ADENOVIRUS 7: A STUDY OF THE HAEMAGGLUTININ AND THE HAEMAGGLUTINATING SYSTEM

A Thesis

Presented to

the Department of Bacteriology and Immunology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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August 1964

ACKNOWLEDGEMENTS

The author very gratefully acknowledges the patience and fortitude that Dr. Charles E. Mannau has demonstrated toward him in their year of association. He is also grateful to Dr. J. G. Wilt for help and advice, to Frank Goldberg for technical assistance, and to the Medical Research Council grant MA-1217 for financial assistance. Furthermore, the author is grateful for the help tendered him by the staff of the Department of Bacteriology and Immunology and especially by Miss Lorna Campbell.

ABSTRACT
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Adenovirus 7: a study of the haemagglutinin and of the haemagglutinating system.

Harvests of Adenovirus type 7 (Gomen strain) grown in KB cells have been found to give a much higher haemagglutinin titre than do harvests from primary human amnion cells. Attempts to increase haemagglutinin production in human amnion cells by either adaptation or selection were not successful. Furthermore, the settling patterns in haemagglutination titrations of human amnion-grown virus were not of the classical type due to a non-specific effect of the proteins present. The optimal system for assay of the Gomen haemagglutinin grown in KB cells has been determined. Gomen haemagglutinin is inactivated completely within ten minutes at 56°C.; however, partially purified preparations are inactivated more rapidly than are crude. It is also inactivated at pH values below 4.2 and above 10.6; by treatment with chymotrypsin and periodate; but not by urea or by trypsin. Adsorption of the haemagglutinin to erythrocytes is proportional to erythrocyte concentration, and occurs at temperatures above 18°C. in the pH range 6 to 9. Elution occurs rapidly and completely at 4°C. and to a significant degree at temperatures up to 22°C. Haemagglutinin recovery is not decreased by varying the volume of the eluting fluid. The erythrocyte receptors for the Gomen haemagglutinin are destroyed by both trypsin and chymotrypsin, but not by urea or periodate. They are not destroyed by three hours exposure to pH 5.5.

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INTRODUCTION

The interaction between many of the human Adenovirus serotypes and the host cell has been studied and the associated growth kinetics and morphological alterations have been described. However, studies of the soluble antigens, their relationship to one another and to the infectious virus particle, and studies of the biochemical changes in the host cell associated with infection have received detailed attention only with regard to types 1, 2, 4, 5, and 6. All but two of the twenty-eight human serotypes agglutinate the erythrocytes of one animal species or another, but apart from the optimal agglutination temperatures and a limited amount of data on the physico-chemical nature of the various haemagglutinins, little is known. Evidence that the haemagglutinin is at least partly separate from the infectious particle is available for many serotypes but its precise relationship to other soluble antigens is known only in relation to the subgroup containing types 1, 2, 4, 5, and 6.

The Adenoviruses, as a group, show many common properties, e.g. ultrastructure, possession of a common complement-fixing antigen, intracellular location of cytopathic effect, and some chemical properties. In spite of this, the group can be subdivided on the basis of their activities, viz., type of infection, growth cycle kinetics,

haemagglutination characteristics, etc. Furthermore, it has been reported that two (and perhaps three) members are oncogenic for neonatal hamsters. In other words, for a group which seems to be remarkably homogeneous on preliminary examination, it seems to span almost the entire range of viral activities and functions.

Studies are being conducted in this laboratory in an attempt to determine the relation of differences in biological activity of the Adenoviruses to differences in other attributes. At present, however, attention is being concentrated on the subgroup of which types 3 and 7 are members, and with particular reference to haemagglutination relative to the virus particle and other soluble antigens, as knowledge concerning haemagglutination by this subgroup is meagre.

The aim of this project concerns the nature of the type 7 Adenovirus haemagglutinin and the factors which affect its reactions with susceptible erythrocytes. Four major aspects were investigated: production and assay of the haemagglutinin; stability of the haemagglutinin to various physico-chemical treatments; characteristics of the process of adsorption to and elution from erythrocytes; and stability of the erythrocyte receptors to various physico-chemical treatments. In some experiments, the Adenovirus type 3 haemagglutinin was also included for contrast with type 7. A considerable amount of work was done with a strain of the type 14 virus (also for

LITERATURE REVIEW

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The first members of the Adenovirus group were reported in 1953 (Rowe et al., 1953). Since then, discovery of new members has resulted in the current recognition of forty-four serotypes (Pereira et al., 1963) which are divided into six subgroups called the human, simian, bovine, canine, murine and avian Adenoviruses. The three criteria for inclusion in this group are:

"(a) group-specific antigenicity, (b) ether resistance and (c) production of a characteristic cytopathic effect in continuous cell cultures of epithelial origin" (Binsberg, 1962).

The Adenoviruses contain DNA[1] (Pereira et al., 1963). The capsid is of icosahedral cubic symmetry and its diameter has been estimated by various techniques as being between 60 and 85 mu. The capsid is composed of 252 capsumeres with a centre-to-centre spacing of approximately 7 mu. A peri-capsidal membrane has not been demonstrated. These viruses are very stable at low temperatures and over a wide range of pH values but are readily inactivated at 56°C. Most of them have not been demonstrated to be pathogenic for animals other than their natural host. Haemagglutination of the

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[1]DNA and RNA are the abbreviations for deoxyribonucleic acid and ribonucleic acid respectively.

erythrocytes of various species is demonstrated by many of these viruses.

There are twenty-eight recognized serotypes among the human Adenoviruses (Pereira et al., 1963). Although early studies were primarily concerned with their medical significance and with diagnosis, some interesting epidemiological considerations were noted. Adenovirus types 1, 2, 5, and 6 cause sporadic or inapparent infections primarily in childhood, and can be grouped together on this basis (Ginsberg, 1959). In contrast, types 3, 4, 7 and 8, which can be considered as another group, are more generally responsible for epidemics and occur primarily among adults, especially military recruits.

The human Adenoviruses cause intranuclear cytopathic lesions in susceptible cells. The lesions produced fall into one of two general types (Boyer et al., 1959) and when the serotypes studied were grouped according to the type of lesions produced, the two groups were the same as obtained by grouping on the basis of epidemiology. In one type of lesion, small eosinophilic inclusions gradually increase in size, become granular, basophilic, and Feulgen-positive, and aggregate to form an irregularly shaped body surrounded by large clear vesicles (Boyer, Leuchtenberger, and Ginsberg, 1957). The Feulgen-positive inclusions are composed of virus. This type of intranuclear inclusion is characteristic

of types 1, 2, 5, and 6. Type 5, and more rarely type 6, form crystals relatively late in the course of infection which appear as slender, eosinophilic, Feulgen-negative columnar structures devoid of virus. Types 3, 4, and 7 produce intranuclear inclusions of a different type. Eosinophilic inclusions are rarely seen, their shape is less regular when they are present, and vesiculation is less prominent. The inclusions formed are Feulgen-positive masses and the nuclear chromatin is arranged in rosette, network, or honeycomb formations. These inclusions contain virus particles as well as do the basophilic, Feulgen-positive crystals.

Other differences between types 1, 2, 5, and 6 and types 3, 4, and 7 have been noted. The degree of neutralization by type-specific antisera of any of these serotypes is directly proportional to the dilution of antiserum. However, the slopes of the regression lines for the degree of neutralization versus the dilution of antiserum for types 1, 2, 5, and 6 are twice those of types 3, 4, 7, and 14 (Ginsberg, 1957; Denny & Ginsberg, 1961). The growth cycles of these viruses exhibit differences as well. The eclipse period of types 1, 2, 5, and 6 is approximately twenty-one hours long, whereas that of types 3, 4, 7, and 14 is approximately nineteen hours long (Ginsberg, 1957; Denny & Ginsberg, 1961). Ginsberg's estimate of the length of the eclipse

phase is based on experiments which did not use one-step growth. A truer estimate of the length of the eclipse phase of type 2 is probably that of Green and Dawson (1961) who did use one-step growth. They found that the eclipse period of type 2 when grown in KB cells in suspension culture was fourteen hours long. This does not, however, negate the fact that Ginsberg found differences in the eclipse periods of types 1, 2, 5, and 6 and types 3, 4, 7, and 14.

The most comprehensive classification of the human Adenoviruses has been performed by using haemagglutination characteristics and is described in the section on haemagglutination.

I. HAEMAGGLUTINATION

Rosen (1958) first reported that the human Adenoviruses are capable of haemagglutination. However, the indirect haemagglutination test had been applied to the Adenoviruses prior to this and was already being used for serological diagnosis of Adenovirus infections. Friedman and Bennet (1957) adsorbed Adenovirus material to sheep erythrocytes treated with tannic acid and found that, although the indirect haemagglutination test was not reliably type-specific, it was easier to employ and more useful than the complement-fixation test. Prier and LeBeau (1958) found that higher serum titers were obtained with the indirect haemagglutination

test than with the complement-fixation test when these tests were used to measure antibody levels and that the indirect haemagglutination test tended to be more type-specific than the complement-fixation test or the conglutination test. Ross and Olnsberg (1958) developed the indirect test further. They found that, by using chick erythrocytes and by adsorbing the test sera with a pool of three Adenovirus serotypes, they were able to obtain a type-specific reaction in 50% of the serum titrations. The antigen which is adsorbed to the erythrocytes is soluble as it stays in the supernate on centrifugation at 30,000 r.p.m. while the infectivity is sedimented (Prier & LeBeau, 1958). The indirect haemagglutination test has been superseded by the direct haemagglutination and haemagglutination-inhibition tests for routine serological investigations.

The first animal Adenovirus to be reported to haemagglutinate was Infectious Canine Hepatitis virus (Pastler, 1957). Since then, most of the forty-four Adenovirus serotypes which are now recognised have been tested for direct haemagglutination and only three of them have been reported as not haemagglutinating. These three are the murine Adenovirus (Pereira et al., 1963) and human Adenovirus types 12 and 18 (Rosen, 1960). No report has yet been published as to the haemagglutination or lack of it for the avian Adenovirus (called GAL virus) and some of the simian

Adenoviruses (Pereira et al., 1963). Thus, of forty-four human and animal serotypes, at least thirty-four agglutinate erythrocytes of one animal species or another.

Although Rosen reported that human Adenoviruses can agglutinate erythrocytes in 1958, it was not until 1960 that he reported being able to subdivide the human Adenoviruses into four groups on the basis of their haemagglutination characteristics (see Table I).

Group 1 agglutinates rhesus monkey erythrocytes; the group 2 serotypes agglutinate completely erythrocytes from Osborne-Mendel and Sprague-Dawley rats; the group 3 serotypes agglutinate rat erythrocytes partially; and two serotypes, types 12 and 18, have been reported to not agglutinate erythrocytes. Rosen's original classification of the eighteen serotypes then known has been extended to include the twenty-eight human serotypes currently recognized. This classification correlates well with previous attempts at classification mentioned above. However, it groups type 4 with types 1, 2, 5, and 6 as group 3. By all other criteria, type 4 resembles types 3, 7, and 14 (vide supra).

Adenovirus types 12 and 18 have not yet been observed to agglutinate erythrocytes (Rosen, 1960). They also differ from the other human Adenoviruses in other properties. The DNA's of both types differ from those of Adenovirus types 2 and 4 in buoyant densities and in denaturation temperatures.

TABLE I

CLASSIFICATION OF THE HUMAN ADENOVIRUSES ON THE BASIS OF HAEMAGGLUTINATION CHARACTERISTICS[1]

GROUP	SEROTYPES	AGGLUTINATE RHESUS MONKEY ERYTHROCYTES	AGGLUTINATE RAT ERYTHROCYTES
1	3, 7, 11, 14, 16, 20, 21, 25, 28,	+	-
2	8, 9, 10, 13, 15, 17, 19, 22, 23, 24, 26, 27,	-[2]	+
3	1, 2, 4, 5, 6,	-	partially
Unclassified	12, 18,	-	-

[1] Rosen, 1960

[2] Some types agglutinate rhesus cells, but to a lower titre.

The fact that their DNA is very similar by these criteria to the DNA of Polyoma and of Shope papilloma virus (Green & Pina, 1963) is of interest when related to the fact that both types 12 and 18 are oncogenic in neonatal hamsters (Trentin et al., 1962; Huebner et al., 1962). Very recently, suggestive, but inconclusive evidence has been reported which indicates that type 7 Adenovirus is also oncogenic in neonatal hamsters (Girardi et al., 1964).

Although haemagglutination has been demonstrated with several simian Adenoviruses, both of the two known bovine Adenoviruses, and Infectious Canine Hepatitis virus (Pereira et al., 1963), only the Infectious Canine Hepatitis virus haemagglutinin has been characterized to any degree. Three reports have been published concerning the Infectious Canine Hepatitis virus haemagglutinin. One (Pautler, 1957), from New Zealand, states that only fowl erythrocytes are agglutinated. A second (Espmark & Salonatedt, 1961), from Sweden, states that only human "O" and albino rat erythrocytes are agglutinated. The third report (Shimizu et al., 1960), from Japan, claims that human erythrocytes, of all blood groups, and guinea pig erythrocytes are agglutinated to high titer while domestic fowl erythrocytes are agglutinated to low titer only. All three reports agree that elution occurs within one hour at 37°C. but not at 4°C. or at room temperature and all three therefore performed haemagglutination titrations at 4°C. or

at room temperature. Pastier (1957) claims that agglutination occurs only between pH 7.5 and 8.0; Kapsenberg and Kalmanstedt (1961) claim a pH range of 6.5 to 7.5; and Shimizu and his colleagues (1960) claim a pH range of 6.0 to 7.0. Pastier (1957) and Shimizu et al. (1960) agree that agglutination occurs at sodium chloride concentrations between 0.3 and 0.02 M. Pastier (1957) was able to obtain partial separation of the haemagglutinin from the infectious particle on filtration through a Berkefeld W candle filter which would indicate that at least part of the haemagglutinin is non-infectious.

Both bovine Adenoviruses have been observed to agglutinate rat erythrocytes and bovine Adenovirus type 2 agglutinates mouse erythrocytes (Pereira et al., 1963).

Rosen's classification of the human Adenoviruses into three groups based on haemagglutination characteristics (Table I) is used henceforth and the terms group 1, group 2, and group 3 refer to his classification.

Haemagglutination by the Group 1 Adenoviruses. Of the nine serotypes which constitute this group (Table I), only types 3, 7, 11, 14, and 16 have been studied in any detail. Rosen (1960) found that types 3, 7, and 14 agglutinate rhesus monkey erythrocytes to a higher titre at 37°C. than at 4°C. while the titers obtained with types 11 and 16 are equal at both temperatures. This has been confirmed by

Simon (1962) using rhesus monkey erythrocytes and by Henry and her colleagues (1963) using grivet monkey erythrocytes. Rosen (1960) also found that the haemagglutination titres obtained with types 3, 7, and 14 varied with the individual rhesus monkey blood donor whereas the titres with types 11 and 16 remained constant. This, too, has been confirmed by Simon (1962) using rhesus monkey erythrocytes. Henry and her colleagues (1963), using grivet monkey erythrocytes, reported, however, that type 14 would not agglutinate grivet monkey erythrocytes. Also, while the haemagglutination titer obtained with type 7 varied with the individual donor, that obtained with type 3 remained constant. They reported further that type 3 agglutinated at 37°C. and 20°C. but not at 4°C. while type 7 agglutinated at 37°C. but not at 20°C. or at 4°C.

