place throughout the entire serum series, the serum is diluted 1:10 in the urea medium and the test is then repeated with diluted serum. The level is calculated in the same manner, multiplied by the dilution factor.

Experimental results. It was found that the standard inoculum was consistently inhibited by 0.8 μg of the standard aureomycin. Thus the minimum assayable concentration of aureomycin in the serum is 0.8 μg per ml of serum. Although this is not as sensitive as methods in current use, it is adequate for clinical purposes, since the minimum detectable level is well within the range of blood concentrations regularly found with the doses commonly employed.

To test the accuracy of this procedure, 36 samples of normal human serum were fortified with known concentrations of aureomycin just prior to testing and then assayed. The results of the assay proved accurate in 28 out of 36 tests, or 77.8%. In the remaining tests there was a discrepancy between the prepared and calculated values, but in no instance was there any error greater than one tube, except in one, which was two tubes under. Considering the experimental error arising from the preparation of the test specimen as well as the performance of the assay, a high correlation was found between the concentration of the prepared specimens and the result of assay.

Summary. A rapid method for the determination of the aureomycin concentration of the blood is described. Sterile specimens and sterile technic are not required. The procedure is based upon the inhibition of the test organism, *Proteus vulgaris* OX-19 by the antibiotic, thereby preventing the reaction and color change produced in the urea-phenol red medium when urea is split to ammonia by the test strain. The minimum assayable level is 0.8 μg per ml of serum. A high degree of correlation was found between the actual value and the results of assays with 36 prepared specimens.

I am greatly indebted to Miss Joan Greenwald for her capable technical assistance.

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Characterization and Separation of an Inhibitor of Viral Hemagglutination Present in Urine. (17825)

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Hemagglutination caused by influenza, mumps and Newcastle disease viruses is inhibited by a number of biological materials which possess such activity in varying degree(1-3). The various inhibitors have certain properties in common: when tested with appropriately heated viruses, they are active in high titer, but their activity is destroyed by the unheated viruses; it is also destroyed by cholera vibrio extract, crystalline trypsin, or periodic acid. The inhibitors are substances of high molecular weight and are thought to be mucopolysaccharides or mucoproteins. The interaction between inhibitors and viruses has been studied in considerable detail by numerous workers(4-7), but purification of the inhibitors has met with difficulties which have not been completely overcome(7-14). Among the most

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TABLE I.
Inhibition of Hemagglutination by Different Viruses with Normal Human Urine.

Virus	Units, final	Urine* inhibition titer	Virus	Heated, °C	Units, final	Urine* inhibition titer
PR8	3	16		56 30′	2	1024
FM1	2	8		″ ″	2	512
Lee	2	4		″ ″	2	512
Swine	2	1024		52 ″	2	64000
Mumps	2	128		46 20′	2	128
NDV	3	4		50 ″	2	32

* A single specimen of urine from a normal adult male was employed. It was neither dialyzed nor heated.

active of the viral inhibitors is a purified erythrocyte extract which, in an amount of 0.1 μg, inhibits hemagglutination by 1 unit of influenza virus (PR8) at 4°C(8).

The present study is concerned with the isolation and characterization of a substance present in normal urine which possesses in extraordinary degree the capacity to inhibit hemagglutination by influenza, mumps and Newcastle disease viruses. Not only is the substance present in the urine of human beings, but it is found in that of the cat, the rabbit, and the guinea pig as well.

Materials and methods. Urine. Urine from normal men was used in most experiments. It was collected in sterile flasks and stored at 4°C.

RBC. Blood from individual chickens was collected into ACD(15) and stored at 4°C. RBC were washed 3 times with 0.85% NaCl buffered at pH 7.2 with 0.01 M phosphate, and diluted to a 1% suspension.

Viruses. FM1, Lee, PR8, swine or NDV was inoculated in 10-day chick embryos. Allantoic fluid was harvested 48 hours later, centrifuged at 3,000 r.p.m. for 20 minutes, the supernate distributed in plastic tubes, frozen, and stored at −70°C. For each test a tube was thawed and the contents used but once. Mumps virus was inoculated in 7-day chick embryos and allantoic fluid was harvested 5 days later.

Hemagglutination tests. Serial 2-fold dilutions of infected allantoic fluid were made in 0.4 cc of buffered saline and 0.4 cc of RBC suspension was added to each tube (final concentration = 0.5%). Tests were read after 1 hour. The last tube showing strong agglutination (2-3+) was considered as the endpoint.

