

**Herpes simplex virus:**  
**the isolation and typing of strains**

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# ABSTRACT OF THESIS

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1. The literature concerning the pathogenesis of herpes simplex infections is reviewed, and the main features of infection of the mouth, skin, eye, genital tract and central nervous system are described in detail.
2. A review of the composition and growth of the virus is followed by a detailed discussion of the methods available for the allocation of strains to type 1 and type 2, including antigenic analysis by neutralisation, complement fixation and other methods.
3. The following biological markers of the two virus types are studied; (i) the type of cytopathic effect produced in cell cultures; (ii) the appearance of the pocks developed on the chick embryo chorioallantoic membrane; (iii) the comparative thermal stability of the two types; (iv) the production of filaments or microtubular structures within infected cells, especially in the nucleus, by type 2 strains; and (v) pathogenicity for experimental animals. The established differences are summarised.
4. The recovery of viruses from the main clinical sites of infection in the present study is described, with special reference to the detection of infection in young adults.
5. Virus was isolated from children and young adult patients with a diagnosis of stomatitis; serological evidence was obtained that a considerable number of these patients were experiencing a primary infection. The younger patients were seen in a general practice while the older ones had been referred to or attended the Periodontal Department of a Dental Hospital.
6. Patients infected on the skin of the face, trunk and limbs were examined and virus isolated; the age distribution of the patients was found to be similar to that of patients with oral infections.
7. The reported finding that herpetic infection of the cornea occurs in older patients, (over the age of 50) was confirmed.
8. The features of the patients with genital tract infections were found to confirm the reported age distribution; in female patients, involvement of both the external genitalia and the cervix-uteri was diagnosed; in the male, infection was usually of the glans or shaft of the penis. One quarter of the patients tested were experiencing primary infection.
9. To test the validity of the association of virus type and site of infection, virus strains isolated from the sites listed above were submitted to antigenic typing and biological characterisation.
10. The microquantal neutralisation method was selected and the conditions of the test investigated; the procedure was adapted to BHK 21 cells, and the incubation period was shortened to three days. With this modified technique, the reproducibility of virus infectivity and serum neutralising antibody titrations was found to be comparable with published results.

11. Rabbit antisera prepared against reference strains HF (type 1) and MS (type 2) were examined for their ability to type reference strains. It was found that a high titre, type specific anti-MS serum had been produced. As this had not been reported previously, an investigation of the cross-reactivity of antisera produced by immunising rabbits with different doses of virus was undertaken. "High dose" sera produced in response to virus inocula containing more than  $10^{3.0}$  TCID<sub>50</sub> per 0.02 ml cross-reacted with both virus types, but "low-dose" sera resulting from the inoculation of virus of titre less than  $10^{2.0}$  TCID<sub>50</sub> per 0.02 ml were more specific for the homologous virus.
12. Alternative serological methods of comparing strains were investigated. A quantal neutralisation test in conventional tissue culture tubes was not sensitive enough, and although chessboard complement fixation tests with the rabbit antisera described above did give some indication of type, it was not thought to offer any advantage over the microquantal test.
13. To allow an economical plaque or focal assay of virus infectivity, a titration system using monolayer cultures of cells in standard culture tubes was developed. Viral foci were counted by low power microscopy after incubation for 34-44 hr in ordinary growth medium. The reference rabbit antisera were tested against the homologous and heterologous viruses by a short incubation procedure. It was concluded that the method was not suitable for routine application.
14. Investigation of IgM and IgG fractions of the same antisera by the microquantal, complement fixation and kinetic methods described failed to show any advantage compared with the use of whole sera.
15. Before the microquantal technique was applied to a series of fresh isolates, the duration and temperature of incubation of the neutralisation reaction were examined, and the defined conditions were confirmed as most appropriate.
16. Two-hundred and eight strains of herpes simplex virus were tested against two sets of reference sera and divided into two types, confirming reported findings that HF-like or type 1 strains were associated with oral, eye and skin infections, whereas MS-like or type 2 strains were isolated from infections of the genital tract and adjacent skin sites. No antigenic variation of type 1 strains with age or site of recovery was found.
17. Eleven of the 66 (16.7 per cent.) genital isolates were classified as type 1; this included 2 of 36 isolations from male patients and 9 of 30 from female patients, including two viruses recovered from the cervix uteri.
18. To confirm that the antigenic type of these strains agreed with other characters, pock production, type of cytopathic effect, and filament production were investigated.
19. The pock diameters of 99 freshly isolated strains of herpes simplex virus were measured: the association between type 1 strains and small pocks, and type 2 and large pocks was confirmed.
20. Seventy-eight strains were passed in fibroblast cell cultures and the type of cytopathic effect assessed: type 1 strains produced cell rounding, but in addition to this, type 2 viruses gave rise to small fusiform syncytia.

