was generally of low titer. Future study must determine whether small amounts of SV-40 stimulate antibodies which effectively inhibit further propagation of SV-40 or whether the virus is capable of continued multiplication until it reaches a concentration sufficiently high to cause a neoplasm.

Summary. Continued multiplication of SV-40 has been demonstrated in 2 continuous lines of human cancer cells, in HeLa for approximately 8 months and in HEp-2 cells for a period of 6 months. Infection of the HeLa cells and to a lesser extent the HEp-2 cells was accompanied by changes in the gross appearance of the cultures depending upon the concentration of SV-40 in the inocula. The concentration of SV-40 in the fluid from the human cell cultures was high and neoplasms were induced in hamsters by fluid from 2 different cultures. SV-40 was also shed into the nutrient fluid of CE cultures kept for 6 months. The infectivity titers were low as determined in CMK and hamsters injected with fluid from a 91-day-old infected CE culture failed to develop neoplasms within a 160-day period. It was determined that approximately $10^{5.5}$ ID_{50} virus was necessary to induce neoplasms in hamsters within a period of 215 days.

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Mechanism of the Effect of Trypsin on Specific Hemagglutination with Incomplete Rh Antisera.* (27903)

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Certain Rh antisera, as well as those of other blood group systems, react with but do not agglutinate homologous cells suspended in saline. Among the methods utilized to detect these incomplete (cryptagglutinoid) Rh antibodies are (1) suspension of RBC in colloidal medium, (2) Coombs antiglobulin reaction, (3) complement fixation, (4) agglutination inhibition, and (5) enzyme treatment of cells. This report is concerned with the mechanism by which trypsin, one of the active enzymes, produces this serologic effect.

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Materials and methods. Crystalline trypsin was obtained from Nutritional Biochemicals Corp. Incomplete anti-C (rh′), anti-D (Rh_o), anti-E (rh″), and anti-c (hr′) were obtained from Wadley Research Inst. and from Ortho Pharmaceutical Corp. The experimental methods largely parallel those employed in studies with papain(1). Serologic tests were performed using the 2 minute procedure previously described(2). Cells are treated with enzyme for 2 min at 37°C,

washed, treated with antiserum for 2 min, centrifuged, and examined microscopically for agglutination. Enzymatic assay of trypsin was performed with tosylarginine methyl ester (TAME) as substrate(3). For certain experiments trypsin was inactivated by reaction with diisopropyl fluoro phosphate (4). Trypsin-I-131 was prepared by the iodination procedure of Rajam and Knorpp (5) except that carrier NaI was eliminated to increase the specific activity of the product. Diisopropyl phosphoryl trypsin (DIP-trypsin) was similarly iodinated. In experiments to determine the binding of enzyme to cells, packed RBC were added to an equal volume of labelled protein. After 2 min incubation, the supernatant was removed, and the cells were washed 6 times (until wash counted at background level). The residual I-131 on RBC was then determined with Nuclear-Chicago scintillation well detector DS-202 and scaler model 172. Serial passage experiments were conducted by removing the supernate after contacting trypsin with an equal volume of RBC and adding it to a second batch of RBC of volume equal to that of the trypsin solution. This process was repeated 8-10 times.

Results. It has been shown(1) that the 2-minute incubation was sufficient for trypsin to produce the serologic effect of interest. This effect may be defined as the specific agglutination of a saline suspension of human enzyme-treated red cells by an incomplete antibody.

Using enzyme labelled with I-131, experiments were performed to determine whether trypsin became bound to RBC under conditions producing the serologic effect. The specific radioactivity of the trypsin ranged from 83-250 CPM/mg and from 96-224 CPM/mg for DIP-trypsin. Three different cell donors were used; their blood type and probable Rh genotype were O, CDe/cde; O, cDe/cde; and A, cDE/cde. The results of these experiments are summarized in Table I. Values for enzyme concentrations of 1.0 mg/ml or greater are arithmetic means of 2-5 determinations. It is seen that both trypsin and DIP-trypsin became firmly attached to the cells. Despite the increased quantity of the latter which was bound, only trypsin produced the serologic effect. DIP-trypsin failed to do so with anti-C, anti-D, anti-E, or anti-c. Enzymatic activity of DIP-trypsin with TAME was less than 5% the activity of untreated enzyme. At concentrations greater than 0.5 mg/ml, the fraction of enzyme bound was independent of its concentration in the incubation mixture, but total quantity was proportional to total amount of trypsin in contact with the RBC.

