striction of uterine growth is obtained on the combined treatment of 0.1 μg estradiol 17β plus 10 μg 16 epi-estriol. Of particular significance is the fact that a summation of the two estrogens does not obtain, even though the maximal growth potential of the uterus is at least 50% more than that reported for 0.1 μg estradiol 17β(3).

This substance does not seem to alter the vaginal cornifying effects of estradiol 17β. The vaginal smears of those animals on 0.1 μg estradiol 17β with and without 16 epi-estriol seem to be relatively indistinguishable, both showing a preponderance of cornified epithelial cells with degenerative nuclei. Thus, the restrictive action of 16 epi-estriol seems to be mainly on the uterine growth-promoting effects of estradiol 17β.

These results quite clearly parallel those of Hisaw, Velardo, and Goolsby(3), who reported that estriol likewise inhibited estradiol 17β on the growth of the uterus. Furthermore, Huggins and Jensen(4) showed that 16 epi-estriol was quite efficacious in restricting the effects of estrone on the uterus in hypophysectomized rats. Therefore, these results seem wholly consonant with the thought that the various estrogens and/or their metabolites act *in concert* to produce the end organ result (3). These experiments provide additional data that give credence to the concept that an endocrine response is accomplished through a precise ratio of the hormone and/or its metabolites.

Summary. The adult, ovariectomized rat was used to ascertain the effects of a new estrogen, 16 epi-estriol, on the activity of estradiol 17β. These results indicate that when 3 daily doses of 5 to 25 μg of 16 epi-estriol are subcutaneously injected into rats receiving 0.1 μg estradiol 17β daily for the 3-day period, the response of the uterus to estradiol is restricted. Normally, 3 daily doses of 0.1 μg estradiol 17β increase the uterine wet weight from the value of the ovariectomized rat from approximately 116 mg to 227 mg. The simultaneous administration of 5, 10, or 25 μg of 16 epi-estriol in addition to the estradiol treatment reduces the response to 191 down to 173 mg. The inhibitory action is likewise reflected in the dry weight data. No apparent modification of the vaginal cornifying action of estradiol could be induced by 16 epi-estriol.

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Hemolysis of Sheep Erythrocytes Sensitized with Leptospiral Extracts. (22113)

CHARLES D. COX.

Department of Microbiology, University of South Dakota School of Medicine, Vermillion.

Serodiagnosis of leptospirosis presently is based on agglutination, agglutination-lysis (AL), and complement-fixation procedures. The agglutination test employing killed leptospirae has been found to parallel the AL test in its type specificity(1). Gochenour(1) found that complement-fixation tests employing sonically-disrupted leptospiral antigens(2) showed a broader spectrum than AL procedures. Various modifications of AL procedure, such as that of Stoenner(3), may alleviate some difficulties associated with AL technic, in this case the hazard of working with living leptospirae. However, several serotypes of leptospirae known to be involved in leptospirosis make it necessary to employ several antigens in the above technics used as screening tests, the choice of serotypes depending on specificity of procedure and the purpose of the investigation(4). The need

for screening procedure employing antigen with specificity sufficiently broad to manifest antibodies produced against all serotypes of leptospirae would seem apparent. Recent progress was made by Chang and McComb (5), who described a hemagglutination (HA) procedure using human blood group O erythrocytes. Their HA test exhibited fairly broad specificity, based upon titers which were, however, very moderate in comparison with those of the highly type-specific AL reaction. Several reports(6-10) appeared concerning microbial substances capable of sensitizing erythrocytes in the presence of homologous antiserum to lytic action of complement (C'). Preliminary studies in this laboratory demonstrated that ethanol extracts of leptospirae were capable of sensitizing sheep erythrocytes to subsequent agglutination by homologous rabbit antisera, confirming the work of Chang (5) using human group O cells. Further investigation showed that sensitized sheep erythrocytes were susceptible to lysis in the presence of high dilutions of homologous rabbit antisera and complement.

This report describes the performance of the hemolytic test (HL) and results of studies to ascertain its specificity and sensitivity in comparison with the AL and HA procedures.

