of the normal serum components and also whether they are added to the urine after completion of glomerular filtration or originate in the tissues of the urinary tract(6,18).

Since 7S γ-globulin is present in normal human urine it is probable that the antibodies demonstrated in the present study reside in that fraction. However, Porter's isolation of biologically active fragments with sedimentation coefficients of about 3.5S and molecular weights of 50,000 to 80,000 from a papain digest of rabbit γ-globulin antibody suggests that the small globulins in normal urine may act as antibodies(20).

The biological significance of the urinary antibodies is not clear. Since the antibodies in the urine of healthy persons are constantly diluted by the large amounts of urine, a marked protective effect locally seems unlikely. It is conceivable, however, that even such small amounts of specific antibodies in the urine might interfere with the isolation of certain pathogens, *e.g.*, Leptospira(2), viruses(16), by their direct effect on the organisms or by acting as opsonins. In the presence of gross proteinuria such as might accompany a febrile illness, such an effect may be even more likely.

Summary. The presence of precipitating antibody was demonstrated to diphtheria toxoid in the urine of patients immunized with that antigen and to pneumococcal polysaccharide in the urine of patients convalescing from pneumococcal pneumonia.

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Virological Studies on Acute Respiratory Disease in Young Adults. I. Isolation of ECHO 28.* (26749)

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The JH and 2060 viruses were originally isolated from cases of mild acute respiratory disease and have been reported to induce this type of illness in human volunteers(1,2,3,4). Recent laboratory investigations have failed to detect any significant antigenic differences between these 2 viruses(5,6), which have now been designated ECHO 28 by the Committee on ECHO Viruses(6). No additional isolations of ECHO 28 have been reported and its incidence has been deduced entirely by serological methods(5,7). The present report describes the isolation of 8 strains of ECHO 28 from young adults with naturally occurring mild acute respiratory infections.

Materials and methods. Study population. One hundred and one freshmen and sophomore medical students, 5 of whom were women, were enrolled as volunteers in this project. The range in age was from 20 to 31 years. At approximately 6-week intervals throughout the academic year the total group was sampled as described below and served as asymptomatic controls. Each individual reported to the laboratory at the very first signs of acute respiratory disease, when additional samples were taken. They returned 2 to 3 weeks later for convalescent specimens.

Samples. Separate dry throat and nose swabs were taken in duplicate. One set was placed in 0.5% bovine serum albumin in phosphate buffered saline for virus isolation. The second nose and throat swabs were placed in individual tubes of broth for bacteriologic studies. A portion of the samples for viral study were inoculated into tissue cultures on the same day, and the remainder was frozen at –40°C. Serum specimens were also obtained and stored at –20°C.

Tissue cultures. The H.Ep. 2 line was carried in our laboratory on Eagle's medium (MEM) with 10% calf serum. Cultures used for virus isolation were maintained on Eagle's medium with 5% inactivated chicken serum and were incubated in stationary slanted racks at 37°C for at least 14 days after inoculation. Monkey kidney tissue cultures were prepared from cells that had been grown in bottles, trypsinized and frozen according to the method of Stulberg *et al.*(8). The cell suspension, stored at –90°C, was quickly thawed and diluted in growth medium containing 0.5% lactalbumin, 2% calf serum and 0.2% SV-5 rabbit antiserum to provide cultures with islands rather than a complete monolayer of cells. These cultures were maintained on 50% Medium 199 and

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