Simon (1962), on further investigating the failure of types 3 and 7 to haemagglutinate at 4°C., found that after adsorption to the erythrocytes at 42°C., elution of the haemagglutinin from the erythrocytes occurred when the erythrocyte-haemagglutinin complex was incubated at 0°C. to 4°C. Elution occurred at this temperature at ionic concentrations between 0.15 and 0.5 M and between pH 5.0 and 9.0. On decreasing the volume in which elution occurred, relative to the volume in which adsorption occurred, Simon recovered less haemagglutinin. Adsorption and elution does not seem

to destroy the erythrocyte receptors as, after the type 3 haemagglutinin had adsorbed to and eluted from the rhesus monkey erythrocytes, type 3 or 16 haemagglutinins would still adsorb to the erythrocytes.

Types 11 and 16 do not elute at 4°C., although each is adsorbed at 37°C. (Simon, 1962). Simon found, however, that after adsorption to erythrocytes, the haemagglutinin elutes at pH 3.0. The temperature at which elution occurred was not included in his report. To demonstrate elution, it was necessary to use red cell stroma as the rhesus monkey erythrocytes lysed at pH 3.0.

Although Simon (1962) found that 0.25% trypsin had no effect on the type 3 haemagglutinin when incubated at 37°C. for one hour, Bauer and Wigand (1962) reported that the type 11 haemagglutinin was reduced in titre when exposed to 1% trypsin for one hour. Perhaps this indicates another difference between the type 3 and 11 haemagglutinins. Buckland and Tyrrell (1963) reported that papain at 1% concentration and bisulphite at 0.01 M do not inactivate the type 7 haemagglutinin. Formalin at 20% concentration reduced the haemagglutination titer considerably, as did periodate at 0.046 M and exposure to 56°C. for twenty minutes. Simon (1962) reported that the type 3 haemagglutinin is also inactivated at this temperature. However, Bauer and Wigand (1962) reported that ten minutes exposure to 65°C. is necessary

to inactivate the type 11 haemagglutinin completely.

RDE[2], at concentrations that destroy receptors for the PR8 strain of Influenza A virus, has no effect on the erythrocyte receptors for the group 1 Adenoviruses (Simon, 1962). However, Buckland and Tyrrell (1963) reported that undiluted Vibrio cholerae filtrate, which may contain other enzymes than RDE, did reduce the susceptibility of rhesus monkey erythrocytes to agglutination by Adenovirus type 7. Formalin (1%), periodate (0.0056M), and papain (2%) also reduced agglutinability by type 7 appreciably, while chymotrypsin (0.01%) produced only a small reduction. Similarly, Simon (1962) reported that trypsin and periodate decreased the susceptibility of rhesus monkey erythrocytes to agglutination by the Adenovirus type 3 haemagglutinin. The evidence above would indicate that the haemagglutinin and the erythrocyte receptors are both glycoproteins. Thus, N-acetyl neuraminic acid may be involved in the structure of the erythrocyte receptors.

Haemagglutination by the Group 2 Adenoviruses. The twelve members of group 2 (Table I) all agglutinate Osborne-Mendel and Sprague-Dawley rat erythrocytes completely. Henry and her colleagues (1963) reported that types 9, 10, and 15

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[2]RDE is the abbreviation for the Receptor-destroying-enzyme of Vibrio cholerae.

agglutinate Wistar rat erythrocytes as well, although type 13 does not. These three serotypes also agglutinated human "O" Rh-positive erythrocytes and types 9, 13, and 15 agglutinated rhesus monkey erythrocytes, but to lower titre than rat erythrocytes (Rosen, 1960). For haemagglutinin assay, the optimal incubation temperature seems to be 37°C. Some of the serotypes in this group, however, agglutinate equally at 37°C. and 4°C. (e.g. types 8 and 10), but others (e.g. types 9 and 15) agglutinate to lower titres at 4°C. than at 37°C. (Henry et al., 1963; Rosen, 1960). One report has been published which claims that types 9, 10, and 13 agglutinated to higher titre at 4°C. and room temperature than at 37°C. (Longrel et al., 1963).

As papain and formalin partially inactivate the type 9 haemagglutinin and inactivation by urea occurs to a degree depending upon the concentration up to a maximum at 2.0 M (Buckland & Tyrrell, 1963), the type 9 haemagglutinin must be at least partly protein. Similarly, the partial inactivation of the type 9 haemagglutinin by periodate indicates that polysaccharide may be essential to its action as well. The type 9 haemagglutinin is inactivated by exposure to 56°C. for twenty minutes. However, exposure to 65°C. for five minutes is necessary to inactivate the haemagglutinin completely (Wigand & Bauer, 1962).

As Vibrio cholerae filtrate reduces the agglutinability

of human "O" erythrocytes by the type 9 haemagglutinin (Buckland & Tyrrell, 1963), N-acetyl neuraminic acid may be an integral part of the erythrocyte receptors.

Haemagglutination titres obtained with types 10 and 13 are markedly reduced when the human "O" red cells used in the titration have been previously treated with the erythrocyte-modifying factor which is associated with types 1, 2, 4, and 15 (Kasel et al., 1960; vide infra).

Haemagglutination by the Group 3 Adenoviruses. Adenovirus group 3, which consists of types 1, 2, 4, 5, and 6, also agglutinates Osborne-Mendel and Sprague-Dawley rat erythrocytes, but only partially (Rosen, 1958). The reaction is such that all positive tubes in a titration appear as ± reactions, i.e. in all the positive tubes, some of the erythrocytes sediment in the negative ring or button pattern. However, in the presence of antiserum against any other member of the subgroup, types 1, 2, 5, and 6 demonstrate complete agglutination (Rosen, 1960). Rosen claimed that type 4 would not agglutinate completely, even in the presence of heterotypic antiserum. Pereira and de Figueiredo (1962), on investigating this phenomenon, found, however, that heterotypic antiserum did enhance haemagglutination by type 4, but more heterotypic antiserum was necessary than with types 1, 2, 5, and 6.

By column chromatography of crude type 1, 2, 5, and 6 virus suspensions on DEAE-cellulose[3], two haemagglutinating fractions are obtained. One fraction elutes at a sodium chloride concentration of 0.075 M and the other elutes at 0.175 M concentration (Pereira & de Figueiredo, 1962). The fraction which elutes at 0.175 M sodium chloride behaves as a true haemagglutinin in contrast to the second fraction and is probably responsible for the partial agglutination observed with these viruses. It is adsorbed to rat erythrocytes at 37°C., partially adsorbed at 22°C., and not adsorbed at all at 4°C.

The other haemagglutinating fraction, which elutes at 0.075 M sodium chloride, does not agglutinate erythrocytes directly but requires the inclusion of heterotypic antiserum in the system. Homotypic antiserum, on the other hand, abolishes haemagglutination entirely. Although the role of the haemagglutinin in this reaction would seem to resemble that of the antigen in the indirect haemagglutination reaction, one obvious difference exists, viz., homotypic antiserum does not cause haemagglutination but rather inhibits it. Pereira and his colleagues theorised that the haemagglutinin is removed from the erythrocyte surface by the homotypic antiserum. In spite of this explanation, however, they

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[3] DEAE-cellulose is the abbreviation for diethyl-aminoethyl-cellulose.

were able to demonstrate no adsorption of this factor by rat erythrocytes at 4°C., 22°C., or 37°C.

II. SOLUBLE ANTIGENS

In the previous section on haemagglutination, emphasis was laid on the activity and stability of the Adenovirus haemagglutinins and on some of the characteristics of the haemagglutinating systems. The relationship between infectious virus and haemagglutinin was omitted as it is described in this section.

A number of non-infectious but virus specific antigens have been found in Adenovirus-infected tissue culture fluids in addition to infectious virus. The three that have been most characterised are the group-specific complement-fixing antigen, the toxin, and the type-specific complement-fixing antigen. Two other particles have been described as well, viz., the erythrocyte receptor-modifying factor and a viral inhibitor. Haemagglutinin activity is at least partly separate from the infectious virus but it has been demonstrated that haemagglutination by the group B serotypes is a function of the toxin and the type-specific complement-fixing antigen.

The soluble particles found in virus-infected tissue culture fluids seem to be normal virus components produced in excess. Wilcox and Ginsberg (1961), on studying the production of the group-specific and type-specific complement-

fixing antigens and of the toxin, found that these antigens are produced even when the synthesis of infectious virus has been decreased by the incorporation of proflavine in the maintenance medium. The antigens are synthesized two to three hours after synthesis of DNA begins and two hours before the appearance of infectious virus (Flanagan & Ginsberg, 1962). Ginsberg and his colleagues only employed Adenovirus types 4 and 5 but, in the absence of evidence to the contrary, these findings may be considered to indicate that the Adenovirus soluble antigens are normal virus components synthesized in excess which are released from the cells without being assembled into infectious virus particles.

The Adenovirus Group 2 Soluble Antigens. In 1958, Pereira reported that tissue culture fluids containing Adenovirus types 1, 2, and 5 caused two cytopathic effects, one due to viral reproduction and the other, which occurred earlier, due to a non-infectious particle. The factor which caused the early cytopathic effect was separable from the infectious particle by ultracentrifugation. Pereira (1958) labelled this particle as the early cytopathic factor. In 1959, Pereira, Allison, and Farthing found that, by submitting Adenovirus type 5 preparations which were partially purified by treatment with the fluorocarbon, Arcton 113, to agar gel-diffusion and immunoelectrophoresis, they were able to

separate three non-infectious antigens which they labelled A, B, and C. Antigen B was identical to the early cytopathic factor, antigen A was a group-specific complement-fixing antigen, and antigen C was a type-specific complement-fixing antigen (Kleaperer & Pereira, 1959). Wilcox and Ginsberg (1961) confirmed the existence of the three antigens, which they labelled L, T, and S corresponding to A, B, and C, respectively. They were able to separate the antigens by column chromatography on DEAE-cellulose as well as by gel-diffusion. For greater convenience, Pereira's labelling system for these antigens is used henceforth (see Table II).

Antigen A is common to all the Adenoviruses, human and animal, except GAL[4] virus. Antigen B, as well as being called the early cytopathic factor (Pereira, 1958) and antigen T (Wilcox & Ginsberg, 1961), has been referred to as the toxin (Everett & Ginsberg, 1958) and the cell-detaching factor (Rowe et al., 1958). It is responsible for the early cytopathic effect which occurs when a concentrated virus inoculum is applied to a tissue culture monolayer. This cytopathic effect appears within a few hours, is not related to virus reproduction, and is reversible by replacement of the virus

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[4]GAL (Gallus,adeno-like) virus is now considered to be an Adenovirus and is classified as the avian Adenovirus (Pereira et al., 1963) but was previously excluded from the group by some authors because it does not possess the group-specific complement-fixing antigen (Ginsberg, 1962).

TABLE II

ANTIGENIC COMPOSITION AND ALTERNATE NAMES OF THE ADENOVIRUS GROUP SOLUBLE ANTIGENS

ANTIGEN	ALTERNATE NAMES	ANTIGENICITY	COMPONENTS[6]
A	group-specific complement-fixing antigen, L antigen[1]	group-specific	α (DNA?)
B	toxin[2], cell-detaching factor[3], early cytopathic factor[4]	group-specific type-specific	$\beta\gamma$ (RNA?)
C	type-specific complement-fixing antigen, S antigen[1]	type-specific subgroup-specific	$\gamma\delta$ (DNA?)
Viral Inhibitor	----------	type-specific	γ
ERV	erythrocyte receptor-modifying factor[5]	type-specific	?

[1] Wilcox and Ginsberg, 1963a.

[2] Everett and Ginsberg, 1958.

[3] Rowe et al., 1958.

[4] Pereira, 1958.

[5] Found in types 1, 2, 4, and 15 only, Kasel et al., 1960.

[6] According to Pereira & de Figueiredo, 1962.

inoculum with fresh maintenance medium (Pereira, 1958). When tissue culture fluids containing antigen B are subjected to high speed centrifugation, the infectious virus is sedimented but the antigen is not.

The three antigens A, B, and C, are separable from each other and from the infectious particle by column chromatography on DEAE-cellulose (Wilcox & Ginsberg, 1961). The three types of particles recovered are not homogeneous antigenically. On gel-diffusion, antigens B and C demonstrate reactions of partial identity. Furthermore, when antigen B is treated with trypsin and rechromatographed, its activity is destroyed, but a type-specific particle elutes at the same sodium chloride concentration as antigen C. Since antigen B reacts with heterotypic antiserum on complement-fixation tests and on gel-diffusion, Pereira (1960a) decided that the evidence above indicates that antigen B contains two components, one, group-specific, and the other, type-specific and common to antigen C. As antigen B does not demonstrate a reaction of identity on gel-diffusion with antigen A, Pereira concluded that the group-specific components on A and B are different. He named the group-specific antigen on A, α , that on B, β , and the type-specific antigen shared by B and C, γ (see Table II).

As mentioned above, the haemagglutinating activity of the group 3 serotypes can be separated into two fractions

by chromatography on DEAE-cellulose (Pereira & defigueiredo, 1962). One fraction elutes from the column at 0.175 M sodium chloride, as does antigen B, and the other elutes at 0.075 M concentration, as does antigen C. The haemagglutinin which elutes at the same concentration as antigen B is inhibited by type-specific antiserum. The other haemagglutinin demonstrates haemagglutination only in the presence of heterotypic antiserum to other members of group 3, and not in the presence of heterotypic antiserum to members of groups 1 or 2. Pereira and defigueiredo (1962) concluded that this haemagglutinin is subgroup-specific. Since the antigen C haemagglutinin is subgroup-specific, the antigen B haemagglutinin cannot be the γ component shared by antigens B and C. However, since the antigen B haemagglutinin is inhibited type-specifically, it cannot be the β component present in antigen B either. Pereira and defigueiredo therefore concluded that haemagglutination must be some function of antigen B as a whole and that the antigen C haemagglutinin must be a previously unrecognized subgroup-specific component of antigen C. He labelled this component δ. Pereira postulated the presence of the δ component solely on the grounds of the evidence from haemagglutination enhancement by heterotypic antiserum. As attempts to demonstrate this component by the complement-fixation test and by gel-diffusion were not successful.

Thus, as is seen in Table II, antigen A has the group-specific α component; antigen B contains both the group-specific β component and the type-specific γ component; and antigen C contains the type-specific γ component and the subgroup-specific δ component.