Materials and methods. Cultures—L. pomona, L. icterohemorrhagiae B, L. bataviae, L. grippo-typhosa, L. hebdomadis, and *L. canicola B* were obtained from Dr. Harold S. Bryan, College of Veterinary Medicine, University of Illinois. *L. autumnalis, L. icterohemorrhagiae C, L. canicola C* were obtained from Dr. R. S. Chang, Harvard University School of Public Health. *L. canicola R, L. hyos, L. ballum,* and *L. pyrogenes* were obtained from the Veterinary Division, Army Medical Service Graduate School. All cultures were grown in a medium consisting of 0.2% sterile tryptose-phosphate broth (Difco). Before use, sterile rabbit serum was added to final concentration of 10% and final medium heated in 56°C waterbath for 30 minutes. This medium was simple to prepare and yielded leptospiral growth comparable with other contemporary leptospiral media. *Diluent.* Phosphate-buffered isotonic saline was used in AL and HA procedures. It consisted of 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M KH_2PO_4, yielding pH 7.2. Veronal-buffered saline was prepared as described by Mayer(11) and used as diluent in the HL procedure. *The AL Procedure* followed that of Gochenour(1), except that sera were decimally diluted. *Immune sera* were obtained from rabbits injected with viable 5 to 7-day-old cultures of leptospirae. Before use in HA and HL tests, each serum was inactivated and adsorbed with 0.1 volume of washed sheep erythrocytes. Pre-inoculation sera were collected from each rabbit, and checked for presence of leptospiral antibodies by all 3 procedures. *Leptospiral extracts* were prepared essentially as reported by Chang(5), with the exception that the 90% ethanol precipitate was washed in absolute ethanol, dried, and weighed. Dried, weighed extract was then redissolved in merthiolated saline to final stock concentration of 0.2 mg/ml. In later experiments, incorporation of veronal diluent at pH 7.4 in stock solution maintained extracts at constant activity in the HL test for several months. One fraction has been so maintained with constant activity for over 8 months. Active extracts were prepared from all leptospiral cultures excepting *L. grippotyphosa* and *L. ballum,* from which extracts have been prepared with at most only slight HL activity. Considerable difference occurred between HL activities of extracts obtained from different culture growths of the same organism. *Sensitization of Erythrocytes for HA and HL Procedures.* One volume of 10% suspension of washed sheep erythrocytes was mixed well with 10 volumes of appropriate dilution of leptospiral extract. Erythrocyte suspension and fraction dilution were prepared in appropriate diluents. The mixture was agitated intermittently for 1 hour in 37°C waterbath, centrifuged, and the packed erythrocytes washed once with diluent. The sensitized erythrocytes were then reconstituted to a 1% suspension in appropriate diluent. *HA Procedure.* One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. The cell control contained sensitized

TABLE I. Reciprocal Agglutination-Lysis Titrations.*

	Anti-leptospiral rabbit sera											
Leptospiral antigens	*autumnalis*	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
autumnalis	*4*	1	—	—	—	1	2	—	—	4	2	—
ballum	—	*4*	—	2	2	—	—	—	1	—	—	4
bataviae	—	—	*5*	—	—	—	2	1	1	—	—	—
canicola B	—	2	—	*4*	5	1	1	—	2	—	—	2
canicola C	1	2	—	4	5	2	1	—	3	1	—	2
canicola R	—	2	—	4	*5*	—	—	—	3	—	—	3
grippo-typh.	—	—	—	—	—	*4*	1	—	1	2	1	—
hebdomadis	1	—	—	—	—	—	*5*	—	—	—	—	—
hyos	—	—	2	1	—	1	1	*5*	—	—	—	—
ictero B	1	2	—	3	3	—	—	—	*4*	—	—	3
ictero C	3	—	—	—	1	—	1	—	1	*5*	2	—
pomona	2	—	—	—	—	—	—	—	—	2	*4*	—
pyrogenes	—	4	—	2	2	1	—	1	1	—	—	*5*

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

erythrocytes and diluent. Serum control contained lowest dilution of serum and non-sensitized erythrocytes as a check on adsorption. Tubes were agitated and incubated at 30°C for 16-20 hours, and read by gently flicking the tube. *HL Procedure.* One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. Two exact units of guinea pig C′ in 0.2 ml volume were added to each tube, followed by addition of 0.3 ml of diluent. Serum control contained non-sensitized in place of sensitized erythrocytes. C′ control contained diluent in place of serum, and sensitized cell control contained sensitized erythrocytes plus 0.9 ml of diluent. Tubes were agitated and incubated 1 hour in 37°C waterbath and read for hemolysis in usual manner, recording hemolysis as complete, partial, and negative. *Standardization of Leptospiral Extracts for HA and HL Procedures.* Double dilutions of the extract starting from 0.2 mg/ml were used to sensitize erythrocytes in the HA procedure. Such sensitized erythrocytes were then set up against master dilutions of known homologous immune rabbit sera in HA and HL procedures. Titration of all dilutions of extract against each serum dilution in HL tests was made in presence of 0.2 ml of 1:30 dilution of guinea pig C′ per tube, which was consistently found to represent an excess of C′. The end-point or concentration of extract used in further tests was selected as highest dilution, or smallest amount, above which there was no decrease in activity. Acceptable levels of extract activity demonstrated homologous immune serum HA titers between 1:10 and 1:100 and HL titers of 1:100 to 1:10000. Satisfactory HA and HL activity was usually achieved with extract concentrations ranging from 2.0 to 200 mg/ml. Some extracts in high concentrations hemolyzed erythrocytes in diluent; however, the end-point was always several dilutions higher than that which caused hemolysis. *Titration of C′ for Use in HL Procedure.* Using leptospiral extract which had been diluted according to previous standardization and the usual decimal dilutions of homologous immune serum, series of titrations were set up, each with increasing 0.05 ml increments of guinea pig C′ diluted 1:30. Volumes of C′ ranged from 0.05 to 0.3 ml. The end-point or 1 exact unit of C′ was taken as the smallest amount showing complete lysis in highest dilution of immune rabbit serum, and 2 exact units in 0.2 ml volume were used thereafter in HL procedure.

