there be at least 10,000 infectious particles present in the original virus suspension and that the virus be an RNA-containing one.

Several attempts have been made to produce infectious nucleic acid by the same method from vaccinia, a presumed DNA-containing virus. So far all such attempts have been unsuccessful despite the fact that some were carried out in parallel with successful enterovirus experiments, using similar quantities of virus and the same materials throughout.

Summary. 1) RNA-containing fractions have been prepared from crude tissue culture virus suspensions of 6 varieties of enterovirus by the method of Gierer and Schramm. Each RNA preparation has produced plaques on human cell monolayers. The plaque-forming agent is completely inactivated by RNAase but not by DNAase. RNA is, therefore, an essential component for the infectivity of the preparation. 2) The plaques formed by RNA fraction contain intact virus from which RNA is prepared. The RNA directs the cell to replicate not only the specific RNA, but also a new highly specific protein needed for synthesis of intact original virus.

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Assay and Properties of Serum Inhibitor of C'1-Esterase* (25034)

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Evidence has been presented that the first component of human and guinea pig complement (C'1) exists in serum as a proenzyme which may be activated by antigen-antibody complexes(1-5). Human C'1 was also activated by plasmin(1,6) and, in a partially purified state, by autocatalysis(6,7). Two activities were demonstrable for human "activated C'1" (C'1-esterase): hydrolysis of certain synthetic amino acid esters, of which N-acetyl-L-tyrosine ethyl ester (ALTEe) was most susceptible(6-8); and inactivation of the second and fourth components (C'2 and C'4) of human complement(3,7). Both activation of C'1 and enzymatic activity of C'1-esterase were inhibited by a property of fresh human serum which was non-dialyzable, heat-labile (56°-30 min.), and unrelated to any of the 4 recognized components of complement(6,8). However, the precise relationship of the serum inhibitor of activation and of esterolysis could not be defined in the absence of purification studies. The present investigation was undertaken to provide information for subsequent purification of the serum inhibitor of C'1-esterase. The assay and properties of this inhibitor in normal serum are described and a comparison is given of levels of inhibitor in various mammalian sera.

Materials and methods. C'1-esterase—Partially purified human C'1 was prepared by published procedures(7) and activated autocatalytically by adjustment of physico-chemical conditions (pH 7.4, ionic strength 0.15, 37°, 15 min)(6,7). Rabbit C'1 and C'1-esterase were prepared from normal rabbit serum by identical procedures. *ALTEe*—N-acetyl-L-tyrosine ethyl ester, synthesized in the Dept. of Chemistry of Western Reserve Univ., was used as a substrate for C'1-esterase. The ester was dissolved in 2-methoxyethanol (methyl cellosolve) to a stock concen-

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tration of 1.6 M. *Mammalian Sera.* Blood was drawn without anticoagulant, clotted at room temperature for about 1 hr, and held overnight at 1-5°. Serum was separated by centrifugation in the cold and either maintained at 0° for use within 24 hr or frozen at −45°. Blood was obtained from healthy human donors by venipuncture; from rats and guinea pigs by exsanguination from the carotid artery; and from mice, rabbits and monkeys by cardiac puncture. Mouse serum represented a pool from 16 CF-1, female white mice anesthetized intraperitoneally with Nembutal® (25-35 mg/kg) and bled by cardiac puncture after thoracotomy. Monkey (rhesus) serum was obtained through the courtesy of Dr. F. C. Robbins, Cleveland Metropolitan General Hospital, and bovine, porcine, and ovine sera through the courtesy of Swift and Co., Cleveland. *Assay of C'1-esterase* (6,8). Esterase activity was measured by microformol titration of the acid liberated from ALTEe during 15 min. incubation with enzyme at 37°, pH 7.4, ionic strength 0.15, and a final substrate concentration of 0.08 M. Typically, 1.88 ml of pH 7.4 phosphate buffer of ionic strength 0.15 and 0.5 ml of an appropriate dilution of esterase were preincubated at 37° and 0.125 ml of ALTEe at 37° was then added. One ml aliquots were withdrawn at 0 time and at 15 min. and added immediately to one ml of neutralized (phenolphthalein) 37% formaldehyde. Each sample was microtitrated with a one ml microsyringe (Micrometric Instrument Co., Cleveland) using 0.05 N NaOH and one drop of 1% alcoholic phenolphthalein as indicator. Esterase activity was expressed in units based on the difference between the 15 min. and 0 time titrations. One unit was defined as that amount of enzyme which hydrolyzed an amount of ALTEe equivalent to a net titration of 0.01 ml of 0.05 N NaOH.