TABLE I. Adsorption of Trypsin–I-131 and DIP-Trypsin–I-131 by Red Blood Cells.

Enzyme concentration (mg/ml)	Residual I-131 on RBC (%)	
	Trypsin	DIP-trypsin
2.4	20.0	52.8
2.0	22.8	49.4
1.2	24.3	47.8
1.0	21.6	64.7
.6	22.3	61.6
.5	23.3	54.4

In several instances, RBC treated with trypsin were used as a possible source of enzyme in an assay system with TAME. No hydrolysis of substrate due to trypsin bound to cells was detected. In other experiments supernate from cells treated with trypsin at a concentration of 2 mg/ml were serially passed to other cells through 8 passages, and the serologic effect was still detectable. When the initial concentration of trypsin was reduced to 0.1 mg/ml, the serologic activity of the supernate quickly disappeared.

Another approach to the question of enzyme binding to RBC has been to measure residual enzymatic activity in the supernatant recovered from incubation of trypsin with cells. When concentrations of trypsin giving nearly complete hydrolysis of TAME were used with RBC, no significant difference in activity between supernate and enzyme control solution was observed. At lower levels of enzyme, supernate gave decreased hydrolysis of TAME compared to control values. In these data summarized in Table II the values have been corrected for hydrolysis of substrate by red cell supernate without added trypsin. The serologic activity disappeared below the 0.05 mg/ml level.

Discussion. When classified in terms of

TABLE II. Trypsin Activity in RBC Supernates.

Trypsin concentration (mg/ml)	TAME hydrolysis (% of control)	Serologic activity
.05	58	+
.01	24	—
.001	9	—

the mechanism by which proteolysis is effected, trypsin is a serine protease while papain, ficin, and bromelin are thiol proteases (6). Despite this basic difference a number of features in the present data parallel the findings with the latter 3 enzymes(1). Like the other enzymes, trypsin becomes bound to red cells during the 2-minute incubation period as indicated by studies with trypsin-I-131. Trypsin must bind at a site different from its enzymatic site or the inactivated DIP-trypsin would not become attached. That the latter does bind without producing the serologic effect indicates that the enzymatic site must be intact in order to effect agglutination. These findings are similar to the ones for thiol proteases in that papain inhibited by *p*-chloromercuribenzoate also bound to the red cells but did not produce the serologic effect until the enzyme was activated.

The loss in enzyme activity noted when assaying trypsin in RBC supernates by TAME hydrolysis is in accord with the concept of enzyme remaining bound to the cells. The fraction of enzyme bound at these low concentrations (Table II) is greater than that bound at higher trypsin concentrations. The quantitative aspect of these studies, however, is probably less accurate than are the data with trypsin-I-131 because of the dilution of supernate by the residual fluid on even tightly packed cells.

The serial passage experiments are in accord with the data for adsorption of labelled trypsin. If approximately 20% of the enzyme is adsorbed when contacted with an equal volume of red cells, then a trypsin solution initially at a concentration of 2 mg/ml would still have a concentration of about 0.20 mg/ml after 10 passages, if the effect of dilution is ignored. This concentration is more than adequate to produce the serologic effect which was still strong at 0.05 mg trypsin/ml. On the other hand, if the initial trypsin concentration was 0.1 mg/ml, the serologic effect would be expected to disappear rapidly with serial passage, as indeed it did.