The presence or absence of nucleic acids in these antigens is in controversy. Pereira and his colleagues (Allison et al., 1960; Pereira, 1960a) claim that antigens A and C contain DNA since the particles fluoresce yellow-green on staining with acridine orange and are labelled when grown in cells in a medium containing $Na_2P^{32}O_4$. Wilcox and Ginsberg (1963a) claim that there is no DNA present for three reasons. The first is that these antigens band together with a buoyant density of 1.2832 gm./cm.^{3} when centrifuged in a cesium chloride equilibrium density gradient. This buoyant density is less than that of a deoxyribonucleoprotein. Secondly, no inactivation was demonstrated by treatment with deoxyribonuclease. Finally, in contrast to the findings of Pereira and his colleagues, Wilcox and Ginsberg (1963a) could demonstrate no labelling with P^{32}.

Pereira and his co-workers (Allison et al., 1960) claim that antigen B does not contain any nucleic acid as it does not become labelled when virus is grown in cells maintained in maintenance medium containing $Na_2P^{32}O_4$.

Opposing this view is that of Wilcox and Ginsberg (1959) who claim that antigen B is a ribonucleoprotein which loses its activity in parallel with its dissociation into protein and RNA by the cationic exchange resin XE-64.

Pereira and his colleagues (Allison et al., 1960) determined the diffusion coefficients in agar and in gelatin of preparations of antigens A, B, and C purified by column chromatography. They found that they have diffusion coefficients equal to those of spheres of 8, 17, and 10 mu diameter, respectively. Antigens A and C are resistant to acid (pH 2.0), proteolytic enzymes, ether, and periodate. Antigen B is resistant to acid, ether, and periodate, too, but looses its toxic activity when treated with trypsin, papain, chymotrypsin, or pepsin. Antigen B is inactivated by exposure to 70°C. for ten minutes, antigen C by exposure to 80°C. for ten minutes, and antigen A by exposure to 90°C. for ten minutes.

Wilcox and Ginsberg (1963b) have found, on electron microscope examination of antigen preparations purified by chromatography on DEAE-cellulose, that the antigen A particles are morphologically closely similar to or identical to the capsomeres of the intact virus. Furthermore, it is apparently the major antigen of the capsid. Antigen C has the shape of "thread-like strands" both free and on the capsid.

Antigen A is assumed to contain only the α antigenic

component (Pereira, 1960a). Recently, however, Wilcox and Ginsberg (1963c) have found that purified antigen A elicits the production of not only group-specific but also type-specific antibodies in rabbits. Whether this is due to two distinct groupings on the antigen or to the elicitation of a "heterogeneous population of antibodies of which less than 50% can cross-react with heterologous antigens" is not yet known (Wilcox & Ginsberg, 1963c).

Infection of HeLa cells with Adenovirus types 1 to 6 leads to the production of a viral inhibitor. The action of the inhibitor is expressed in reducing the total yield of Adenovirus type 5, Poliovirus type 1, and Vaccinia virus in HeLa cells when the cells are treated with the inhibitor prior to infection (Pereira, 1960b). Three inhibitory effects are demonstrated against Adenovirus type 5, viz., less extensive cytopathic effect, decreased production of soluble antigens, and less virus synthesized in inhibitor-treated cells than in control HeLa cell cultures which had been treated with uninfected HeLa cell extracts. The main effect manifested against Poliovirus and Vaccinia virus is a reduction in the yield of infectious virus.

The viral inhibitor had no effect when added simultaneously with the challenge virus, but when added to cells a few hours prior to infection, it demonstrated inhibition. If the inhibitor was incubated with cells and then removed,

no traces of inhibition were demonstrable twenty-two hours later. Purified antigens B and C demonstrate the same inhibitory effects although antigen A does not. Pereira (1960b) has therefore suggested that the inhibitory action is a function of the type-specific component γ shared by antigens B and C. The activity of the inhibitor is abolished by type-specific antiserum which is further evidence for this hypothesis. The inhibitor is inactivated at 70°C., as is antigen B. Pereira has concluded that it is not an interferon, although it shares many properties with them, firstly, because it is not released spontaneously by infected cells in the first three days after infection and secondly, because it is virus-specific.

Adenovirus types 1, 2, 4, and 15 also produce a soluble antigen which has been called the erythrocyte receptor-modifying factor or ERM (Kasel et al., 1960). This is a type-specific antigen which modifies the receptors on human "O" erythrocytes in such a manner that the haemagglutination titers obtained with types 10, 13, and RAH-2 are decreased from eight to sixty-four fold when ERM-treated erythrocytes are used for assay. These receptors are also destroyed by RDE. It seems, however, that the ERM does not destroy N-acetyl neuraminic acid since it has no effect on the receptors for the haemagglutinins of Influenza A virus (strain PR8), Influenza B virus (strain

Lee), Parainfluenza virus type 3, and Polyoma virus.

The ERM demonstrates maximum activity at 37°C. and its effects are increased with the time of incubation with erythrocytes. The activity of the ERM is not diminished by repeated exposure to human erythrocytes. It is not adsorbed by rat erythrocytes either and it is inhibited by type-specific antiserum. The haemagglutinin which Pereira and deFigueiredo (1962) have labelled as the δ antigen shares these characteristics and one is led to wonder whether the erythrocyte receptor-modifying effect is not another manifestation of the δ component. However, this factor is not inactivated by exposure to 80°C. for thirty minutes whereas antigen C, which contains the δ component, is destroyed by incubation at 80°C. for ten minutes (Allison et al., 1960). Ultraviolet light, ether, deoxyribonuclease, ribonuclease, trypsin, and chymotrypsin, all do not inactivate the erythrocyte receptor-modifying factor (Enzel et al., 1960).

The majority of the published literature about the Adenovirus soluble antigens concerns research performed on the group 3 Adenoviruses. The literature concerning the human Adenoviruses of groups 1 and 2 serves only to establish that the complement-fixing activity and the haemagglutinins are at least partly separable from the infectious particle.

The Soluble Antigens of Group 1. When a suspension of Adenovirus type 3 or 7 is mixed with an erythrocyte suspension, the infectious virus particles and the haemagglutinins are adsorbed to the erythrocytes while the complement-fixing activity is not (Simon, 1962; Zuschek, 1961).

Zuschek (1961) reported further evidence for the separability of the haemagglutinin and the group-specific complement-fixing antigen. He was able to sediment the haemagglutinating activity by high speed centrifugation while the complement-fixing activity remained in the supernate. Also, the haemagglutinin passed through a Seitz S-1 filter pad while the group-specific complement-fixing antigen did not.

Evidence is also available that the haemagglutinin can be at least partly separated from the infectious particle. On high speed centrifugation, Zuschek (1961) found that the infectious particle sedimented faster than the haemagglutinin which in turn sedimented faster than the complement-fixing antigen. Bauer and Wigand (1962) found that on high speed centrifugation of an Adenovirus type 11 suspension, they were able to separate a part of the haemagglutinating activity from the infectivity.

The evidence above indicates strongly that the haemagglutinin, complement-fixing antigen, and the infectious

particle are separate entities.

Soluble Antigens of the Group 2 Adenoviruses. When Adenovirus type 9 suspensions are mixed with rat erythrocytes, both the haemagglutinating activity and the infectivity are completely absorbed from the suspension (Wigand & Bauer, 1962). This would suggest that the virion haemagglutinates since it adsorbs to erythrocytes. However, on high speed centrifugation, all the infectivity, but not all of the haemagglutinating activity is sedimented (Wigand & Bauer, 1962; Bauer & Wigand, 1962). This indicates once again that at least some of the haemagglutinin is not infectious.

Although the erythrocyte receptor-modifying factor is produced primarily by the group 3 Adenoviruses (Kasel et al., 1960; vide supra), one of the members of group 2, type 15, has also been found to stimulate its production).

MATERIALS AND METHODS

MATERIALS AND METHODS

Virus Strains. The virus strain used was the prototype Gomen strain of Adenovirus type 7 (Berge et al., 1955) which was obtained from the American Type Culture Collection, Viral and Rickettsial Registry and Distribution Centre (ATCC) where it had been passed ten times in HeLa cells. The virus used in these experiments had a further four to five passages in primary human amnion cell monolayers followed by one passage in KB cell monolayers. In certain experiments, Adenovirus type 3 was included for comparison with the type 7 virus. The Adenovirus type 3 strain used was the prototype G.B. strain (Howe et al., 1955) and was also obtained originally from ATCC. It had been passed seven times in HeLa cells and since receipt in this laboratory has been passed a further seven to eight times in primary human amnion cells.

Virus Production. Five to ten sixteen ounce prescription bottles containing complete monolayers of KB or primary human amnion cells were infected with 10^6-10^7 $TCID_{50}$[1] of virus and were examined periodically for cytopathic effect. The maintenance medium (Eagle's-Earle's-2% calf serum; see below) was replaced approximately once a week

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[1] 50% Tissue Culture Infectious Doses.

until the cytopathic effect covered more than half the monolayer. The bottles were frozen when the cytopathic effect became complete. With KB cells, this usually occurred within three to seven days. With primary human amnion cells, the effect did not become complete for one to three weeks.

Virus was released from the infected cells by three cycles of alternate freezing at -20°C. and thawing at room temperature. The harvests from these cultures were pooled, centrifuged at 2000 r.p.m. for thirty minutes to remove particulate cell debris, aliquoted, and stored in a mechanical freezer operating at -20°C.

Cell Cultures. Two types of cell culture was employed, viz., primary human amnion cells for infectivity titrations and production of Adenovirus type 3 pools and KB cells[2] (Eagle, 1955), for the production of Adenovirus type 7 pools with high haemagglutinin titers.

Primary human amnion cell monolayers in tubes and in sixteen ounce prescription bottles were prepared by the techniques described by Wilt et al., (1964). The growth medium consisted of 60% Hank's Balanced Salts Solution (BSS), 20% Tryptose phosphate broth (Difco), and 20% inactivated

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[2]Originally derived from human epidermoid carcinoma of nasopharynx. A calf serum-adapted line was obtained from Microbiological Associates Inc., Bethesda, Md.

calf serum adjusted to pH 7.4 with 1.4% sodium bicarbonate and containing 200 IU of penicillin, 100 γ streptomycin, 100 γ chloramphenicol, and 50 γ mycostatin per milliliter. Complete monolayers were formed within six to eight days.

KB cells were grown in sixteen ounce prescription bottles. Bottles containing complete monolayers were washed and treated with 0.25% trypsin[3] in Hank's BSS for ten to fifteen minutes at 37°C to detach the cells from the glass. The action of trypsin was stopped by the addition of fresh growth medium and the cell suspension was centrifuged at 1000 r.p.m. for ten minutes. The supernate was removed and the cell pellet was resuspended in sufficient growth medium so that each bottle seeded received an aliquot of forty-five ml. of cell suspension at a cell concentration of approximately 2-3 x 10^5 per ml. The growth medium used was Eagle's Minimal Essential Medium (Eagle, 1959) containing 10% inactivated calf serum, 100 IU of penicillin, and 50 γ of streptomycin, adjusted to pH 7.4 with 1.4% sodium bicarbonate. A complete monolayer formed within two to four days and the medium was removed and replaced with maintenance medium.

Cell cultures were maintained in Eagle's Minimal Essential Medium with Earle's BSS substituted for Hank's.

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[3]Trypsin 1-300, Nutritional Biochemicals, Cleveland, Ohio.

The calf serum supplement was 5% for primary human amnion cells and 2% for HEp.

Virus Assay. Infectivity titrations were performed in tube cultures of primary human amnion cells. The cells were maintained in 95% Syverton-Scherer's Maintenance Medium (Syverton, Scherer, & Ellwood, 1954) and 5% inactivated calf serum adjusted to pH 7.4 with 1.4% sodium bicarbonate and containing 200 IU of penicillin and 100 γ of streptomycin per ml. Serial ten-fold dilutions of virus were prepared in Hank's BSS and, from each dilution, four tube cultures were inoculated with 0.2 ml. each.

Final readings were taken after three weeks and the log. $TCID_{50}$ was calculated by the Reed and Muench (1938) method.

Erythrocytes. Grivet monkey (Cercopithecus aethiops)[4] erythrocytes were employed. These were used in preference to rhesus monkey erythrocytes since they have been reported to be more sensitive (Rosen, 1958). After receipt, they were stored at 4°C. and aliquots were removed aseptically when needed. These aliquots were washed three times before use in dextrose-gelatin-veronal (DGV) buffer (Casals & Clarke, 1958) and the erythrocytes not employed the same day were

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[4]Obtained from Connaught Medical Research Laboratories, Toronto, where the blood was drawn aseptically and mixed immediately with an equal volume of Alsever's solution.

stored at 6°C. in the DGV buffer for periods of up to one week. After storage in DGV buffer, the erythrocytes were washed twice more before use. Rosen (1960) claims that there is no loss in agglutinability with such storage.

The packed cell volume was determined by centrifugation at 1500 r.p.m. for twenty minutes in a 12.0 ml. graduated centrifuge tube and the cells were suspended to the required concentration in phosphate buffered saline (Dulbecco & Vogt, 1954) or in physiological saline (0.15 M NaCl). The erythrocytes suspended in saline were used for haemagglutinin assay but for all other purposes, the erythrocytes were suspended in phosphate buffered saline in order to maintain a constant pH.

Haemagglutinin Assay. Serial two-fold dilutions of virus were prepared in 0.4 ml. volumes in physiological saline in 85 mm. x 10 mm. tubes. To each tube was added 0.1 ml. of a 0.5% suspension of erythrocytes in saline[5]. The racks of tubes were then shaken and placed in a 35°C. incubator for two to three hours. Readings were made according to the pattern of the settled cells (Salk, 1944). The haemagglutinin titre was taken as the highest dilution of virus, prior to addition of the erythrocyte suspension, which

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[5]Normal rabbit serum was added to the erythrocyte suspension at the final concentration of 0.5% to prevent spontaneous agglutination.

produced partial agglutination of the erythrocytes. The patterns were scored as + (complete agglutination), ± (partial agglutination), or - (no agglutination).

Purification of Haemagglutinin. Adenovirus type 7 haemagglutinin was partially purified by adsorption to erythrocytes at 37°C. followed by elution at 4°C. A 10% suspension of erythrocytes in phosphate buffered saline was centrifuged and the supernate removed. The packed cells were resuspended in the virus material to the final concentration of 5% in a total volume of 10-20 ml. The mixture was incubated in a water bath at 37°C. for twenty to forty minutes. Simultaneously, centrifuge cups containing 2.0 - 5.0 ml. of water were incubated as well. The haemagglutinin-erythrocyte mixture was then centrifuged at 2500 r.p.m. in a centrifuge at room temperature for five minutes. The temperature of the mixture usually dropped to 20°C. during this process. The supernate was removed and discarded and the cells were resuspended to their original volume with phosphate buffered saline. They were then incubated at 37°C. for a further twenty to forty minutes and again centrifuged. After being resuspended to the original volume again with phosphate buffered saline, the erythrocyte suspension was incubated in a water bath at 2-4°C. for twenty to forty minutes and centrifuged at 2500 r.p.m. in

a refrigerated centrifuge at 8°C. The specific activity of the haemagglutinin in terms of protein was found to have increased up to forty fold when purified in this way.