Results. 1) Assay of serum inhibitor of C'1-esterase. The studies to be described were confined entirely to inhibition of esterolytic activity of C'1-esterase. Accordingly, the assay for serum inhibitor was a modification of the assay for esterase activity(8). Various volumes of human serum, taken to 0.5 ml with 0.15 M NaCl, were preincubated

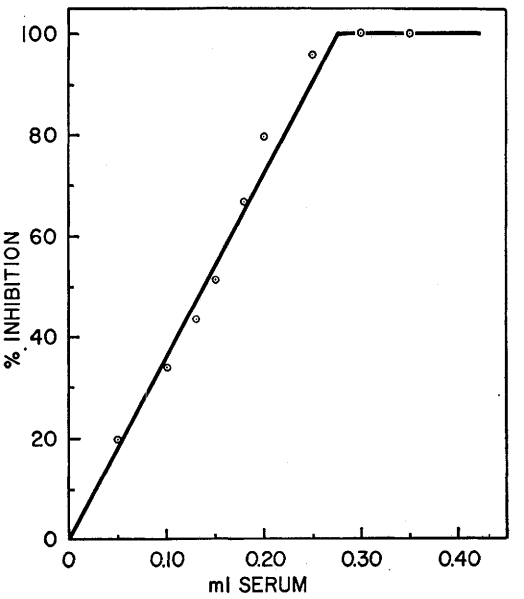

FIG. 1. Proportionality curve for inhibition of human C' 1-esterase (38 units/ml) by normal human serum.

at 37° with a constant amount of human C'1-esterase and phosphate buffer at a final pH of 7.4 ± 0.4 and ionic strength of 0.15. ALTEe was added, the amount of residual free enzyme measured by microformol titration, and the amount of esterase inhibited calculated. The assay was found to be linear over the entire range of inhibition (Fig. 1). The effect of varying length of preincubation time of esterase and inhibitor was studied by reacting enzyme, buffer, and serum at 37° in the absence of ALTEe. At intervals of 1, 10, 20, 30, 60, and 120 min., aliquots were withdrawn, substrate added, and esterase inhibition determined. Maximal inhibition was found within 1 min. and was unaffected by further incubation(8). The effect of varying the concentration of esterase was also studied. In every case, a given amount of serum inhibited the same *number* of units of esterase regardless of initial concentration of enzyme, indicating that inhibition was stoichiometric (Table I). One unit of inhibitor was defined as that volume of serum which inhibited 10 units of esterase, as measured by micro-formol titration.

On the basis of these studies, the following assay procedure was adopted: 1.38 ml of pH

TABLE I. Stoichiometry of Interaction of Serum Inhibitor with C' 1-Esterase: Various Concentrations of Esterase with Constant Serum Inhibitor.*

Esterase added, units/ml	Esterase inhibited, units/ml
16.4	11.4
23.8	10.8
32.8	10.7
36.9	10.8
41.0	12.6

* 0.1 ml of human serum, taken to 0.5 ml with 0.15 M NaCl.

7.4 phosphate buffer at ionic strength 0.15 was preincubated at 37° with 0.5 ml of human C'1-esterase at a concentration of 40 ± 2 units/ml and various volumes of the serum to be assayed for inhibitor, taken to 0.5 ml with 0.15 M NaCl. After 5-10 min., 0.125 ml of 1.6 M ALTEe at 37° was added, the mixtures were incubated at 37° for 15 min., and the residual free enzyme was determined by titration in the usual manner. Number of units of inhibitor in the unknown serum could then be calculated. For example, if 0.25 ml of serum inhibited 15 units of enzyme, the serum contained 1.5 units of inhibitor in 0.25 ml or 6 units/ml. Since the enzyme-inhibitor interaction was stoichiometric, inhibitor levels could be determined at any point on the inhibition curve. An enzyme control in the presence of substrate and absence of inhibitor was always included to reestablish the potency of the enzyme.