Hemagglutination-inhibition tests. Serial 2-fold dilutions of urine or inhibitor preparation were made in 0.2 cc of buffered saline. Allantoic fluid, diluted so as to yield 2 units of virus in the *final* mixture, was added in 0.2 cc amount to each tube. The mixtures were kept at room temperature for 1 hour, then 0.4 cc of the RBC suspension was added and the tests were read after another hour. The tube showing complete inhibition of hemagglutination was taken as the endpoint.

Experimental. Results of a representative experiment on the hemagglutination-inhibition titers of urine determined with different viruses, both unheated and heated, are shown in Table I. Of the viruses tested, swine influenza was most susceptible to inhibition. PR8, FM1, and Lee gave low inhibition titers before and 64-fold higher titers after heating. NDV yielded the lowest titer of all. With mumps virus 20 minutes of heat-

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TABLE II.
Effects of Treatment of Urine on Hemagglutination-Inhibition Titer.

Treatment of urine*	Inhibition titer†
None	128
Dialysis vs. 0.14 M NaCl	128
" " dist. H_2O	2048
" " " " followed by paper filtration	2048
Paper filtration, undialyzed urine	128
" " , followed by dialysis vs. dist. H_2O	128
Undialyzed urine, 7000 g, 30′, supernate	128
" " " " ", sediment in 0.14 M NaCl	0
" " " " ", " " dist. H_2O	2048
" " 56°C, 30′ (after adjustment of pH to 6.5)	128
" " 70°C, 30′ (after adjustment of pH to 6.5)	2048

* Specimens of urine from 1 normal adult male were used.
† Determined with Lee virus, heated at 56°C, 30′; final concentration, 2 units.

ing at 46°C (heat tolerance of hemagglutination) did not increase susceptibility to inhibition. In addition, urine inhibited hemagglutination with GDVII virus(16) in moderate titer but did not affect hemagglutination with PVM.

Inhibitor titers with RBC from different chickens. Anderson(17) showed that RBC from certain chickens were unsuitable for demonstrating the inhibitory activity of normal serum. A similar phenomenon had been encountered previously(18). RBC from 11 individual chickens were used in inhibitor titrations with a single urine specimen. Both heated Lee and PR8 were employed. With the RBC from 9 chickens, all inhibitor titers fell between 128 and 1024. RBC from 2 chickens showed no capacity to act as indicators for the inhibitor against Lee, although with PR8 usual inhibitor titers were obtained. These observations were confirmed on repeated testing. In all experiments reported in this paper, RBC from individual chickens known to be satisfactory in inhibitor titrations were employed. Guinea pig and human erythrocytes also can be used satisfactorily in inhibitor experiments with urine.

Effects of treatment of urine. In most instances the actual concentration of inhibitor is higher than is indicated by the titer of the untreated urine. Table II shows that only about 6% of the inhibitor could be accounted for when fresh untreated urine was employed. The titer after dialysis against water or after heating at 70°C when the pH was adjusted to 6.5 was 16 times higher than prior to such treatment. However, the titer did not increase after dialysis against 0.14 M NaCl, or when dialysis was preceded by filtration through paper. Centrifugation of untreated urine at 7,000 g caused no reduction in the inhibitor titer, but yielded an appreciable amount of mucoid sediment which gave no inhibition when resuspended in saline. When the sediment was resuspended in distilled water, it showed a high inhibitor titer. Urine of low specific gravity (*e.g.* 1.005) showed no rise in inhibition titer on dialysis against water. On the average such urine showed a titer of 512. Urine with high specific gravity (*e.g.* 1.028) showed marked increase in titer on dialysis, *e.g.*, from 16 to 8192. Thus, specific gravity can be used as a criterion for predicting the change in inhibitor titer which occurs on dialysis.

Comparison of inhibition titers of heated (70°C) or dialyzed morning urine from 7 normal men against Lee and PR8 revealed that the titers did not differ markedly, ranging from 1024 to 8192. The differences were correlated directly with variation in specific gravity. Inhibition titers of dialyzed urine obtained on different days from the same persons showed similar variation. Urine

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from normal women gave inhibition titers comparable to those from men. In addition, urine from cats showed inhibition titers of moderate degree; that from rabbits and guinea pigs gave lower titers. When sediments obtained by centrifugation of fresh urine were dissolved in distilled H_2O, they gave high inhibitor titers. However, when to such a solution NaCl was added to 0.58 M and the mixture held at 4°C for 24 hours, no free inhibitor could be detected. Under these circumstances the solution became turbid; if the inhibitor concentration was sufficiently high, precipitation of the inhibitor occurred. As the amount of salt added was reduced so as to yield 0.29 M or less, increasing amounts of free inhibitor were demonstrable and the titer at 0.036 M NaCl was equal to the maximum titer of the urine after dialysis against water. The progressive decrease in inhibitor titer in the presence of salt concentrations of 0.14 M or more is a slow process and may not reach equilibrium for long periods (1-2 days); the higher the salt concentration, the shorter the period required. However, the increase in inhibitor titer at low salt concentrations is very rapid and occurs in a few minutes.