Results. Reciprocal AL titrations between various leptospiral serotypes and their homologous and heterologous antisera were carried out. It is apparent from Table I

TABLE II. Reciprocal Hemolytic Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera: *autumnalis*	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
autumnalis	3	3	4	3	3	2	4	4	3	2	3	3
bataviae	2	3	3	2	2	2	2	3	2	2	2	2
canicola B	2	3	2	2	2	2	2	3	2	2	2	2
canicola C	3	4	4	3	4	3	4	4	3	3	3	4
canicola R	3	3	3	3	3	2	3	4	3	3	3	3
hebdomadis	2	2	2	2	2	2	2	2	2	2	2	2
hyos	3	3	3	2	3	2	3	3	3	2	2	2
ictero B	3	4	3	3	3	2	3	4	3	3	3	3
ictero C	3	3	4	4	4	3	4	4	4	4	3	4
pomona	3	4	5	4	4	3	4	5	4	5	3	4
pyrogenes	2	2	2	2	2	2	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction.

that AL reaction is quite type-specific as is generally recognized. The same master dilutions of immune rabbit sera were used to set up similar reciprocal HL titrations (Table II). HL reaction is extremely group specific. Antibodies produced against any one of the leptospiral types reacted broadly with all leptospiral fractions studied. The finding of higher heterologous than homologous titers with some antisera is felt to reflect differences in activity between fractions obtained from different leptospiral types. For example, highly active fractions were obtained consistently from our cultures of *L. pomona, L. icterohemorrhagiae,* and *L. canicola C.* However, as mentioned previously, only the slightest activity was obtained from extracts prepared from *L. ballum* and *L. grippo-typhosa.* Fractions obtained from *L. pyrogenes* were consistently low in HL activity. On the basis of results in Tables II and III, *L. pomona* was selected as the culture from which we obtained extracts for use in screening human sera for leptospiral antibodies by HL and HA procedures.

Selected leptospiral extracts were used to sensitize sheep erythrocytes which were then cross-titrated in HA procedure with homologous and heterologous immune rabbit sera (Table III). Although only selected extracts were used in HA titrations, it should be mentioned that, as in HL titration, extracts prepared from *L. ballum, L. grippo-typhosa,* and *L. pyrogenes* consistently possessed minimal activity or were completely inactive. The HA reaction is apparently similar in spectrum to the hemolytic reaction; however, HA titers were consistently lower, which causes the degree of crossing to appear less significant than in HL procedure.

TABLE III. Reciprocal Hemagglutination Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera: *autumnalis*	*ballum*	*bataviae*	*canicola B*	*canicola R*	*grippo-typh.*	*hebdomadis*	*hyos*	*ictero B*	*ictero C*	*pomona*	*pyrogenes*
autumnalis	2	2	1	2	2	1	2	2	2	1	2	2
canicola C	2	2	2	2	2	1	2	2	2	1	2	2
canicola R	1	1	1	1	1	1	1	1	1	—	1	1
ictero C	2	2	2	2	2	1	2	2	2	1	2	2
pomona	2	2	2	2	2	1	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

TABLE IV. Comparative Titrations on Human Sera.

No. of sera showing result		Result using following titrations: AL	HL	HA
505		—*	—	—
9		+†	—	—
10		—	+	—
23		—	—	+
1		+	+	—
24		—	+	+
0		+	—	+
8		+	+	+
Total sera	580			
Total positive	75	18	43	55
% positive	12.9%	3.1%	7.4%	9.5%

* Negative = No reaction in dilution of 10^{-1} or higher.

† Positive = Complete reaction in dilution of 10^{-1} or higher.