Protein Measurement. Protein measurements were performed in order to determine the relative increase in specific activity of haemagglutinin preparations after purification. A modification of the Lowry method (Kabat & Mayer, 1961; Lowry et al., 1951) was employed and, since no attempt was made to remove non-proteinaceous materials, measurements were of any materials that react with the Lowry reagents. Crystalline bovine serum albumin fraction V[6] was used as standard and readings were taken colorimetrically on the Klett-Summerson photoelectric colorimeter at 660-740 mu (filter no. 69).

Buffers for pH Studies. The BDH Universal buffer[7] (Prideaux & Ward, 1924) was used in most of these experiments. At pH 7.0, when diluted according to instructions, this buffer has an ionic concentration of 0.093 M. At other pH values, the ionic concentration varies since sodium hydroxide is used to adjust the pH. To avoid lysis of erythrocytes from hypotonicity in the haemagglutination

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[6]Nutritional Biochemicals Corp., Cleveland, Ohio.

[7]British Drug Houses, Toronto, Ontario.

titrations, the buffer was diluted 1:9.3 with physiological saline, regardless of the pH desired. Thus, when adjusted to pH 7.0 for haemagglutinin assay, the buffer had the ionic concentration of 0.01 M in saline. In some experiments, phthalate-HCl, phthalate-NaOH, and carbonate-bicarbonate buffers were employed. These, too, were diluted in physiological saline so that when adjusted to pH 7.0, their ionic concentration would be 0.01 M.

Chemicals and Enzymes. Trypsin, chymotrypsin, urea, and periodate were employed in inactivation studies. Crude trypsin[8] was employed after solution in PBS and clarification by filtration through a Millipore membrane[9] at 2%, 1%, and 0.5% concentrations. Crystalline trypsin[10] and chymotrypsin[11] were dissolved in 0.01 M HCl to 1% concentration and stored frozen at -20°C. After thawing at room temperature, further dilutions to 0.2% and 0.1% were made in tris (trishydroxymethylaminomethane) buffer at pH 7.5.

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[8] Trypsin, 1-300, Nutritional Biochemicals Corp., Cleveland, Ohio.

[9] Grade HA, 0.45 u mean pore diameter, Millipore Filter Corp., Bedford, Mass.

[10] Trypsin, salt-free, 2x crystalline, Nutritional Biochemical Corp., Cleveland, Ohio.

[11] α chymotrypsin, 3x crystalline, Worthington Biochemical Corp., Freehold, N.J.

Urea was dissolved in PBS at concentrations of 4 M, 1 M, and 0.5 M. Sodium metaperiodate was dissolved in PBS at 0.005 M and 0.00005 M concentrations.

Crude soybean trypsin inhibitor[12] was employed at a concentration of 1% in PBS to stop the action of both the crystalline trypsin and chymotrypsin.

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[12]Crude soybean trypsin inhibitor, Worthington Biochemical Corp., Freehold, N.J.

EXPERIMENTAL PROCEDURES AND RESULTS

EXPERIMENTAL PROCEDURES AND RESULTS

I. HAEMAGGLUTININ PRODUCTION AND ASSAY

Haemagglutinin Production. Early in the course of the investigations reported here, it was observed that harvests of Sozen virus grown in primary human amnion cells contained little or no haemagglutinating activity. Furthermore, the haemagglutinating patterns obtained were difficult to read because they were not of the classical type (vide infra). On the other hand, when Sozen was grown in KB cells, harvests of much higher haemagglutinin titre were obtained.

Tables III and IV summarize the haemagglutinin titres obtained on the various passes in the two cell lines in two ounce and in sixteen ounce prescription bottles, respectively. Two ounce prescription bottles were inoculated with 10^5-10^6 $TCID_{50}$ of virus and the sixteen ounce prescription bottles were inoculated with 10^6-10^7 $TCID_{50}$. In both cases, the virus inoculum was grown in human amnion cells. The bottles were frozen when the cytopathic effect became complete, this period of time being indicated in the tables in the columns labelled "incubation period". Table III shows the haemagglutinin (HA) titres of the individual bottles whereas the HA titres reported in Table IV are the titres of the pooled harvests of a number of bottles. In two

TABLE III

HAEMAGGLUTININ TITRES OF INDIVIDUAL CULTURES OF STRAIN GOWEN GROWN IN PRIMARY HUMAN AMNION AND IN KB CELLS

	NO. OF BOTTLES	INCUBATION PERIOD (IN DAYS)	HA TITRE
HUMAN AMNION CELLS	6	10-16	16 16 16[1] 32 32 32
	4	14-16	8 16 16 16
	3	16	<2 <2 <2
KB CELLS	4	5-6	256 256 128 32
	4	3	128 128 128 64
	5	4	>1024 >1024 >1024 >1024 >1024

[1]The patterns of the settled cells were non-classical.

TABLE IV

HAEMAGGLUTININ TITRES OF POOLED HARVESTS OF STRAIN GOSEN GROWN IN PRIMARY HUMAN AMNION AND IN KB CELL CULTURES

	NO OF BOTTLES	INCUBATION PERIOD (IN DAYS)	HA TITRE
HUMAN AMNION CELLS	6	--	0[1]
	3	13	<2
KB CELLS	9	10	4000
	5	4	1000
	4	4	>1024

[1]The patterns of the settled cells were non-classical.

experiments, cultures were maintained in medium supplemented with either horse serum or calf serum. The object of this procedure was to determine whether the serum supplement affects the HA titres obtained. No significant variation in titre with serum was observed. The experiments were all performed at different times.

Selection for a Haemagglutinating Strain of Semen. Burnet and his colleagues (Beveridge et al., 1944) were able to demonstrate a change in the haemagglutinating properties of Influenza virus isolates after repeated passage at low dilution in the chick embryo amniotic cavity; this is the well-known O-D variation. Maurseth, Flugarud, and Labelle (1960) were able to develop a haemagglutinating variant from the non-haemagglutinating D'Amori strain of ECHO 6 virus. Virus was serially passaged in primary human amnion cells, with adsorption to erythrocytes and lysis of the erythrocyte-virus complex between passages. Thus the haemagglutinating variant was obtained apparently by a process of selection.

Two experiments were conducted to determine whether it was possible to derive, by either adaptation or selection, a variant strain of Adenovirus type 7 which produces higher HA titres when grown in primary human amnion cells. The first experiment consisted of repeated passage of type 7

virus in human amnion cells in order to determine whether further adaptation to human amnion cells results in increased haemagglutinin production. The original Gomen material had previously been passed four times in primary human amnion cells. Serial ten-fold dilutions of the material from each passage were prepared and six amnion cell cultures were inoculated with 0.2 ml. of each dilution. The titrations were incubated for two weeks and the tubes of the highest dilution which showed a strong cytopathic effect were frozen. The cells were disrupted by three cycles of freezing and thawing and the tissue culture fluid was clarified by centrifugation at 2500 r.p.m. for thirty minutes. The entire process was then repeated. In all, five passes in primary human amnion cells were performed. The final material represented a 10^{-15} dilution of the original. In each passage, the dilution harvested lay between 10^{-1} and 10^{-3}. The highest haemagglutinin titre obtained after any passage was 1:4.

In the second experiment, advantage was taken of the fact that type 7 adsorbs to erythrocytes at 37°C. and elutes from them at 4°C., a process which is more fully described in a later section. It was theorized that any virus material which adsorbs and elutes will probably haemagglutinate as well and therefore adsorption and elution between passes would select for haemagglutinating variants.

Therefore, after each passage, the harvest was allowed to adsorb to erythrocytes at 5% final concentration at 37°C. for twenty minutes. The erythrocytes were centrifuged, the supernate removed, and the red cells resuspended to their original volume with phosphate buffered saline (PBS). The erythrocyte suspension was then incubated at 2-4°C. for twenty minutes and centrifuged in the cold and the supernate, which contained eluted haemagglutinin was withdrawn. This eluate was usually diluted two to five times in PBS and 0.2 ml. inoculated into each of ten tube cultures of human amnion cells. The tubes were incubated for one to two weeks until the cytopathic effect was complete and were then frozen. The original material employed was the harvest from the final passage of the adaptation experiment just described. In all, four passages were performed and the HA titre was never higher than 1:4 after any passage.

The Haemagglutinin Washing Agent. Classical haemagglutination patterns were not manifest in titrations of Gomen virus grown in primary human amnion cells. Instead, erythrocytes settled as a central amorphous ring in much the same fashion as the negative pattern, but with a ragged edge, in contrast to the compact ring and defined edge of the negative pattern. Figure 1 is a photograph of three haemagglutinin titrations, one of Gomen grown in human amnion cells (uppermost row of patterns in the figure).

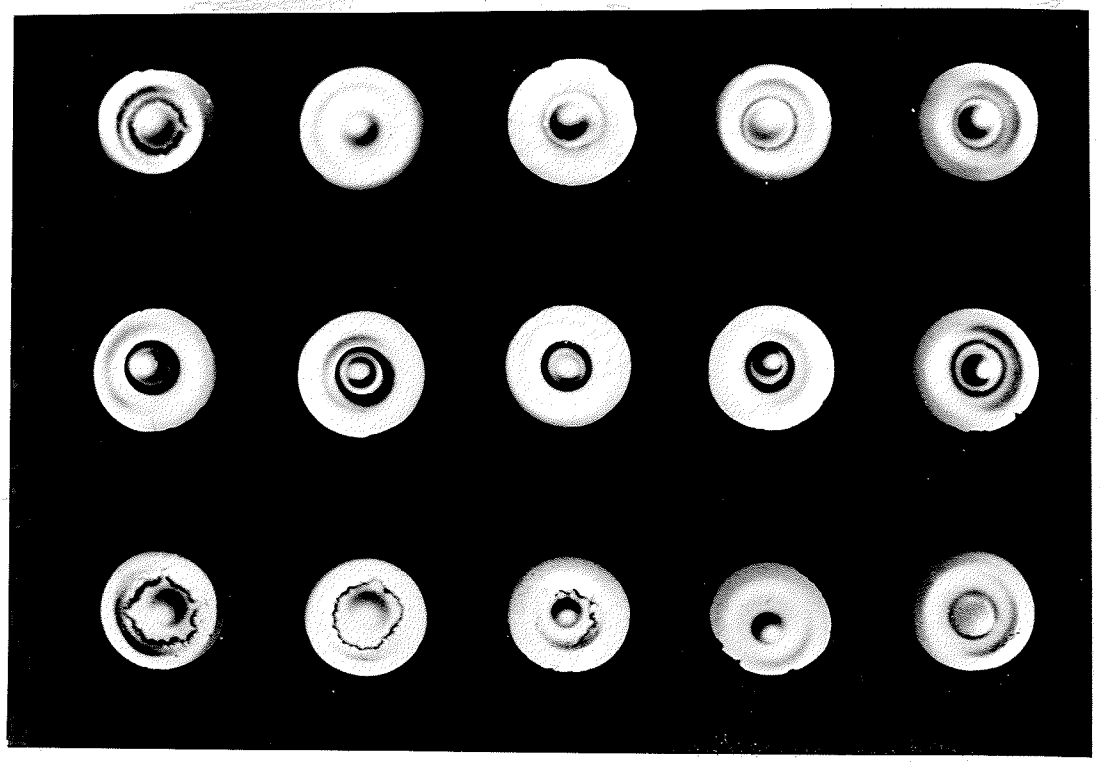

FIGURE 1

SETTLING PATTERNS IN HAEMAGGLUTINATION TITRATIONS

Upper row : Genes grown in primary human amnion cells.
Middle row: uninfected primary human amnion cell extract
Lower row : Genes grown in KB cells.

The innermost and outermost rings, best exemplified in the pattern in the top right corner, are reflections of light and are to be ignored.

one of Semen grown in KB cells (lowermost row), and the third of uninfected human amnion cell extracts (central row). In the human amnion-grown Semen titration (uppermost row) the patterns, reading from left to right, are interpreted as modified, classical, ±, negative, negative. The patterns of the titration of uninfected human amnion cell extracts (central row) are all read as negative. The first three patterns from the left of the titration of Semen material grown in KB cells (lowermost row) are interpreted as modified but differ slightly from the modified pattern of human amnion-grown virus. The fourth pattern from the left is a classical one and the last pattern is a negative pattern inserted for contrast.

Except for a few rare occasions, such as the one demonstrated in the figure, the modified patterns were the only indication that harvests from human amnion cells infected with Semen virus contained any haemagglutinin. Although the first few dilutions of KB-grown virus also presented a modified settling pattern, the HA titre of these materials was high enough so that classical haemagglutination occurred in the higher dilutions in which this effect had presumably been lost by dilution to extinction.

It was found that after partial purification of human amnion-grown virus by adsorption and elution, classical haemagglutination patterns were obtained routinely to a

titre of 1:2 to 1:16. An experiment was designed to determine the origin of the factor, or masking agent, responsible for the modified patterns in the unpurified materials. Adsorption of human amnion-grown material by erythrocytes at 2.5% final concentration was allowed to proceed at 37°C. in a total volume of 1.2 ml. in ten replicate tubes. After thirty minutes, the tubes were centrifuged and the supernates removed. The packed erythrocytes of two tubes were resuspended in 1.2 ml. of PBS; two others in Earle BSS; two in Eagle-Earle medium without calf serum supplement; two in Eagle-Earle with 5% calf serum; two in uninfected human amnion cell extracts; and two in the supernatant fluids recovered after adsorption. All the solutions had been previously adjusted to pH 7.0 with 0.1 N NaOH or HCl. Elution was allowed to proceed for thirty minutes at 4°C. and the tubes were centrifuged in the cold. The original material did not give classical haemagglutination on titration. Table V records only the classical patterns obtained.

Classical patterns appeared only in the eluates which contained no calf serum. Furthermore, elution into the Eagle's-Earle's medium resulted in classical HA patterns while elution into the Eagle's-Earle's-5% calf serum medium did not. The only difference between the two solutions is that one contains calf serum. It was therefore concluded

TABLE V

REMOVAL OF THE MASKING AGENT
BY ADSORPTION AND ELUTION

ELUTING FLUID	HA TITER
Supernate after Adsorption	<2
Amnion Cell Extract	<2, <2
Eagle-Earle-5% Calf Serum	<2, <2
Eagle-Earle	2, 4
Earle's BSS	2, 4
PBS	2, 2

that (1) Dawson grown in primary human amnion cells haemagglutinated erythrocytes to low titre only and that (2) there was present in unpurified virus suspensions, an agent, apparently associated with calf serum, which prevented either the expression of classical haemagglutination or the elution of the haemagglutinin from the erythrocytes. A duplicate experiment yielded the same results.