Quantitative relationship between inhibitor and virus. When the quantities of urine and heated Lee virus employed in inhibition experiments were varied with respect to each other, the results illustrated in Fig. 1 were obtained. The relationship between the two variables was one of multiple proportions only when both were in high concentration. At lower concentrations the ratio between virus and inhibitor became 2:1 and at extreme dilution may be as great as 3:1. This phenomenon was not due to loss of hemagglutinating activity of virus at high dilution because there was no appreciable drop in virus titer when comparable dilutions of allantoic fluid were kept at room temperature for 10 hours. Similar experiments were carried out with a urine dialyzed against water which caused a 32-fold increase in inhibitor titer. The results were closely similar to those shown in Fig. 1. When mixtures of varying quantities of inhibitor and virus were held for 12 hours before the addition of RBC, the relationship between the two variables had the same form as shown in Fig. 1. However, the inhibitor titers were increased 4-fold. This is in agreement with the progressive rise in inhibition titer with time described below.

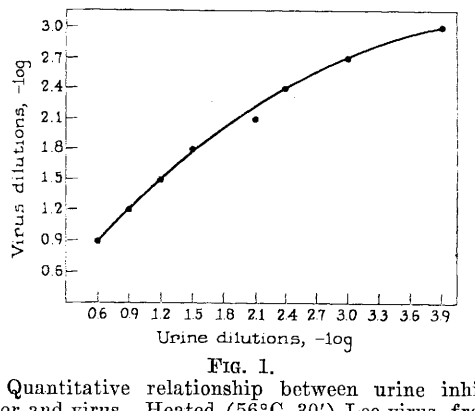

FIG. 1.
Quantitative relationship between urine inhibitor and virus. Heated (56°C, 30′) Lee virus, from 1 to 128 units, and dilutions of normal untreated urine were employed. The urine showed no increase in inhibitor titer on dialysis against distilled H_2O.

Increase in inhibition titer with time. Mixtures of urine dilutions and a constant amount of either Lee or PR8 (4 units per tube) were kept at room temperature for periods up to 21 hours before addition of RBC. Control titrations were carried out simultaneously by mixing inhibitor and virus, each of which had been kept separately in comparable dilutions under identical conditions. In addition, control virus titrations were carried out with comparable dilutions which were held for equally long periods. Results of a typical experiment with Lee virus are shown in Fig. 2. The inhibitor titer showed a rapid rise during the course of the first hour. During the following 11 hours the rise continued, but at a much slower rate. Maximal inhibitor titers were not reached until 12 hours. Control titrations with virus and inhibitor dilutions kept separately for similar periods showed no increase in titer when mixed, nor did the hemagglutination titer of the virus suspension decrease during the course of the experiment. Identical results were obtained with a dialyzed urine that showed a 16-fold rise in titer after dialysis, as well as with a purified prepara-

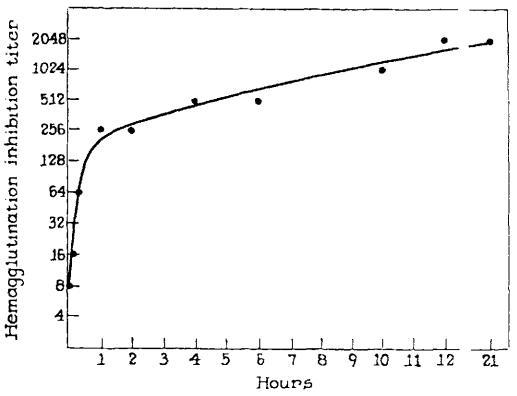

FIG. 2.

Increase in inhibitor titer with increase in time of interaction between inhibitor and virus. Heated (56°C, 30′) Lee virus, 4 units, and dilutions of normal untreated urine were employed. The urine showed no increase in inhibitor titer on dialysis against distilled H_2O.

tion of inhibitor. Moreover, dialyzed urine and the purified preparation, when tested at 4°C, gave closely similar results. Thus, titrations of the inhibitor should be allowed to react for 12 hours before addition of RBC if the system is to reach equilibrium. For practical reasons, a 1-hour period of interaction was employed in this study. Lanni and Beard(6) demonstrated that with egg-white and swine influenza virus maximal titers are obtained after about 30 minutes of incubation at 28°C. Subsequently, they showed(14) that the inhibitor titer of cow's milk increased as incubation was prolonged to 90 minutes.