To compare relative activity of each of the 3 procedures, AL, HA, and HL titrations were performed on 580 human sera submitted at random from hospitals and clinics in this area (Table IV). With none of the patients was an initial clinical diagnosis of leptospirosis considered. All 3 procedures were performed as previously described, and the AL test was performed using 3 types: *L. pomona, L. ictero B,* and *L. canicola B.* It should be mentioned that of the 580 sera, only 2 febrile patients were available for repeat serum titrations. One patient showed progressively increasing titers to both AL and HL procedures to peak at 1:10000 levels. The AL titers were against *L. icterohemorrhagiae* and *L. canicola,* and the HA titer remained at 1:10 throughout. The other patient never showed a positive AL titer with any of the 3 leptospirae used as antigens; however, the HL titer progressively increased during course of the illness to a level of 1:10000, and the HA titer rose to a level of 1:100. Six of the positive sera showed AL titers of 1:1000 or higher, and 3 showed AL titers of 1:10000 or higher. In a similar fashion 21 sera showed HL titers of 1:1000 or higher and 5 were as high as 1:10000. None of the sera showed HA titers above 1:100.

It should be noted that the apparent advantage of the HA procedure in detecting antibodies missed by the other two procedures (23 sera) was based upon an HA reaction in serum dilutions of only 1:10, without which the positive HA reactions would drop to 1.1%. The reason for this number of weak HA reactions and their connection with leptospirosis is not known. The sera had been adequately adsorbed with sheep erythrocytes, as evidenced by negative controls. In contrast, positive reactions in AL and HL procedures were based predominantly upon complete reactions in serum dilutions of 1:100 to 1:10000.

Ability of HL procedure to detect antibodies in titers of 1:100 or higher missed by AL procedure could be readily explained on the basis of differences in specificity of the reactions. Thus, it is felt that a total of 31 of the 34 sera in Table IV may be showing high HL titers stimulated by serotypes of leptospira other than the 3 used in these AL tests. Ability of the AL test to detect antibodies missed by the other 2 procedures (9 sera) is at present unexplained; however, it is possible that this reflects differences in persistence of antibody levels detectable by the 3 procedures. Clarification of this point would depend upon the difficult task of coupling similar studies on selected human cases of leptospirosis with adequate cultural studies, extending over many months from very early in the course of the illness.

If leptospiral extracts should prove to remain stable over long periods of time with respect to HL activity by other workers, it would seem that the HL procedure would show promise as a convenient and useful tool with which to screen human sera for leptospiral antibodies. Although it requires an extra step in the addition of complement over the HA procedure, it is as easily read, more rapid in performance, and considerably more sensitive. It is certainly less hazardous to the operator than the AL procedure. HL procedure would seem to warrant further study as a screening tool by those laboratories where adequate bacteriological control studies as well as patient followups would be available.

Summary. Leptospiral extracts are capable of sensitizing sheep erythrocytes to the HL action of homologous and heterologous lepto-

spiral antiserum and complement, and description of the HL procedure is presented. The HL procedure was shown to be broadly specific, in sharp contrast to the high degree of type specificity seen in the AL procedure. In comparison with HL titrations, the HA reaction was also found to have broad specificity but to be considerably less sensitive than the HL reaction. All 3 procedures were compared in a study involving 580 randomly selected human sera, from which data it would seem that the HL procedure warrants further study as a possible screening tool for the rapid detection and titration of leptospiral antibodies in human sera.

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Failure of Synthetic 5-Hydroxytryptamine Creatinine Sulfate to Enhance Clot Retraction of Platelet-Poor Human Plasma.* (22114)

SERGIO I. MAGALINI† AND MARIO STEFANINI.‡

Saint Elizabeth's Hospital and Department of Medicine, Tufts University School of Medicine.

Many platelet constituents with specific effects on the hemostatic mechanism have been recently identified(1). Whether they represent intimate constituents of platelets or are carried by them still requires investigation. These agents include a thromboplastic factor (2), constituents accelerating conversion of prothrombin to thrombin and of fibrinogen to fibrin(3), an antiheparin factor(4), possibly antifibrinolysin(5,6). An additional substance, 5-hydroxytryptamine (serotonin), has been identified in dried platelets preparation (7,8) and shown to liberate from platelets during clotting of human blood(9). Serotonin has been shown to possess vasoconstrictor and capillary protecting activity(10). Recently, it has also been suggested that serotonin is able to induce clot retraction of platelet-poor bovine and human plasma(11,12). This observation is of considerable importance since clot retraction has been generally considered to be due to intact platelets rather than to a single platelet constituent. This study is an attempt to evaluate the clot retraction promoting effect of the commercially available synthetic derivative of serotonin, 5-hydroxy-tryptamine creatinine sulfate, the use of which is currently recommended in the treatment of the bleeding tendency of thrombocytopenic states. At the same time, observations have been made on the ability of this compound to influence the rate and extent of prothrombin conversion during clotting of platelet-poor plasma.

Materials. Native platelet-poor plasma.

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† Damon Runyon Fellow in Cancer Research.

‡ Established Investigator, American Heart Assn.