A further experiment was performed to determine the mode of action of the masking agent. Adsorption and elution in 2.0 ml. volumes was conducted as described above. After adsorption, duplicate tubes of packed erythrocytes were suspended in one of the following fluids: PBS containing 2% calf serum; PBS containing 0.3% bovine serum albumin; PBS; or PBS-2% calf serum thrice adsorbed with erythrocytes (2.5% final concentration) for ten minutes at 37°C. The bovine serum albumin was employed to determine whether the masking effect could be duplicated by a crystalline protein; the adsorbed serum to determine whether the factor might interfere with haemagglutination by binding to erythrocytes in which case it might be removed from solution by adsorption to erythrocytes. In addition, 0.5 ml. of each of the duplicate eluates in PBS was mixed with 0.5 ml. of PBS containing 2% calf serum in order to determine whether the serum acted as a masking agent. Table VI presents the HA titres, classical and otherwise, of the

TABLE VI

THE INFLUENCE OF THE COMPOSITION OF THE ELUTING FLUID ON THE MANIFESTATION OF HAEMAGGLUTININ ACTIVITY

ELUTING FLUID	TITRE	HA PATTERNS
PBS	4,4	Classical
PBS +2% Calf Serum	8	Modified
3x Absorbed PBS +2% Calf Serum	4,4	Modified
PBS +0.3% Bovine Serum Albumin	4,4	Modified
PBS (PBS-Calf Serum Added After Elution)	4,4	Modified

various eluates. Classical patterns were obtained only with the eluates in PBS. In all others, the non-classical patterns were quite similar.

Since the materials eluted into PBS gave non-classical patterns when mixed with serum, the serum probably masks the classical pattern. The masking agent does not seem to be appreciably adsorbed to erythrocytes as the test with thrice adsorbed PBS-calf serum mixture demonstrates. Further, since the patterns of virus eluted into bovine serum albumin approximate the patterns of virus eluted into serum, the masking effect may be due to a non-specific effect of protein or to the albumin in calf serum.

A subsequent experiment was performed to determine whether elution is inhibited in the presence of serum. Human amnion-grown Gomen was adsorbed to erythrocytes and eluted into PBS containing 2% calf serum as described above. The eluate was then subjected to a further adsorption to erythrocytes and was allowed to elute into PBS with no addendum. HA titrations revealed that the original material and the eluate into serum gave modified HA patterns while the eluate into PBS gave classical patterns. As all three manifested the same titre, it was concluded that calf serum acts only as a masking agent and does not affect elution. Calf serum obtained from local sources was used in the experiments described, and hence no conclusions can be drawn about calf serum in general.

All further work reported was concerned with EB-grown Gosen material in the crude state unless stated otherwise.

Haemagglutinin Assay. Experiments were undertaken to determine the optimal volume and erythrocyte concentration for the assay of haemagglutinin. Titrations were performed in 0.4 ml. and 0.8 ml. volumes at the same erythrocyte concentrations. The titres were identical in both volumes but the erythrocytes took longer to sediment in 0.8 ml. and the patterns were less clear. As volumes of less than 0.4 ml. are inconveniently small, all subsequent HA titrations were performed in this volume.

A number of experiments were performed to determine the relationship between erythrocyte concentration and HA titre. One-tenth millilitre of each erythrocyte suspension was added to the 0.4 ml. dilutions. The erythrocyte concentrations tested ranged between the final concentrations of 0.6% and 0.05%. At final erythrocyte concentrations below 0.075%, the patterns were indiscernible whereas they were clear at higher concentrations. The titre obtained changed very little between 0.2% and 0.6%. However, between 0.075% and 0.2% the haemagglutinin titre was inversely proportional to the concentration. The rate of increase of the titre with decrease in concentration varied from experiment to experiment; as an example, the ratio

between titres at 0.1% and 0.2% ranged from 2:1 to 16:1. All the experiments, however, did demonstrate higher titres at the lower concentrations. A final concentration of 0.1% erythrocytes (0.1 ml. of 0.3% erythrocytes added to 0.4 ml. of the virus dilution) was selected for use in all further work because the test was rendered very sensitive at this concentration, but yet allowed one to make a clear distinction between a positive and negative settling pattern.

For routine haemagglutination titrations, normal rabbit serum was added to the erythrocyte suspension to the final concentration of 0.5% in order to prevent spontaneous agglutination. It was observed, however, that the erythrocytes did not sediment as clearly as desirable in titrations of partially purified virus. An experiment was conducted in order to determine whether higher concentrations of normal rabbit serum would increase the clarity of the settled patterns. Six titrations were dispensed from a master dilution and erythrocyte suspensions containing 0.5%, 1.0%, and 2.0% normal rabbit serum were added to two titrations each. The patterns were clearer at 1.0% than at 0.5% and there was no difference in titre between the two. At 2%, the patterns were even more clear but the titre was four-fold lower. It was, therefore, decided to use 1% normal rabbit serum in the erythrocyte suspension when purified materials were being titrated.

Variations in the Agglutinability of Erythrocytes.

Bosen (1960) found that the erythrocytes from rhesus monkey blood donors varied, from individual to individual, in their susceptibility to agglutination by types 3, 7, and 14 but not by types 11 and 16. Henry, Chardonnet, and Sohler (1963) found that grivet monkey erythrocytes varied in agglutinability with type 7 but not type 3, and were not agglutinated by type 14.

To test for variation, each blood was tested shortly after arrival and simultaneously with the blood lot received two weeks previously, against standard aliquoted pools of Bosen and type 3 (strain G.S.) stored at -20°C. The titrations of each were dispensed from a master dilution and duplicate titrations were employed to test each blood.

The Bosen dilutions were prepared in saline and received 0.1 ml. of an 0.5% suspension of erythrocytes in saline. Dilutions of the type 3 virus were prepared in PBS and received 0.1 ml. of a 1.0% erythrocyte suspension in PBS since earlier tests of individual bloods with the standard preparation of the virus had been carried out under these conditions. The titrations of both viruses were incubated at 37°C. The titres obtained are presented in Table VII. Differences in haemagglutinin titre greater than two-fold are considered significant. It is evident that there were significant variations in titre with

TABLE VII

VARIATION IN AGGLUTINABILITY OF INDIVIDUAL GRIVET MONKEY ERYTHROCYTES[1]

VIRUS	HA TITRE								TOTAL
	<16	16	32	64	128	256	512	1024	
Type 7 (Gomen)	6[2]	2	2	0	4	1	0	0	15
Type 3 (G.B.)	0	0	0	0	2	0	6	10	18

[1]Titre obtained when blood was tested within 7 days of collection.

[2]Number of individual monkey blood samples agglutinated to the titre shown.

individual blood lots with the Gomen virus. Except for two blood lots which demonstrated titres of 128 with the type 3 preparation, the majority of the individual monkeys donated blood which did not vary significantly in titre.

On correlating the haemagglutinin titres obtained with the blood lots when first tested and when tested two weeks later[1], it was observed that agglutinability with Gomen tended to decrease on storage(Table VIII). The HA titre with most of the bloods had decreased by only a factor of two and would normally be considered insignificant. However, this decrease in titre was a consistent phenomenon and is probably a real decrease. One blood lot seems to have increased sixteen-fold in its titre with Gomen. This increase is probably due to some experimental error since it varies so markedly from the other bloods tested.

The titres of the bloods tested with type 3 also tended to change on storage but no consistent pattern of increase or decrease was noted and no generalisations can be made.

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[1]The bloods were stored at 4°C. mixed with an equal volume of Alsever's solution.

TABLE VIII

CHANGES IN AGGLUTINABILITY WITH AGE OF ERYTHROCYTES[1]

CHANGE IN MA TITRE[2]	TYPE T	TYPE 3
16x+	1	0
8x+	0	0
4x+	0	1
2x+	0	3
0	1	1
2x -	5	1
4x -	1	1
8x -	1	0

[1]Number of bloods which demonstrated the changes in titre shown when tested within 7 days of collection and again 14 days later.

[2] + denotes increase; - denotes decrease.

II. STABILITY OF THE HAEMAGGLUTININ

Thermal Inactivation. Experiments were performed to determine the thermal inactivation rates of crude and of partially purified Gosen preparations at 37°C., 45°C., and 56°C. The same basic experimental techniques were employed in all experiments. The materials to be tested were dispensed in 1.0 ml. volumes in corked Kahn tubes (10 mm. x 85 mm.) and were stored in a water bath at 4°C. until incubation. The sample tubes were then immersed in a thermostatically controlled water bath at the desired temperature at various time intervals. At the completion of the incubation period, all the tubes were removed and immediately cooled in a 4°C. water bath. HA titrations were performed immediately the experiment was concluded. Triplicate samples were employed in the experiment at 37°C., duplicate samples in the 56°C. experiment, and single samples at 45°C. The crude virus in the 37°C. and 45°C. experiments was EE-grown Gosen virus diluted 1:20 with PBS. In the experiment at 56°C. the same virus material, but undiluted, was employed. In all the experiments, the purified virus was EE-grown material partially purified by adsorption to erythrocytes and elution into PBS. Thermal inactivation tests at 37°C. and at 45°C. were performed once only, whereas that at 56°C. was repeated four times.

Table IX shows the results of these three experiments.

TABLE IX

THERMAL INACTIVATION OF CRUDE AND OF PURIFIED PREPARATIONS OF THE TYPE 7 HAEMAGGLUTININ

EXPERIMENT	TEMPERATURE	TIME	HA TITRE	
			CRUDE	PURIFIED
1	37°C.	0	1024[1]	64[1]
		4 hrs.	512	2
		8 hrs.	800	<2
		16 hrs.	512	<2
		24 hrs.	128	<2
2	45°C.	0	256	64
		15 mins.	256	32
		30 mins.	256	2
		60 mins.	64	<2
		120 mins.	64	<2
3	56°C.	0	512[2]	256[2]
		2 mins.	512	92
		4 mins.	512	23
		6 mins.	736	32
		8 mins.	256	11

[1]Geometric means of triplicate samples.

[2]Geometric means of duplicate samples.

In all, the results indicate that the thermal inactivation rate of crude virus is less than that of partially purified material. Of the three other thermal inactivation tests performed at 56°C., a similar difference in the rate of inactivation of crude and of purified materials was observed in two. In the fourth experiment, however, the crude material had been diluted 1:20 with PBS and its inactivation rate was no different from that of the purified haemagglutinin.

The previous set of experiments indicate that unpurified preparations of Sonea are inactivated more slowly than purified preparations. Experiments were performed to determine whether the effect could be duplicated by the incorporation of serum or of crystalline bovine serum albumin fraction V in the purified haemagglutinin preparations. To this end, calf serum and serum albumin were incorporated in preparations of purified HA to final concentrations of 2% and 0.15% respectively. These concentrations were chosen because 2% is the concentration of serum in tissue culture fluids and the 0.15% serum albumin is approximately equivalent to 2% serum in protein content. Inactivation of the haemagglutinin in these preparations was compared to the inactivation at 56°C. of both crude and purified HA preparations with no addendum. It is evident from Figure 2 that addition of serum to the

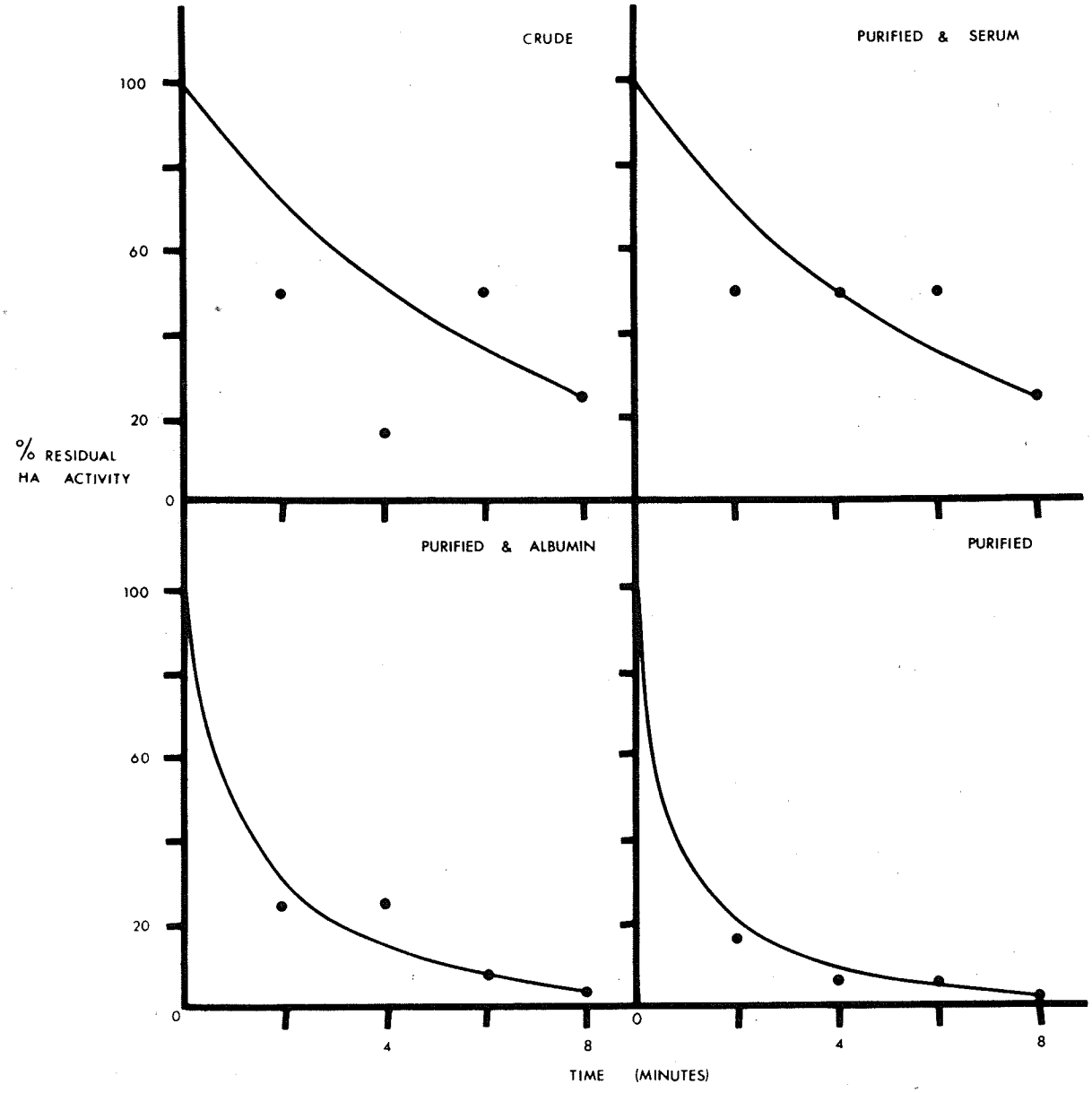

FIGURE 2

EFFECT OF THE COMPOSITION OF THE HA PREPARATION ON INACTIVATION AT 56°C

purified HA stabilises it. Bovine serum albumin did not produce this effect, however.