Pseudo - hemagglutination. When RBC from some chickens are mixed with certain urine specimens, agglutination of RBC occurs in the absence of virus. Usually the titers are low(2-8) but occasionally values of 16 to 32 are obtained. Such pseudo-hemagglutination can interfere with the determination of the inhibitor titer, particularly if the latter is low. The pattern of pseudo-hemagglutination is variable: it can closely resemble that caused by viruses, but more frequently it is coarser in type. Heating urine at 70°C for 30 minutes eliminates pseudo-hemagglutination, regardless of whether the RBC or the urine was the factor of greater importance.

Destruction of inhibitor. Lee or PR8 virus as well as cholera vibrio filtrate destroys the inhibitory activity of urine upon incubation at 37°C for 1-2 hours. Pneumococcal hyaluronidase in a concentration of 0.001 mg/cc eliminates inhibitor activity under similar conditions. Crystalline trypsin in a concentration of 0.5 mg/cc had no effect on inhibitor titer after incubation at 37°C for 24 hours; after 7 days, however, the titer was reduced by 99%. Lithium periodate in a concentration of 0.00025 M reduced the inhibitor titer by 98% in 2 hours at 23°C. Heating dialyzed buffered urine at 70°C for 8 hours caused no drop in inhibition titer. Heating at 80°C for 30 minutes reduced the titer by 98% but after 2 hours there was no further reduction. Similar results were obtained at 100°C; after a rapid drop in titer (marked reduction was demonstrable at 1 minute), no further reduction took place during 2 hours.

Purification of inhibitor. Precipitation with alcohol.* The addition of 6 volumes of 95% ethyl alcohol gave the best yields of inhibitor. The mixtures were held at 22-24°C and the precipitates obtained were redissolved in water. To obtain maximal yields, it was necessary either to use undialyzed urine or to employ 9 times concentrated urine which was dialyzed before precipitation. The results of one experiment were: To 2 liters of urine was added 12 liters of 95% alcohol and the mixture held at 22-24°C for 48 hours. The precipitate was collected in the centrifuge, and dissolved in 200 cc of distilled H_2O. A small amount of inert material was separated by centrifugation and the supernatant removed, concentrated to 40 cc *in vacuo* and dialyzed against distilled H_2O at 4°C. The material (P) was dried from the frozen state: 115 mg of a pale brown powder, readily soluble in H_2O was obtained. The inhibition titer at a concentration of 1 mg/cc was 128,000 against 2 units of heated Lee. Analytical tests yielded: Molisch reaction: negative before but positive after acid hydrolysis; biuret test: negative; nitrogen: 9.8%; phosphorus: 0.94%; ultraviolet absorption

* Dr. Walther F. Goebel, The Rockefeller Institute, kindly cooperated in the purification studies.

curve: peak at 2750 Å.

Fraction P was further purified as follows: a 0.75% solution, containing 85 mg and 5% of sodium acetate, was shaken 5 times with chloroform-octyl alcohol mixture (4:1)(19). The supernatants (S) were collected and pooled. The cakes in the chloroform layer also were combined, mixed with an equal volume of 95% ethyl alcohol, and the precipitate (C) was collected by centrifugation and redissolved in water. This and the pooled supernatants (S) were dialyzed against water. The yields were: S = 23 mg; C = 34 mg. The inhibitor titers at concentrations of 1 mg/cc were 128 and 4,096,000, respectively. Comparing these values with the titer of fraction P: *i.e.* 128,000, it appears that only 0.1% of activity remained in the aqueous phase (S). On the other hand, the precipitate (C), which represented less than half of fraction P on a weight basis, showed an inhibitor activity 32 times greater than that of P.

The analytical data with the highly active fraction (C) were: Molisch reaction: positive without acid hydrolysis; biuret test: positive; nitrogen: 12.5%; phosphorus: 0.00%. Ultraviolet absorption curve showed a peak of the same height as given by fraction P with a maximum at 2800 Å, and considerably accentuated minima on both sides. The relatively inactive aqueous phase fraction (S) was biuret negative, but Molisch positive and the ultraviolet absorption curve showed no peak at 2800 Å. Computations showed that 1 unit of heated Lee was inhibited by 0.004 μg of the original alcohol precipitate (P); by 0.4 μg of the material that remained in the aqueous phase (S); and by 0.0001 μg of the substance obtained from the chloroform phase (C).