The precision of the assay was determined by 10 measurements of the inhibitory activity of a single human serum at each of 3 concentrations (0.10, 0.15 and 0.20 ml of serum). The mean of the 30 determinations was 7.2 units/ml with a range of 6.3-8.1 and a standard deviation of ± 0.42. *2) Some properties of serum inhibitor of C'1-Esterase.* a) *Time-temperature stability.* The inhibitor in human serum was stable for at least 24 hr at 0°, 21° and 37° and for at least 4 months at −45°. It was unaffected by freezing and thawing 2 times. During incubation for 30 min, 20% of the inhibitor was inactivated at 52°, 90% at 56°, and 100% at 60°. b) *pH stability.* Aliquots of human serum were adjusted to various pH values with 0.15 N HCl or 0.15 N NaOH, brought to constant volume with 0.15 M NaCl, and allowed to stand overnight at 0°. Each sample was then readjusted to neutrality and assayed. The inhibitor was stable under these conditions between pH 6 and 10 but progressive inactivation occurred at pH values below 6. Complete inactivation was observed at pH 3.6; 65% inactivation at pH 4.8. c) *Effect of alcohols and ammonium sulfate.* The inhibitor was increasingly labile to methanol concentrations greater than 15% at −5°. Similarly, as reported previously(8), the inhibitor did not survive fractionation of human serum with ethanol by procedures of Cohn *et al.*(9). However, essentially all of the inhibitory activity of human serum could be recovered from the supernatant fraction resulting from precipitation with 40% ammonium sulfate. 3) *Comparative levels of serum inhibitor of C'1-Esterase in various mammalian sera.* Serum from 4 of the 9 species tested contained demonstrable inhibitor of human C'1-esterase (Table II). Human, monkey and guinea pig sera had comparable and relatively high titers of inhibitor, while rabbit serum was only about one-third as active. Rat and mouse sera contained spontaneous esterase activity and therefore no measurable inhibitor. Bovine, porcine and ovine sera demonstrated neither spontaneous esterase nor inhibitory activity. The inhibitor in rabbit and guinea pig serum had the same thermal lability as in human serum. Monkey serum was not tested.

Since human C'1-esterase was used in these experiments, the possibility existed that the variation in inhibitor levels among different species was a reflection of species specificity. A partial answer to this problem was provided by preparing and activating partially purified

TABLE II. Serum Inhibitor of Human C' 1-Esterase in Various Mammalian Sera.

Serum	Avg inhibitor titer, units/ml
Human (6 pools of 25)	6.4 (5.6-7.2)
Monkey (2)	5.0
Guinea pig (3)	5.2
Rabbit (3)	1.8
Rat (3)	0
Mouse (pool of 16)	0
Bovine (1)	0
Porcine (1)	0
Ovine (1)	0

TABLE III. Species Specificity of Rabbit and Human Sera as Sources of Inhibitor *vs* C'1-Esterase Prepared from Rabbit or Human Serum.*

Inhibitor source	C'1-Esterase source	Inhibitor titer, units/ml
Rabbit serum	Rabbit	2.0
" "	Human	3.0
Human "	Rabbit	5.0
" "	Human	7.7

* Each serum was assayed against equivalent amounts of rabbit and human C'1-esterase under the usual conditions.

rabbit C'1 by the same procedure used for human C'1(6.7). The inhibitory activity of rabbit and human sera *vs.* rabbit and human C'1-esterase was then compared (Table III). Rabbit serum was again found to have a lower titer of inhibitor than human serum even when tested against rabbit esterase. It was concluded, therefore, that at least with respect to these two species, no marked species specificity existed in the interaction of C'1-esterase and serum inhibitor.

Discussion and Summary. An assay is described for measurement of a serum inhibitor of an esterase derived from preparations of the first component of complement (C'1-esterase). Esterolysis of N-acetyl-L-tyrosine ethyl ester by C'1-esterase is inhibited instantaneously and stoichiometrically by fresh human serum. Units of C'1-esterase and of serum inhibitor are defined. The inhibitor in human serum is stable at 4° for at least 4 months, at 37° for at least 24 hr. and at 48° for at least 30 min. It is completely inactivated during 30 min. incubation at 60° and is labile at 0° at pH values below 6. The inhibitor is inactivated at −5° in the presence of methanol concentrations of 15% or greater but is recovered quantitatively in a 40% ammonium sulfate supernatant fraction. These data are being applied to purification of the inhibitor from human serum. Purification procedures and properties of the purified inhibitor will be reported elsewhere.

Monkey and guinea pig sera inhibit human C'1-esterase to about the same extent as human serum, while rabbit serum is about one-third as active. No marked species specificity exists in the interaction of inhibitor in human or rabbit serum with human or rabbit C'1-esterase. These observations are being employed on possible role of inhibitor in experimental hypersensitivity.

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Relationship of Infectious Canine Hepatitis Virus to Human Adenovirus (25035)

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A virus strain (Utrecht) was isolated from a fatal case of hepatitis contagiosa canis (HCC) in trypsinized dog kidney cell culture. Inclusions as described by Leader(1) were found in nuclei of these cells when stained with hematoxylin and eosin. In cross complement-fixation tests with a reference dog serum* and a reference virus strain,† the iso-

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