In an earlier experiment, the same procedures were employed except that bovine serum albumin was employed at a final concentration of 2% rather than 0.15% and was dissolved in water rather than PBS. Under these conditions, the inactivation rate was identical to that of unpurified material. Stabilization may have been due either to the higher concentration of serum albumin or to the lower salt concentration of the test mixture.

It is, therefore, concluded that one of the serum components stabilizes the Adenovirus type 7 haemagglutinin against thermal inactivation. This component is probably not an albumin although the effect can be duplicated by a fourteen-fold higher concentration of albumin in water.

The Influence of Hydrogen Ion Concentration. Simon (1962) claims that the Adenovirus type 3 haemagglutinin is inactivated at pH 3 and 4 but not at pH values between 5 and 9. A set of experiments was performed in order to determine the pH range in which the types 7 and 3 haemagglutinins are stable.

The same procedure was followed in all experiments, viz., 4.0 ml. of crude virus was mixed with 12.0 ml. of Universal buffer, at different pH values, to give 16.0 ml.

aliquots of 1:4 dilutions of virus in buffer. As the pH value was altered toward neutrality on mixing buffer and virus, it was adjusted to the desired level by cautious addition of a small amount (not more than 0.5 ml.) of 0.2 N NaOH or 0.2 N HCl. When the pH of each mixture was thus adjusted, 9.0 ml. was transferred to another container and the amount of 0.02 N HCl or 0.02 N NaOH required to adjust it to pH 7.0 was determined. The remaining 7.0 ml. was incubated at 37°C. for three hours in a screw capped bottle of 25 ml. capacity. At the end of the incubation period, 4.5 ml. aliquots were removed and adjusted to pH 7.0 with the predetermined amount of acid or alkali; sufficient pH 7.0 buffer to bring the total volume to 9.0 ml. was then added. The pH of the final adjusted mixtures was measured to give assurance that each was neutral: In all experiments, the adjusted values were pH 7.0 $\pm$ 0.2. HA titrations in pH 7.0 buffer were performed immediately the aliquots had been adjusted.

Table X shows the results of two experiments with Semen virus, carried out at different times. Taken together, and though not in absolute agreement, these results indicate that the Semen haemagglutinin is stable in the range pH 4.2 to pH 9.6. In the acid range, inactivation apparently occurs at a level between pH 3.6 and pH 4.2, and in the alkaline

TABLE X

INACTIVATION OF THE TYPE 7 HAEMAGGLUTININ BY CHANGES IN HYDROGEN ION CONCENTRATION

Experiment 1		Experiment 2	
pH	HA Titre	pH	HA Titre
3.1	<2	3.2	<2
3.6	<2	3.5	8
4.2	368	7.2	64
7.2	256	9.6	64
9.7	92	10.5	32
11.1	<2		

range, at a level between pH 9.7 and pH 10.5. The results of control experiments (see page 65) indicate that the limits are closer to pH 3.6 and pH 9.7, respectively. With the limited data available, it is not possible to draw a firmer conclusion.

For the sake of comparison, the stability of the type 3 (G.B.) haemagglutinin was examined in two similar experiments, and with very similar results. Thus, the haemagglutinins of both type 3 and type 7 Adenovirus are stable over a wide range of pH values, the limits being of the same order as the stability of the infective particle (Ginsberg, 1957). None of the type 3 or 7 materials tested were purified and no statement can be made as to the effect of hydrogen ion concentration on purified material.

The buffer used in the experiments reported above was the BDH Universal buffer (see Materials and Methods). Two experiments were performed to determine whether the inactivation observed in the presence of this buffer was due to the pH <u>per se</u> or to the buffer constituents. In the first experiment, Gomen virus was diluted 1:16 with either PBS

or Universal buffer at pH 7.0 and the mixtures were incubated at 37°C. for three hours. HA titrations of the mixtures at the completion of the incubation period revealed no significant difference in titre between the two. It was, therefore, concluded that, at pH 7.0, the buffer ions did not increase inactivation at 37°C. as compared to the salts in PBS.

In the second experiment, the procedure outlined for the experiments on pH-inactivation was followed, using the Gomen haemagglutinin, with the exception that a phthalate-HCl buffer was employed for the lower pH values and a carbonate-bicarbonate-NaOH buffer was employed for the higher values. PBS was used as diluent in the HA assay.

The results of the titrations indicated that the Gomen HA is inactivated between pH 2.8 and pH 3.6 in the presence of phthalate buffer and between pH 9.7 and pH 10.5 in the presence of carbonate buffer. Since this range agrees with that obtained by using Universal buffer, and neither phthalate, carbonate, nor bicarbonate are present in the Universal buffer, it was concluded that the inactivation of the haemagglutinin observed was due to the pH to which it was exposed.

Enzyme Inactivation. Simon (1962) reported that trypsin at 0.25% concentration does not inactivate the Adenovirus type 3 haemagglutinin. However, Wigand and

Bauer (1962) claim that 1% trypsin does inactivate the Adenovirus type 11 HA. Buckland and Tyrrell (1963) found that chymotrypsin at 0.1% inactivated the Adenovirus type 7 haemagglutinin. Two experiments were performed to test the effect of trypsin and of chymotrypsin on crude Adenovirus type 7 preparations which had been grown in KB cells.

The preliminary experiment, in which crude trypsin was employed in concentrations as low as 0.5%, indicated that the haemagglutinin was inactivated. However, the results were not so conclusive that the possible damaging effect of trypsin on the erythrocytes used in the assay could be excluded.

The second experiment was performed with crystalline trypsin and chymotrypsin (see Materials and Methods). One-half millilitre aliquots of virus material were dispensed to ten corked Kahn tubes (10 mm. x 85 mm.). A further 0.5 ml. of 0.2% trypsin in tris buffer was added to two tubes and 0.1% trypsin to two others. Similarly, 0.5 ml. of 0.2% and 0.1% chymotrypsin was added to two tubes each. To the last two tubes was added 0.5 ml. of 0.2% trypsin which had been inactivated by heating at 90°C. for thirty minutes: these tubes served as a virus control. An inactivated chymotrypsin control was not included. Half of the tubes were incubated at 37°C. for one hour and the other half for three hours. The reaction in each tube

was stopped by the addition of 1.0 ml. of 2% crude soybean trypsin inhibitor in PBS.

It is evident from the results shown in Table XI that 0.1% trypsin does not inactivate the Cohen haemagglutinin in three hours at 37°C. while 0.05% chymotrypsin does and in one hour. The titres of 512 and 1024 obtained after trypsin treatment may be due to experimental error or perhaps to disaggregation of "clumped" haemagglutinin since trypsin has been demonstrated to disaggregate "clumps" of type 3 virus as determined by infectivity titrations (Hannan, 1963).

The possibility exists that the soybean trypsin inhibitor, which does not inactivate chymotrypsin as readily as it does trypsin, did not neutralize the chymotrypsin completely and the inactivation observed is due to the removal of the erythrocyte receptors in the haemagglutinin assay by active chymotrypsin. This possibility could only be eliminated by performing an assay specific for chymotrypsin on the test mixture after the addition of the inhibitor.

Chemical Inactivation. Buckland and Tyrrell (1963) found that the degree of inactivation of the type 9 haemagglutinin by urea increased with the urea concentration up to 2 M and remained at the same high level at greater concentrations. They also reported that sodium periodate

TABLE XI

INACTIVATION OF THE TYPE 7 HAEMAGGLUTININ BY CRYSTALLINE TRYPSIN AND CHYMOTRYPSIN

ENZYME	FINAL ENZYME CONCENTRATION	INCUBATION PERIOD (HRS.)	HAEMAGGLUTININ TITRE
Trypsin	0.1%	1	1024
		3	256
	0.05%	1	128
		3	512
Chymotrypsin	0.1%	1	<2
		3	<2
	0.05%	1	2
		3	2
Heat-inactivated trypsin (control)	0.1%	1	128
		3	128

inactivated the haemagglutinin at 0.025 M final concentration. However, Dixon (1963) claims that periodate has no effect on the Adenovirus type 3 haemagglutinin.

Experiments were performed to determine the effects of urea and of periodate on the type 7 haemagglutinin. One-half millilitre of Dixon material was dispensed to each of five corked Kahn tubes. To one tube was added 0.5 ml. of PBS to serve as a virus control and to each of the other four was added 0.5 ml. of one of the following solutions: PBS containing 4 M urea, 1 M urea, 0.25 M urea, and 0.005 M sodium metaperiodate. The Kahn tubes were incubated at 37°C. for one hour and their contents were then transferred to dialysis tubing of 8 mm. diameter. Dialysis against 500 volumes of PBS was allowed to proceed at 4°C. for three hours and the virus suspensions were immediately titrated. The HA titre of the virus control was 512 as were the titres of all the virus suspensions which had been mixed with urea. The mixture which contained periodate in a final concentration of 0.0025 M exhibited a titre of 2. Inactivation of the type 7 haemagglutinin by urea at final concentrations up to 2 M was therefore not demonstrated. Inactivation by periodate was demonstrable, which agrees with the findings of Buckland and Tyrrell (1963), but not with those of Dixon (1963). However, Dixon tested the type 3 haemagglutinin and this may differ from the type 7 haemagglutinin in its

susceptibility to inactivation by periodate.

III. ADSORPTION AND ELUTION

Simon (1962) reported that, after Adenovirus types 3, 7, and 14 had adsorbed to rhesus monkey erythrocytes at 42°C., haemagglutinin would elute at 0-4°C. After establishing that Adenovirus type 7 adsorbs to grivet monkey erythrocytes at 37°C. and elutes at 4°C., and thus confirming Simon's findings, a set of experiments was performed in an attempt to determine the factors which influence adsorption and those which influence elution. In the following experiments, the erythrocyte concentration was varied from experiment to experiment depending on the degree and rate of adsorption required and on the relative agglutinability of the individual lot of blood.

The Influence of Erythrocyte Concentration on Adsorption Rate. Twelve centrifuge tubes containing one-half millilitre aliquots of Gomen material were immersed in a thermostatically controlled water bath at 37°C. for ten minutes. Then, 0.5 ml. aliquots of a warmed 15% erythrocyte suspension in PBS were added. Duplicate tubes were removed after 1, 3, 7, 15, 25, and 40 minutes and were immediately centrifuged for one minute at room temperature in cups containing warmed water. The supernates were removed and titrated at the completion of the

experiment. The same procedure was repeated twice more, but substituting 7.5% and 4% erythrocyte suspensions to yield final erythrocyte concentrations of 3.75% and 2%.

Figure 3 shows the results of this experiment. It is evident from the graph that the adsorption rate is greater at higher erythrocyte concentrations.

The Influence of Temperature on Adsorption. A number of experiments were performed to test the influence of temperature on adsorption of Dosen virus to erythrocytes. In a preliminary experiment, adsorption to erythrocytes at a final concentration of 2.5% in 1.0 ml. volume was allowed to proceed in two sets, one at 37°C. and the other at 4°C. After forty minutes incubation at 37°C., the HA titre of the supernates had decreased sixteen-fold; it did not decrease significantly after forty minutes incubation at 4°C.

A second similar experiment was performed with incubation at 38°C., 18°C., 12°C., and 4°C. Duplicate tubes containing Dosen material and a final erythrocyte concentration of 5% in 1.0 ml. volumes were incubated at each temperature for forty-five minutes. After centrifugation for one minute, the supernates were recovered and titrated. The original material and the virus control (virus incubated at 38°C. for forty-five minutes) had HA titres of 512. The supernates of the mixtures incubated

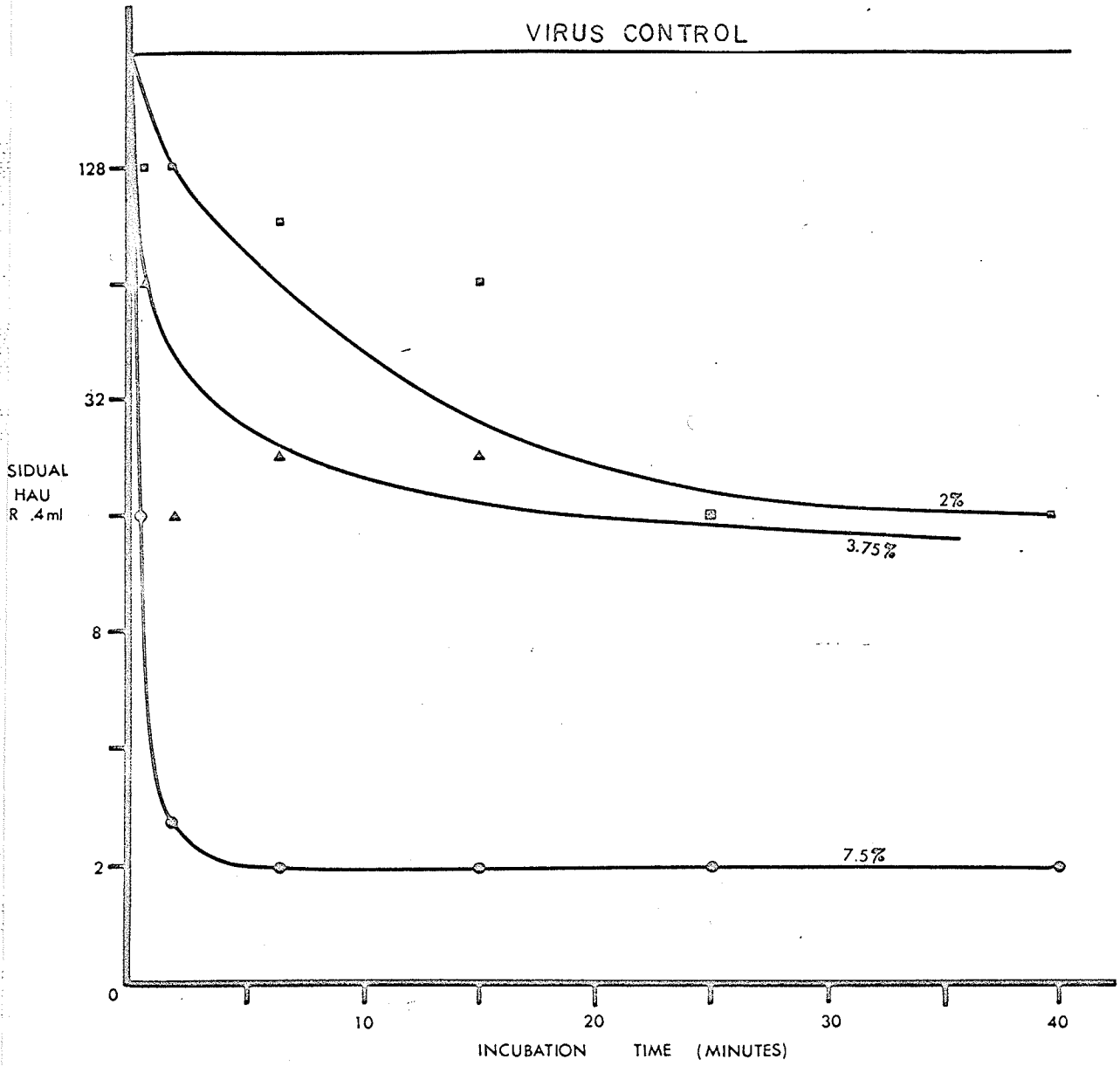

FIGURE 3

EFFECT OF ERYTHROCYTE CONCENTRATION ON RATE OF ADSORPTION OF GOMEN HA

at 38°C. had HA titres of <2 whereas the supernates of the mixtures which had been incubated at 18°C., or at lower temperatures, all had titres of 512 or 1024. Thus, adsorption of the Somen haemagglutinin to erythrocytes does not seem to occur at temperatures of 18°C. or below. This conclusion was confirmed by another experiment in which the adsorption rates at 37°C. and 17°C. were determined.