21. Sixty-four strains were tested for their ability to produce filaments or microtubules in infected cells. With one exception, all type 2 strains were found to do this, and type 1 strains did not.
22. With additional evidence from a comparison of 14 strains of virus for their thermal stability at 4°C, it was concluded that the antigenic typing results were confirmed by the biological characters of the viruses.
23. The features of infection at each of the main sites are discussed, with special attention to the genital tract. Possible explanations for the isolation of a significant number of antigenic type 1 strains are discussed, and it is concluded that this is related to the susceptibility of the young adult population to infection with either type of virus.
24. The relevance of this finding to sero-epidemiological studies to link cervical type 2 infection with carcinoma of the cervix is discussed and it is suggested that some of the studies that have failed to show an association between antibody to type 2 virus and the malignancy might have been performed on populations comparable to that in Edinburgh.
25. Finally the stability of the two virus types is reviewed, and it is suggested that among a population in which both virus types can be isolated from the genital tract, recombination between viruses could occur, and strains with intermediate properties could arise.

PREFACE

An extensive review of the literature on infection with herpes simplex virus confirms that the virus can infect the skin, mucous membranes of the mouth and genital tract, the eye and the central nervous system. Infections can be divided into primary and recurrent types on the basis of the serological response. Primary infection occurs in children, but results of sero-epidemiological studies in Edinburgh indicate that adults can also suffer from this form of the disease.

Infection is usually acquired by close personal contact, the most common sites being the mouth and genital tract. Virus isolation and serological studies over a period of years have confirmed that a significant proportion of infections in adults are primary in nature.

An association between virus type and clinical site of infection has been postulated by several investigators, in particular that antigenic type 2 strains are recovered from the genital tract. To confirm these observations, antigenic typing of virus isolates was undertaken with a quantal neutralisation test performed in microtitre plates. Development of this microquantal method established optimal test conditions and led to a study of the effect of varying the dose of virus inoculated into rabbits to produce the reference type 1 and 2 antisera.

Rabbit antisera were examined by a kinetic neutralisation test, both as whole sera and after fractionation by ultracentrifugation in sucrose gradients. To allow a more economical application of this method to a large number of isolates, a microfocal assay in tube

cultures of cells was developed. It was concluded that for the purposes of antigenic typing this method was too sensitive and the microquantal neutralisation test was used to investigate 208 strains of virus isolated from several sites of infection.

These studies confirmed the association of type 1 strains of virus with oral, skin, and corneal sites, and type 2 strains with the genital tract and adjacent areas of skin; however, a significant number of genital isolates were found to be type 1, especially from genital infections in females. To confirm that these strains were type 1, the genital isolates and a number of other strains were examined by biological tests reported to be associated with virus type. Passage of the viruses in the chorioallantoic membrane of the developing chick embryo confirmed the agreement reported between type and pock size. Further characterisation of isolates by the appearance of the cytopathic effect in cell cultures and by the electron-microscopical examination of infected cells confirmed the reliability of these methods and that the antigenic analysis was in good agreement with the biological marker studies.

The results of the virus isolation and typing studies in relation to the main sites of infection are discussed and an explanation for the high isolation rate of type 1 strains from the female genital tract is postulated. The evidence linking genital herpetic infection and carcinoma of the cervix is discussed, and the related implications of a high rate of genital type 1 infection are considered. Finally, the stability and possible origins of strains of herpes simplex virus are discussed.

Acknowledgements

It is a pleasure to acknowledge the assistance and cooperation of all those who have made this study possible. In the Department of Bacteriology, I am indebted to Professor B. P. Marmion and Dr. R. H. A. Swain for encouragement, and to Dr. J. G. Collee and Dr. J. M. K. Mackay for advice. Over a period of several years, Dr. Isabel Smith has been a patient and loyal colleague. Skilled technical support was provided by Mrs Ann McGowan, and the electron microscopy and ultrathin sectioning were the work of Mr. E. Gowans.