While the thiol proteases hydrolyze additional bonds, the 4 serologically active enzymes have common specificity insofar as they catalyze hydrolysis of derivatives of arginine and lysine. Thrombin and plasmin which also exhibit this specificity are inactive(1). This specificity may then be necessary but insufficient for production of the serologic effect. As yet, production of the effect has not been found to correlate with any known chemical change.

Seaman and Heard(7) reported that a significant decrease in the negative charge on RBC occurs during the first 10 minutes of incubation with trypsin. This charge reduction may lead to sufficiently decreased repulsion between sensitized cells to permit agglutination to proceed. The rapidity with which the change in charge occurs correlates well with the speed of appearance of the serologic effect. Thermodynamic effects might also be considered. Analysis of a number of immunologic systems has shown that they are attended by rather large positive increases in entropy. An explanation offered for this observation is the release of bound water. If enzyme-treated cells bind more water than untreated ones at the antigenic sites and their vicinity, then at the time of the antigen-antibody reaction, there could be a greater release of this water with a consequent greater entropy increase (and free energy decrease) to provide the driving force for agglutination. These hypotheses have been discussed previously(1).

Summary. Under conditions of trypsin treatment of RBC which produce specific agglutination by incomplete Rh antisera, the enzyme binds firmly to the cells. The enzymatically inactive DIP-trypsin binds to an even higher degree but fails to produce the serologic effect. Enzymatic assays of trypsin in RBC supernates support the concept of binding of enzyme to cells. Serial passage of RBC supernates and determination of their ability to produce the serologic effects are also in agreement. An hypothesis con-

cerning thermodynamic considerations of the reaction is discussed.

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Emergence of Competence (for Transformation) of Three *Hemophilus* Species in a Chemically Defined Environment.† (27904)

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A number of factors determine the proportion of cells in a bacterial population in which a new genetic trait may be conferred by biologically active deoxyribonucleic acid (DNA)(1). Among these, the method of cultivation of recipient populations plays a significant role. Under our original method of propagation of *Hemophilus influenzae*, the highest frequency of transformation to streptomycin (SM) resistance was approximately 0.1%. Goodgal and Herriott(2) have shown that the proportion of competent (transformable) cells in R*d* *H. influenzae* populations may be increased to 1% or more if the population is aerated during exponential growth and then, after a period of incubation with reduced aeration, is diluted 20-fold into NaCl containing DNA and incubated for 30 minutes. Some unknown factor(s) in the culture medium appeared to be essential for the high degree of competence of the diluted population; in our experience dilution of even 1:100 in saline leads to a marked decrease in the transformability of the population. This paper reports the use of this "dilution effect" as a tool for development of a chemically defined medium in which a high degree of competence emerges even in 10^4-fold dilution in 3 species of *Hemophilus: H. influenzae, H. aegyptius* and *H. parainfluenzae*. The results suggest that 2 amino acids, L-aspartic and L-glutamic, play a significant role. The process required for emergence of competence in the defined medium is temperature dependent and is inhibited by chloramphenicol.

Materials and methods. Recipient populations: Strains R*b*, R*d* and S*d* *H. influenzae* (3), *H. parainfluenzae* # 7 (Boss)(3), and *H. aegyptius* # 15 (4). *DNA donor populations:* Spontaneously occurring mutants resistant to streptomycin were selected from members of the above 3 species for isolation of DNA. The method used has been reported(5). Homologous species DNA was used in final concentrations of 1 or 2 μg/ml. *Medium used for growth of recipient populations:* Elev. broth(2) was used for the growth of test populations. Levinthal agar was used for pour plate preparations. The Levinthal stock was sterilized by filtration through Seitz filters. *Synthetic medium used for expression of competence:* Components of the medium were: 1) potassium phosphate buffered saline, pH approximately 7.5 (final concentration 0.015 M with respect to the buffer, 0.15 M with respect to NaCl); 2) L-aspartic and L-glutamic acids* in concentrations of 0.5% to 1.0% (w/v). One percent aqueous solutions were adjusted to a pH in

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