Two other experiments were conducted in an attempt to determine the effect of temperature on the adsorption rate in the range 12°C.-37°C. The procedures followed were the same as described previously (p. 70). In the first experiment, the adsorption rate of the Somen haemagglutinin to erythrocytes at 2.5% final concentration was determined at 37°C., 26°C., 22°C., and 12°C. Table XII shows the HA titres of the supernates recovered after incubation for various time intervals.

It is evident from the table that adsorption to erythrocytes occurs, and to a degree which increases with time, at 22°C., 26°C., and 37°C. Furthermore, the higher the temperature, the more rapidly does adsorption occur. The fact that, at 12°C., all the supernates had HA titres four-fold lower than the original material does not seem to agree with the previous experiments mentioned above. However, since the titre of all remained the same, this could possibly be due to a rise in temperature on centrifugation

TABLE XII

INFLUENCE OF TEMPERATURE ON THE ADSORPTION RATE OF THE TYPE 7 HAEMAGGLUTININ TO CHICK ERYTHROCYTES

INCUBATION TIME (mins.)	RESIDUAL HA (HAU/0.4 ml. of supernate)			
	12°C.	22°C.	26°C.	37°C.
Virus Control (40 mins. at 37°C.)	256	256	256	256
1	64	128	64	16
3	64	64	32	8
6	64	32	32	6
10	64	32	23	6
20	64	32	23	8
40	64	32	11	3

sufficient for adsorption to occur to a limited degree.

In the second experiment, the final concentration of erythrocytes was 0.5% to see what effect a lower concentration would have on adsorption at the various temperatures. The tests were incubated at 43°C., 37°C., 27°C., 24°C., and 16°C. Adsorption was demonstrable at 43°C. and 37°C. but not at 27°C. and below. Since the only differences between this experiment and the one in Table XII are in the blood lot and the erythrocyte concentration, these results are suggestive, but not conclusive, that at temperatures between 22°C. and 27°C. adsorption only occurs at a relatively high erythrocyte concentration.

The conclusions reached from the data presented above are as follows: adsorption of the Osmen haemagglutinin to erythrocytes occurs at 37°C., even at relatively low erythrocyte concentrations such as 0.5%. It does not occur between 4°C. and 13°C. even when the erythrocyte concentration is relatively high, e.g., 5%. The relationship between adsorption, temperature, and erythrocyte concentration at temperatures between 22 C. and 27 C. has not been determined conclusively.

The Influence of Hydrogen Ion Concentration on Adsorption. An experiment was performed to determine the pH range in which the Osmen haemagglutinin adsorbs to erythrocytes. Since preliminary experiments had

indicated that the McIlvaine Universal buffer did not have sufficient buffering capacity, phthalate-NaOH and carbonate-bicarbonate-NaOH buffers were employed for the acid and basic pH ranges, respectively, and PBS was employed for the test at pH 7.0. Five millilitres of virus material was mixed with 15.0 ml. of buffer at different pH levels and the pH was adjusted back to the original buffer level with 0.2 N NaOH or 0.2 N HCl as described above (see p. 61). Four millilitre aliquots of a 5% erythrocyte suspension in physiological saline were dispensed to six centrifuge tubes and the erythrocytes were sedimented by centrifugation. The supernates were removed and the temperature of the packed erythrocytes was allowed to equilibrate to that of a 37°C. water bath for ten minutes. The dilutions of virus in buffer were similarly warmed. The erythrocytes were resuspended to 2.5% concentration with 6.0 ml. of the virus-buffer mixtures and incubation was allowed to proceed for ten minutes at 37°C. The mixtures were then centrifuged for one minute at room temperature and the supernates were removed. The final pH of the supernates was determined and samples were adjusted to neutrality with 0.2 N NaOH or 0.2 N HCl. HA titrations in PBS were then performed on the adjusted samples.

Table XIII shows the residual haemagglutinin levels after adsorption. The pH values recorded are those of the

TABLE XIII

EFFECT OF HYDROGEN ION CONCENTRATION ON ADSORPTION OF THE COMBS HAEMAGGLUTININ TO ERYTHROCYTES

BUFFER	FINAL pH[1]	RESIDUAL HAEMAGGLUTININ (HAU/0.4 ml. of supernate)
Phthalate	6.1	2
Phthalate	6.3	8
PBS	7.2	8
Carbonate	7.8	8
Carbonate	9.0	32
Original in PBS (control)	-	256

[1]After incubation with erythrocytes.

supernates; the pH values of the original virus dilutions in buffer had altered by approximately one pH unit toward neutrality during incubation with the erythrocytes. It is evident that adsorption to erythrocytes occurs at pH values between 6.1 and 9.0. Furthermore, it appears that a greater degree of adsorption occurs at pH 6.1 than at 7.2, and a lesser degree at pH 9.0, but this observation would have to be confirmed to be conclusive.

Elution of Haemagglutinin. Simon (1962) reported that complete elution of the type 3 haemagglutinin from erythrocytes to which it had adsorbed, occurred within five minutes after exposure to temperatures between 0°C. and 4°C. An experiment was performed to determine the influence of two factors on the degree of elution: i) the length of time allowed for adsorption and ii) the length of time allowed for elution. To this end, four replicate adsorptions of the Gomen haemagglutinin to 7.5% erythrocytes (in PBS) in a volume of 1.0 ml. were allowed to proceed for forty minutes at 37°C. Two other similar tests were begun thirty-nine minutes later so that adsorption would only proceed for one minute. In all six, the virus material and the erythrocyte suspension had been warmed to 37°C. prior to mixing. All the mixtures were centrifuged together for one minute at room temperature, but in warmed centrifuge cups containing 1.0 ml. of water to minimize the effect of

any drop in temperature. After removal of the supernates, the centrifuge tubes containing the packed erythrocytes were immersed in a water bath at 3°C. and the temperature was allowed to equilibrate for five minutes. Cold PBS was then employed to resuspend the erythrocytes to their original volume. After five minutes further incubation at 3°C. two tubes in which adsorption had been allowed to proceed for forty minutes and the two tubes in which it had been allowed to proceed for only one minute, were centrifuged for one minute in the cold. The supernates were recovered. The last two tubes were incubated at 3°C. for another thirty-five minutes and were then centrifuged, too. All the supernates recovered were titrated. Table XIV shows the geometric mean titres of each set of duplicates. It is evident that the length of the adsorption period and the length of the elution period have no effect on the degree of elution within the limitations of the experiment.

The Influence of Temperature on Elution. Simon (1962) reported that complete elution of the type 3 and 7 haemagglutinins occurred only at 0°C. to 4°C. although partial elution was observed at 20°C. Five experiments were performed in order to determine the temperature range in which elution can occur. The procedure described below was followed in all.

Adsorption of the Gozen haemagglutinin to 5%

TABLE XIV

INFLUENCE OF THE LENGTH OF THE ADSORPTION PERIOD AND OF THE ELUTION PERIOD ON DEGREE OF ELUTION

LENGTH OF ADSORPTION PERIOD (mins.)	HA TITRE AFTER ADSORPTION	LENGTH OF ELUTION PERIOD (mins.)	HA TITRE AFTER ELUTION[1]
1	6	5	256
40	4	5	308
40	8	40	256

[1]The initial titre at the same dilution was 256.

erythrocytes in 1.0 ml. volumes was allowed to proceed for twenty minutes at 37°C. The suspensions were then centrifuged and the supernates removed. Duplicate tubes containing the packed erythrocytes were distributed in water baths adjusted to various temperature levels and the temperature was allowed to equilibrate for five minutes. The erythrocytes were then resuspended in 1.0 ml. of PBS prewarmed or precooled to the test temperature and were incubated for ten to twenty minutes. The suspensions were centrifuged for one minute at room temperature. In all the experiments, the tubes were centrifuged in cups containing water, the temperature of which had been allowed to equilibrate in the corresponding water bath. The HA titres of the supernates recovered after adsorption were usually <2 although an occasional supernate would demonstrate a titre of 1:4.

For greater convenience, the HA titres of the eluates have been expressed as a percentage of the titre of the eluate at 2-4°C. The percentage values were related to the eluate at 2-4°C. rather than to the original material in order to eliminate the fact that complete elution was not obtained at any temperature in one of the five experiments and because the object was to determine the relative degree of elution at various temperatures. Complete elution was also not obtained in a similar preliminary experiment.

The reason is unknown.

The individual percentage values are shown in Figure 4. It is evident from the figure that a considerable degree of elution is obtained at temperatures up to 18 to 22°C. At temperatures above this, haemagglutinin was recovered but to lower titre. It is also evident that the degree of elution, at the various temperatures tested, varied considerably from experiment to experiment and within the individual experiments as well. The reason for this, too, is unknown. The elution found at temperatures above 20°C. might be due to residual haemagglutinin although this is unlikely since the HA titres of the supernates recovered after adsorption were extremely low and haemagglutinin was consistently recovered in tests at the higher temperatures.

The Influence of the Volume of the Eluting Fluid on Elution. Dixon (1962) reported that, on reduction of the volume in which elution occurred relative to the adsorption volume, a reduction in the amount of haemagglutinin eluted was obtained.

An experiment was performed to test the effect of changes in volumes on elution. Twelve replicate adsorptions of the Queen haemagglutinin to 2% erythrocytes in 5.0 ml. volumes were allowed to proceed for thirty-five minutes at 37°C. The tubes were centrifuged for three minutes and the supernates removed. The packed erythrocytes of four

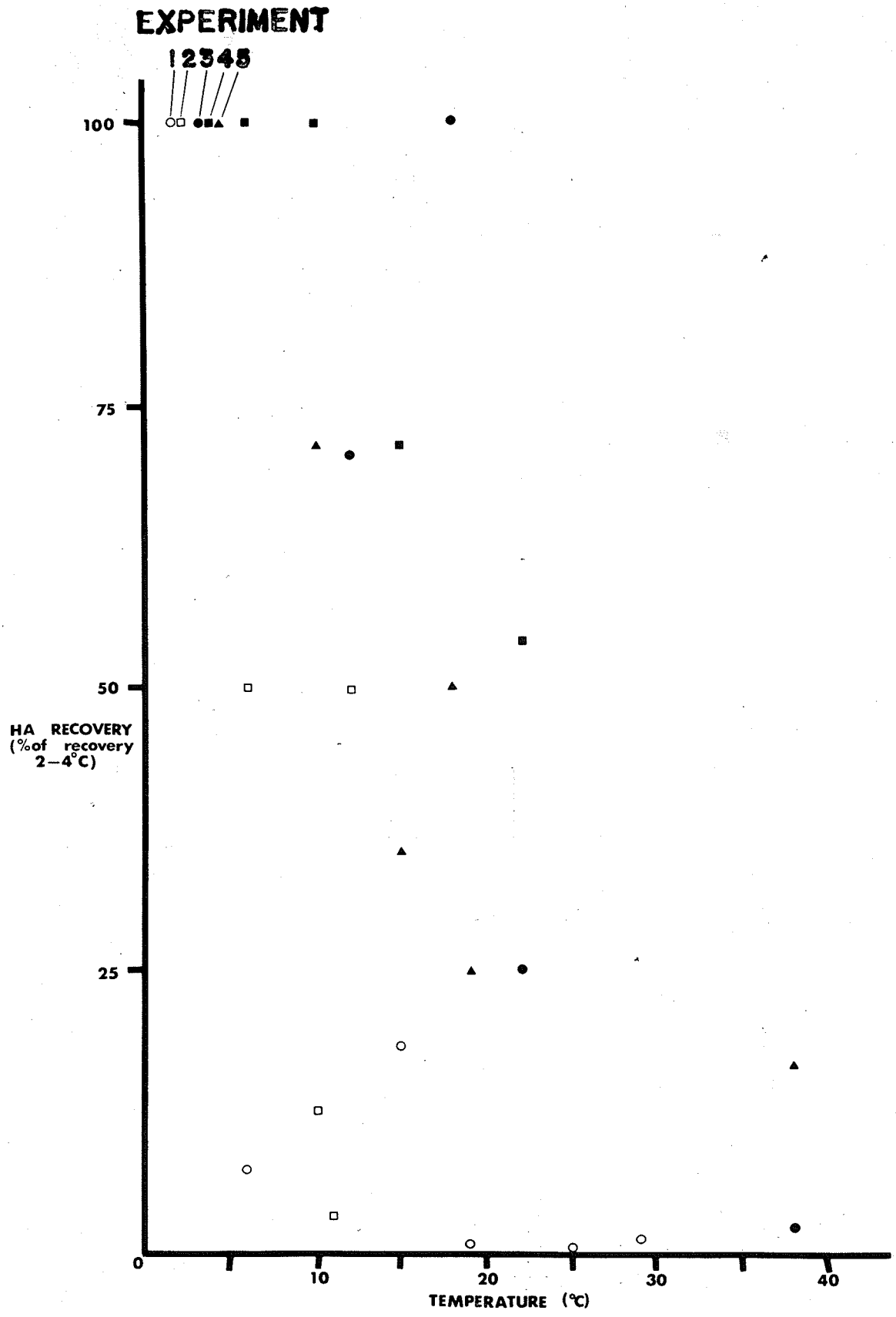

FIGURE 4

THE INFLUENCE OF TEMPERATURE ON ELUTION

replicate tubes were resuspended to 0.5 ml. with cold PBS; those of four tubes to the original volume of 5.0 ml.; and the last four tubes to 15.0 ml. All were incubated at 2°C. for thirty-five minutes. The tubes were then centrifuged and the supernates removed.

Table XV shows the results. The titres of the supernates after adsorption were all <2. It is evident from Table XV that there was no significant difference in the recovery of haemagglutinin at the original volume of 5.0 ml. and at one-tenth the original volume (0.5 ml.). However, two and a half times the recovery at 5.0 ml. was obtained at 15.0 ml. volume.