Many clinical colleagues have provided specimens and information, especially Dr. J. F. Cullen of the Ophthalmology Department, Royal Infirmary of Edinburgh; Professor G. Cowley and Professor J.H.P. Main when at the Edinburgh Dental Hospital and Mr. Kenneth Muir of the Edinburgh Dental Hospital; and Professor J. D. E. Knox while in general practice in Edinburgh. Dr. D. H. H. Robertson and Dr. Margaret Taylor-Brown of the Department of Sexually Transmitted Diseases, Royal Infirmary of Edinburgh, have been most helpful over a number of years.

The illustrations are the work of Mr. J. Paul and the staff of the Medical Illustration Department of the Audio-Visual Services Unit, and Mr. David Brown of the Department of Pathology. Mrs. Elizabeth Welsh patiently and carefully typed the manuscript.

Finally, my thanks are due to my wife and family for their patience during the writing of this thesis.

The results of some of the work incorporated in this thesis have already been reported. The relevant references are:-

1. PEUTHERER, J. F. 1970. The specificity of rabbit antisera to Herpesvirus hominis and its dependence on the dose of virus inoculated.  
J. Med. Microbiol., 3; 267-272
2. SMITH, Isabel W., PEUTHERER, J. F., and ROBERTSON, D. H. H. 1973. Characterization of genital strains of Herpesvirus hominis.  
Br. J. Vener. Dis., 49; 385-390.

Reprints of these papers are bound in Appendix E.

In addition, papers have been read to:-

1. The meeting of the Pathological Society of Great Britain and Northern Ireland, July 1969.
2. The meeting of the Society for General Microbiology, Virology Section, September 1972 (in association with Dr. Isabel W. Smith).



Some of the work included in this thesis is the result of cooperative studies with Dr. Isabel W. Smith of this department.

These are:-

- 1) the sections dealing with the isolation of virus and serological studies on patients infected at various sites;
- 2) the sections concerning the classification of virus isolates by examination of cytopathic effects and the electron-microscopical examination of infected cell cultures.

The work recorded in the sections concerning the antigenic typing and characterisation by pock production of isolates was performed by the author.

The thesis was composed solely by myself.

CONTENTS

	<u>Page</u>
Preface	i
Acknowledgements	iii
Summary	x
<u>Introduction</u>	
Infection with herpes simplex virus	1
(i) Pathogenesis of recurrent infection	3
(ii) Epidemiology	7
Infection at different clinical sites	
(i) Infections of the mouth	11
(ii) Infections of the skin	17
(iii) Infections of the eye	21
(iv) Infections of the genital tract	25
(v) Infections of the nervous system	36
The morphology and composition of herpes simplex virus	41
The growth cycle of herpes simplex virus	44
Antigenic analysis of herpes simplex virus	
(i) Neutralisation tests	59
(ii) Complement fixation tests	63
Other methods of typing strains	65
(i) Cytopathic effects in cell cultures	66
(ii) Growth on the chorioallantoic membrane of the developing chick embryo	71
(iii) Thermal stability	75
(iv) Production of filaments within infected cells	76
(v) Pathogenicity in laboratory animals	76

	<u>Page</u>
Summary of differences between type 1 and type 2 strains of herpes simplex virus	79
<u>Materials and Methods</u>	82
<u>Experimental results</u>	
Virus isolation studies	101
(i) Oral infections	105
(ii) Skin infections	109
(iii) Eye infections	114
(iv) Genital tract infections	119
(v) Infection of the central nervous system	124
Method of antigenic typing of herpes simplex virus	
(i) Reproducibility of infectivity titrations	125
(ii) Variation of pN value with virus challenge dose	128
(iii) Reproducibility of pN values	130
(iv) Effect of varying the challenge dose of virus used in neutralisation tests on the resulting pN values with homologous and heterologous antisera	130
Reference antisera	
(i) Reference viruses	133
(ii) Production of antisera	134
(iii) Effect of varying the dose of virus inoculated to rabbits to produce reference sera	137
(iv) High-dose (H) sera	139
(v) Low-dose (L) sera	139
Investigation of alternative assay systems for typing strains of herpes simplex virus	
(i) The use of a quantal tube assay	144
(ii) The use of complement fixation tests	146