Isoelectric precipitation at pH 3. A 2.5% solution in water of material obtained by alcohol precipitation gave a precipitate when the pH was lowered to 3 with 1 N HCl. After reprecipitation, the material was dissolved in water, brought to pH 7.0 with 1 N NaOH and frozen and dried *in vacuo*. This acid insoluble fraction represented half of the starting material by weight, but was twice as active in inhibition tests. When undialyzed urine was brought to pH 3, only a small amount of precipitate was obtained which showed low activity. However, when 2 liters of urine was concentrated 40 times, and then precipitated in the same way, 60 mg of a slightly brownish material was obtained which showed an inhibitor titer of 256,000 (1 mg/cc; 2 units heated Lee). Comparing this result with that obtained when 2 liters of urine was precipitated with alcohol, it appears that acid precipitation yields material twice as active as and less pigmented than the alcohol procedure (fraction P).

Sodium chloride precipitation. As stated above, the inhibitor in urine is precipitated in the presence of 0.58 M NaCl. This property was used in separation of the inhibitor by filtration and centrifugation. Filtration through porcelain candles or fritted glass discs in the presence of sufficient salt provided separation of inhibitor and urinary pigment. When a urine (specific gravity 1.010) was filtered through a Coors No. 3 candle, no inhibitor appeared in the filtrate. The filter candle was then washed with 0.14 M NaCl until no pigment appeared in the washings. Such washings contained no inhibitor. When distilled water was passed through the filter, all the inhibitor was released. The final water wash (equal in volume to the urine filtered) showed an inhibitor titer 64 times higher than the untreated urine. Similar results were obtained with Selas No. 2 filter candles and with fritted glass filters (Corning). With large volumes of urine *medium* fritted glass filters gave satisfactory results and high yields of inhibitor. Best results were obtained when urine was diluted with 1 volume of distilled H_2O, NaCl was added in a concentration of 0.58 M, the pH was 5-6, and the urine was held at 4°C for 24 hours prior to filtration. The substance is highly viscous and centrifugation in an angle head may cause part of the material to adhere along the wall of the tube. However, the bulk of inhibitor in urine (specific gravity 1.020) was recovered in the sedi-

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ment after centrifugation at 7,000 g for 30 minutes. After washing with 0.58 M NaCl and recentrifugation, the sediments were frozen and dried. In two experiments a titer of 64,000 (1 mg/cc; 2 units heated Lee) was found.

Discussion. The presence in normal human urine of a potent inhibitor of viral hemagglutination makes readily available a rich source of this substance and one essentially free of protein. Separation of the inhibitor from urine can be accomplished without difficulty and yields of purified material, active at 0.0001 μg/cc, are of the order of 17 mg/liter.

The chemical properties of this substance indicate that it has much in common with similar inhibitors found previously in other biological materials and suggests that, like these, it too is a mucoprotein. The striking effects of salt concentration upon the activity of the inhibitor as well as upon its filterability and sedimentability appear to be referable to its solubility properties in electrolyte solutions. This view is supported by the finding that the inhibitor is precipitated in NaCl solutions of 0.6 M. However, there is also evidence to suggest that a heat labile component in urine may combine with the inhibitor in the presence of salt and lead to some of the observed effects.

Summary. Normal human urine contains a highly active inhibitor of viral hemagglutination, effective against PR8, Lee, FM1, swine, mumps, NDV, and GDVII viruses but inactive against PVM. The inhibitor concentration may approach 2 mg%. Considerable amounts of purified material, active at 0.0001 μg/cc, can be obtained readily. The activity of the inhibitor as it occurs in urine is inversely related to the salt concentration; it should be emphasized that in NaCl solutions of moderate strength it precipitates.

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A Feathering Syndrome in Chicks After Feeding Optimal Levels of Lysine in Absence of Arginine. (17826)

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It has been shown that arginine is important for the prevention of an abnormal feathering syndrome caused by a glycine deficiency in chicks(1) and that a lysine deficiency causes an abnormal pigmentation of the feathers in poults(2). During nutritional studies with milo gluten meal (a 44% protein byproduct of starch manufacture from milo and other grain sorghums) a peculiar feathering abnormality developed in chicks fed a diet containing 50% milo gluten meal supplemented with 2% DL-lysine. Milo gluten meal was the only source of protein in the above diet. This syndrome developed consistently in 5 successive groups of experimental chicks. This study was initiated to determine the nature of the abnormal feathering syndrome.

Experimental. New Hampshire chicks were used in these experiments. The chicks were housed in electrically heated battery brooders with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wingbanded when one day old, observed daily, and weighed weekly for a period of 4 weeks.

The basal diet (MG-1) contained 50% milo gluten meal (calculated to provide 22% crude protein), 4% salts IV, 3% corn oil,

* Supported by a grant-in-aid from Corn Products Refining Company, Argo, Ill.

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