Three other similar experiments were performed. In none was there any significant reduction in the recovery of haemagglutinin after elution at lower volumes than the original. However, recovery after elution at two to three times the original volume ranged from two times to six times that obtained on elution into the original volume. In all the experiments there were no significant differences between the recovery of haemagglutinin after elution into the original volume and the original material. However, the amount of haemagglutinin recovered at higher volumes was greater than the amount present in the material which was being adsorbed. This might indicate that disaggregation of clumped haemagglutinin occurs on adsorption and elution

TABLE XV

THE INFLUENCE OF THE VOLUME OF THE ELUTING FLUID ON RECOVERY OF THE GOREN HAEMAGGLUTININ BY ELUTION

VOLUME OF ELUATE	MEAN[1] HA TITRE OF ELUATE (HAU/0.4 ml.)	CALCULATED TOTAL HAU
0.5	100	125
5.0	16	200
15.0	13.5	500
Virus Control (2.5 ml.)	110	690

[1]Geometric mean of four HA titres.

but except for the data obtained from the experiments on the effect of trypsin on the haemagglutinin (p. 65) no other evidence is available for the existence of clumped haemagglutinin.

The Influence of Hydrogen Ion Concentration on Elution. An experiment was performed to determine the pH range in which elution occurs. Although an attempt was made to determine the pH levels above neutrality on elution, the pH levels of the Universal buffer and of the carbonate-bicarbonate-NaOH buffers used dropped to less than pH 8.0 on incubation with erythrocytes. A further limitation to this experiment was that lysis of the erythrocytes occurs at pH values below 5.0 (see below).

Adsorption of the Conex haemagglutinin to 5% erythrocytes in PBS was allowed to proceed at 37°C. for twenty minutes. Three replicate mixtures and a volume of 10.0 ml. (so that the pH of the eluate could be determined) were employed. The tubes were then centrifuged at room temperature and the supernates were completely removed. The erythrocytes were resuspended to their original volume with 10.0 ml. of phthalate-NaOH buffer at pH 4.6, 5.0, and 5.4. The suspensions were then incubated at 4°C for twenty minutes and the tubes were again centrifuged. The supernates were removed, a sample of each was adjusted to neutrality with 0.2 N NaOH and titrated, and the pH of the

remainder was determined.

The HA titres of the supernates after adsorption were all <2. Table XVI shows the HA titres of the eluates at each pH. The pH levels shown are those of the supernates after incubation at 4°C. Elution occurred at pH 4.8 to 6.0 although only 25% was recovered. Lysis of the erythrocytes was not detected in any eluate.

Two similar experiments were performed and elution always occurred at pH levels from 7.0 to 5.2. Lysis was observed at lower pH values but elution to the same degree occurred, regardless of the lysis.

IV. STABILITY OF THE ERYTHROCYTE RECEPTORS

The Influence of Hydrogen Ion Concentration on Erythrocyte Receptors. An experiment was performed to determine what effect exposure to various pH levels would have on the erythrocyte receptors for the Semen haemagglutinin. Five millilitre aliquots of a 10% erythrocyte suspension in saline were dispensed to six centrifuge tubes. The erythrocytes were sedimented by centrifugation and the supernates were removed. The packed erythrocytes were then resuspended with 10.0 ml. of phthalate-NaOH buffer at different pH levels and were incubated at 37°C. for three hours. The erythrocyte suspensions were centrifuged and the supernates removed. The pH level of the supernates

TABLE XVI

EFFECT OF HYDROGEN ION CONCENTRATION ON ELUTION

FINAL pH OF SUPERNATE AFTER ELUTION	HA TITRE
4.8	32
5.3	32
6.0	32
Original Virus[1]	128

[1]Stored at 4°C. during the experiment.

was measured. The erythrocytes which had been incubated at pH levels at which lysis did not occur (i.e. above pH 5.5) were diluted to 0.5% in physiological saline and normal rabbit serum was added to each suspension to 0.5% concentration. The suspensions were then added to dilutions of Semen material dispensed from a master dilution (0.1 ml. of erythrocyte suspension to each dilution in 0.4 ml.). Simultaneously, an untreated suspension of erythrocytes in PBS at pH 7.3 was also added to a similar row of dilutions. The racks of titrations were shaken and incubated at 37°C. for two hours, at which time they were read. The same HA titres were obtained with all suspensions and the conclusion was drawn that incubation of erythrocytes at pH levels between pH 5.5 and 6.6 does not damage their receptors for Semen virus. This conclusion is based on the assumption that damage to the receptors would be manifested in a reduction in the HA titre obtained.

Enzymic Inactivation of Erythrocyte Receptors.

Buckland and Tyrrell (1963) claim that 0.01% chymotrypsin has no damaging effect on rhesus monkey erythrocyte receptors for Adenovirus type 7. Dixon (1962) found that 0.25% trypsin destroys the receptors of rhesus monkey erythrocytes for the Adenovirus type 3 haemagglutinin.

An experiment was performed to determine whether

trypsin and chymotrypsin damage the receptors of grivet monkey erythrocytes for the Gomen haemagglutinin. One-half millilitre aliquots of a 5% erythrocyte suspension were dispensed to three centrifuge tubes. To one tube was added 0.5 ml. of tris buffer containing 0.01% crystalline trypsin; to the second, 0.5 ml. of tris buffer containing 0.01% crystalline chymotrypsin; and to the third was added 0.5 ml. of tris buffer to serve as a control. The suspensions were incubated at 37°C. for one hour and the erythrocytes were sedimented by centrifugation. The supernates were removed and the erythrocytes were washed with 10.0 ml. of saline (i.e. at least twenty volumes). The cells were sedimented again, the supernate discarded, and the erythrocytes were diluted to 0.5% in saline. Normal rabbit serum was added to 0.5% concentration and the suspensions were used in HA titrations in which the dilutions were dispensed from a master dilution. The titre obtained with the control erythrocytes was 128 while those obtained with the erythrocytes treated with trypsin and chymotrypsin were both 1:2. The conclusion was drawn that trypsin and chymotrypsin both inactivate grivet monkey erythrocyte receptors for the Gomen haemagglutinin.

Chemical Inactivation of Erythrocyte Receptors.

Buckland and Tyrrell (1963) found that periodate at

0.0058 M reduced the agglutinability of rhesus monkey erythrocytes with type 7. Simon (1962) found destruction of rhesus monkey erythrocyte receptors for the type 3 haemagglutinin by 0.001 M periodate. An experiment was performed to test the effect of urea and of periodate on the grivet monkey erythrocyte receptors for the Bowen haemagglutinin. The same procedure was followed as described above, but periodate was used at a final concentration of 0.000025 M (higher concentrations lysed the erythrocytes) and urea was used at concentrations up to 1 M (lysis occurred at 2 M). Neither periodate nor urea demonstrated any inactivation of the erythrocyte receptors at these concentrations, using the titre obtained in HA titrations as an index.

DISCUSSION

The published literature on the haemagglutination of Adenovirus type 7 has been based on research performed with virus grown in KB or Hela cells, in which harvests of high haemagglutinating activity are obtained. Furthermore, in this laboratory, Adenovirus type 3 (U.S. strain), regularly produces harvests of high HA titre when grown in primary human amnion cells. Adenovirus type 7, however, does not. The two experiments which were performed in an attempt to increase haemagglutinin production by repeated passage in human amnion cells and by selection for haemagglutinating virus were unsuccessful. However, although haemagglutinin production to low titre was a characteristic of virus growth in human amnion cells, the infectivity titres of harvests of Cohen grown in these cells are at the same levels as those of harvests recovered from KB cells. Since it has furthermore been demonstrated that a non-infectious haemagglutinin exists in Adenovirus type 7 fluids (Dixon, 1962; Zuschek, 1961), it has been speculated that harvests from KB cells contain a much greater amount of non-infectious haemagglutinin than do those from human amnion cells. To confirm this, it would be necessary to separate the infectious and non-infectious haemagglutinins (if such exist) by some procedure such as density gradient

ultracentrifugation and measure the relative levels of each in both KB-grown and human amnion-grown materials.

The existence of a masking agent which affects the settling patterns in HA titrations of crude human amnion-grown Semen material has been demonstrated. A similar effect also occurs in the first few dilutions of titrations of KB-grown material. The effect can be duplicated by the incorporation of calf-serum, crystalline bovine serum albumin fraction V, and calf serum thrice absorbed with erythrocytes. Thus, the effect is most probably produced non-specifically by protein. The possibility does exist, however, that bovine albumin, which was present in all materials tested, is responsible for the masking effect. To eliminate this possibility, it would be necessary to determine what effect some other crystalline protein, such as a globulin, has on the settling patterns.

A protective effect of serum against thermal inactivation of the Semen haemagglutinin has been demonstrated. Stabilization was not manifested by a corresponding concentration of crystalline bovine serum albumin in PBS. It seems, therefore, that stabilization may be due to some specific component of calf serum, other than the albumin fraction. It is interesting to note that in a second experiment, 2% serum albumin in water did manifest.

similar stabilization. This observation is not significant by itself and must be repeated to be conclusive. However, it appears that either an increased concentration of serum albumin or a decreased salt concentration may reduce thermal inactivation of the haemagglutinin. If so, the correct alternative could be determined by testing 2% serum albumin in water and in PBS simultaneously as well as 0.15% serum albumin and water for stabilization in a similar experiment.

In regard to this stabilization, it is of interest to note that the infective particle of the Enteroviruses is stabilized against thermal inactivation in the presence of the cations, magnesium, calcium, and sodium at concentrations above 0.25 M. (Wallis & Melnick, 1962). The Myxoviruses, Adenoviruses, Sindbis virus, and SV_{40} (Vacuolating virus) are not stabilized but rather are inactivated at a greater rate in the presence of these cations.

It was found that crystalline chymotrypsin at 0.05% greatly reduced the activity of the Bosen haemagglutinin. However, since 0.01% chymotrypsin was also found to inactivate erythrocyte receptors, the possibility exists that neutralization by crude soybean trypsin inhibitor was incomplete and the residual active chymotrypsin destroyed the erythrocyte receptors during the

assay procedure. To determine conclusively whether chymotrypsin inactivates the haemagglutinin, it would be necessary to repeat the experiment and assay residual chymotrypsin activity after addition of the enzyme inhibitor. Similarly, the experiment in which chymotrypsin was found to inactivate erythrocyte receptors should be repeated again. The erythrocytes should be resedimented after washing and dilution and the supernate assayed for active chymotrypsin which might inactivate haemagglutinin in the titration. However, since the erythrocytes were first washed in at least twenty volumes of PBS and were then diluted ten times more, it seems highly unlikely that the inactivation observed was due to residual chymotrypsin.

In the experiment performed to determine the effect of the length of the adsorption period and of the elution period on the degree of elution, the packed erythrocytes were allowed to equilibrate to 3°C. for five minutes before cold PBS was added. The conclusion was drawn that complete elution is obtained within five minutes after being resuspended. However, it was demonstrated, in the experiments on the effect of volume on elution, that complete elution is obtained even at 10% of the original volume. It is probable, therefore, that elution occurred, during equilibration, into the small residuum of fluid present. Therefore, the conclusion drawn must be that complete elution occurs within

15

ten minutes of exposure to low temperatures.

In all the elution experiments performed, some variation in the degree of elution was encountered and in some experiments complete elution was not obtained. It is obvious that some unrecognised factor(s) is affecting elution. The nature or possible identity of this factor is at present unknown.

It is possible to draw some conclusions about the nature of the haemagglutinin from the experiments which were conducted to determine its stability to various physico-chemical agents. It is stable over a wide pH range (approximately 4 to 10) but is inactivated at temperatures of 57°C. or greater. Since it is completely inactivated in ten minutes at 56°C., it is not thermostable and would seem to be a different entity from the soluble antigens of the group 3 serotypes all of which are not inactivated completely by exposures to less than 70°C. for ten minutes (Allison et al., 1960). The results of the thermal inactivation experiments correspond to those obtained by Wigand and Bauer (1962).

Chymotrypsin preferentially hydrolizes amino acid linkages of protein chains which involve L-tyrosine or L-phenylalanine. The fact that the haemagglutinin was inactivated by chymotrypsin indicates that it contains proteins essential to its activity which contain tyrosine

or phenylalanine in a configuration susceptible to chymotrypsin. Periodate is an oxidising agent almost specific for polysaccharides and the inactivation of the haemagglutinin reported indicates that it contains a polysaccharide moiety essential to its activity. As Simon (1962) reported that the type 3 haemagglutinin is not inactivated by periodate, this may indicate that the type 3 and 7 haemagglutinins differ in their composition. The results of the experiment with periodate confirm those of Buckland and Tyrrell (1963). Inactivation by crystalline trypsin and urea could not be demonstrated. Simon (1962) was unable to demonstrate inactivation of the type 3 haemagglutinin by trypsin but Wigand and Bauer, (1962) did demonstrate inactivation of the type 11 haemagglutinin. However, crude trypsin did inactivate the type 7 haemagglutinin and this may explain the results reported by Wigand and Bauer. Trypsin acts specifically on proteins or amino acid chains containing arginine or lysine, and urea denatures proteins by splitting hydrogen bonds. However, since only crude preparations were employed, the proteins in the medium may possibly have interfered with the action of both and it is not possible to state that the haemagglutinin is unaffected by these materials.

Adenovirus types 3, 7, and 14 have been reported to adsorb to erythrocytes at 42°C. and elute at 0-4°C. (Simon, 1962). These findings have been confirmed in this laboratory with regard to types 3 and 7. The only other virus which has been reported to adsorb to erythrocytes at elevated temperatures and elute at low temperatures is the Pelitacosis virus (Inaba, Ozori, & Ishii, 1957). Most viruses which elute do so at higher temperatures. The possible mechanism of the odd type of adsorption and elution of Adenovirus type 7 is unknown at present.

The erythrocyte receptors for the Dosen haemagglutinin do not seem to be markedly sensitive to minor changes in hydrogen ion concentration as they are equally agglutinable after incubation at pH levels between 5.3 and 8.0. Since they are destroyed by both crystalline trypsin and chymotrypsin, they must contain an essential protein moiety(s). This protein, however, does not seem to be denatured by urea. The concentration at which periodate was employed (0.000025 M) is so low that the failure to observe inactivation of the receptors does not contradict the findings of Buckland and Tyrrell (1963) and Simon (1962) who used one hundred-fold greater concentrations (and obtained lysis) and observed inactivation.

SUMMARY

HS cells infected with Adenovirus type 7 (Gomen strain) yield harvests of much higher haemagglutinin titre than do similarly infected primary human amnion cells. It was not found possible to increase haemagglutinin production in human amnion cells by either adaptation or selection.

The proteins in calf serum and crystalline bovine serum albumin have been found to non-specifically modify the erythrocyte settling patterns of haemagglutination titrations.

The optimal system for assay of the Gomen haemagglutinin has been determined: to dilutions of virus in 0.4 ml. volumes of saline is added 0.1 ml. of an 0.5% erythrocyte suspension in saline containing 0.5% normal rabbit serum.

Crude preparations of the Gomen haemagglutinin have been found to be thermally inactivated at a slower rate than partially purified preparations. Such stabilization can be duplicated by the incorporation of calf serum but not of crystalline albumin in purified preparations.

The Adenovirus type 7 haemagglutinin is inactivated at pH 3.6 to 4.2 and pH 10.5 to 11.1. The Adenovirus type 3 haemagglutinin is inactivated at pH 3.2 to 3.6

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