	<u>Page</u>
(iii) Application of neutralisation rate studies	
a) Tube focal assay	148
b) Investigation of the optimal assay conditions	149
c) Neutralisation-kinetic studies	153
(iv) The use of IgM fractions of rabbit antisera in the typing of strains of herpes simplex virus	
a) Serum fractionation	155
b) Assay of IgM fractions by neutralisation-kinetic studies	158
c) Assay of serum fractions by complement fixation	158
Effect of varying the time and temperature of incubation of the neutralisation reaction on the resulting pN values	159
Antigenic typing of laboratory strains of herpes simplex virus	163
Antigenic typing of strains of herpes simplex virus	168
(i) Virus strains typed	
a) Age distribution and sites of infection of patients	168
b) Sex distribution of patients	170
(ii) Reference antisera	170
(iii) Reproducibility and consistency of the method in practice	170
(iv) Results of antigenic typing of unknown strains of herpes simplex virus	
a) Results with first pair of reference antisera	180
b) Results with second pair of reference antisera	187
c) Summary of antigenic typing results	191
d) Serological evidence of nature of infection	194

	<u>Page</u>
Biological classification of strains of herpes simplex virus	
(i) The diameter of pock produced on the chorio-allantoic membrane	196
a) Pock diameters of 99 strains of herpes simplex virus	197
(ii) The cytopathic effect produced in cell cultures	203
a) Plaque morphology	206
(iii) The electron-microscopical examination of infected cells	207
(iv) Thermal stability of type 1 and type 2 strains of herpes simplex virus at 4°C and 37°C	208
Virus isolates showing abnormal features	212
Summary of results of characterisation of herpes simplex virus isolates	215
<u>Discussion</u>	
(i) Isolation of herpes simplex virus from different sites	217
(ii) Characterisation of strains of herpes simplex virus	231
(iii) Association of virus type with site of infection	249
<u>References</u>	
267	
<u>Appendices</u>	
A. Dilution and titre conventions	302
B. Derivation of pN formula	304
C. Lists of virus strains and sources	306
D. Solutions	315
E. Reprints of papers	

SUMMARY

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- (25) Finally the stability of the 2 virus types is reviewed, and it is suggested that among a population in which both virus types can be isolated from the genital tract, recombination between viruses could occur, and strains with intermediate properties could arise.

### ABBREVIATIONS

The following abbreviations are used in the text.

hr = hour; min. = minute; l = litre; ml = millilitre;  
cm = centimetre; mm = millimetre; nm = nanometre; g = gramme;  
G = relative centrifugal force; r.p.m. = revolutions per minute;  
TCID<sub>50</sub> = tissue culture infective dose, 50 per cent. dose;  
f.f.u. = focus forming unit; pN = neutralising potency;  
NpN = normalised neutralising potency; K = neutralisation rate  
constant; NK = normalised neutralisation rate constant;  
CF = complement fixing; SD = standard deviation of the mean;  
DNA = deoxyribonucleic acid; RNA = ribonucleic acid; CPE = cytopathic  
effect; CEF = chick embryo fibroblast; BHK21 = baby hamster kidney  
cells, clone 21; RK = rabbit kidney; CAM = chorioallantoic membrane;  
CSF = cerebrospinal fluid; CNS = central nervous system;  
IgM = immunoglobulin M; IgG = immunoglobulin G; IgA = immunoglobulin  
A.

## INTRODUCTION

Infection with herpes simplex virus

Laboratory studies of herpes simplex over many years since the early work of Grüter (1920) have made available considerable information concerning the replication cycle of this virus and its interaction with man, the natural host. Early studies concentrated on the most obvious clinical forms of infection of the skin, lip and eye, and with the rabbit as the culture medium, it was established that material from the vesicles of herpes febrilis, herpes labialis, herpes genitalis and corneal infections all produced keratitis with the characteristic intranuclear inclusion bodies in the rabbit eye (Lipschütz, 1921). Virus isolated from the central nervous system and mouth behaved similarly (Flexner and Amoss, 1925a and b).

That the vesicular infections of skin were of common aetiology had been implied by the histological descriptions of Unna (1896) of a thick-walled vesicle superficial to a papillary process, and containing a fibrinous network and prickle cell debris. Giant cells are present in the edges of the lesion and cell nuclei may show inclusions (Lipschütz, 1921). Confusion existed in early reports between herpes simplex and zoster, but the sensitivity of the rabbit cornea to herpes simplex virus enabled these two conditions to be separated. One other distinguishing feature was the tendency of herpetic lesions to recur at a particular site.

Antibody specific to herpes simplex virus was estimated by neutralisation tests described by Zinsser and Tang (1926) and Andrewes and Carmichael (1930), and the complement fixation technique was applied to the virus by Brain (1932). The specificity of these tests

was demonstrated by the detection of antibodies in the sera of patients with the disease, although no fluctuation in antibody content was found with recurrences of infection of the eye and skin. Indeed later studies (Burnet and Lush, 1939a) proposed that there was an all-or-nothing response in human sera; antibody was either absent, or was present in high titre. This view has some relevance to the pathogenesis of the disease, although the investigations of Jawetz and Coleman (1952) finally established that if virus-serum mixtures were incubated at 37°C for 1 hr before inoculation, then a range of neutralising titres could be obtained.

Virus was apparently isolated from saliva by early workers, and the reports of Levaditi, Harvier and Nicolau (1921) and Levaditi (1926) indicated that it was present more frequently in patients who suffered from herpetic eruptions. They could not isolate the virus from the salivary glands - observations still relevant to the discussion of the virus-host association. Clinical infection in the mouth was described and virus isolated by Youmans (1932) although herpetic gingivostomatitis in children was not reported by Dodd, Johnston and Buddingh until 1938. The severity of the clinical picture showed that this was a different form of infection from the common recurrent lip or cutaneous manifestations and this was confirmed by Burnet and Williams (1939) who established that neutralising antibody to the virus was not present in sera during the acute phase of this illness but had developed 3 - 4 weeks later. Stomatitis was therefore established as one form of primary infection with the virus. Burnet and Williams proposed as a unifying hypothesis that virus is not eliminated from the body after primary infection; it persists in a latent form in some site, and can

be reactivated to produce localised lesions, despite the presence of high titres of neutralising antibody in the serum. This view is still accepted, although the nature of the persisting association of virus and man is still debated.

#### Pathogenesis of recurrent infection

Three mechanisms to explain the phenomenon of recurrent infection can be envisaged and there is evidence to suggest that only one of these will operate at any one site.

Latent infection of sensory nerves and the trigeminal ganglion could be established during a primary intra-oral infection, and reactivation might lead to a vesicular eruption in the area supplied by the affected nerve. This hypothesis is supported by the recent reports of the isolation of herpes simplex virus from cultures of human trigeminal ganglion cells (Bastian et al., 1972; Baringer and Swoveland, 1973). The earlier observations of Carton (1953) also favour this explanation; he showed that section of the posterior root of the trigeminal nerve for intractable pain can lead to herpes simplex in the second and third divisions of the fifth nerve. These supply the skin of the cheek and mucous membrane of the lip, and reactivation of infection may be seen in as many as 93 per cent. of patients. Section of the sensory nerve prevented the appearance of the eruption. A similar mechanism would appear to be necessary to explain recurrent infection of skin areas remote from the mouth, eye or genital tract - sites in which another mechanism to maintain the virus may operate. Virus has been isolated from the saliva, tears and genital tract secretions of apparently symptom-free individuals. On this basis, it

has been argued that a chronic infection of the salivary glands, lacrimal gland or cervix is established during primary infection and that the intermittent excretion of virus leads to localised re-infections of the mucocutaneous junction, cornea or external genitalia (Kaufman, Brown and Ellison, 1968). This explanation appears acceptable in the eye, but there are some difficulties in accounting for the fairly reproducible localisation of recurrences on the lips or face.

The third possibility is that exogenous re-infection occurs, a proposal that is difficult to substantiate or to refute entirely. It seems an unlikely explanation of regular local recurrences, but could be related to the early recurrences seen in children after primary infections (Knox, 1967) and the changing antibody titres noted in such cases (Buddingh et al., 1953; Dascomb, Adair and Rogers, 1955; Yoshino et al., 1962) before high neutralising titres are established. Reinfection is almost certainly important in the genital tract, although infection is often with the antigenically different but related type 2 strain of virus, despite previous type 1 infection (Nahmias and Dowdle, 1968). Autoinoculation or implantation of virus through a skin abrasion has been recorded (Juel-Jensen and MacCallum, 1972) and close personal contact has been shown to result in outbreaks of skin infections in college wrestlers (Selling and Kibrick, 1964).

The role of antibody in herpes simplex virus infections has not been clearly defined; many studies suggest that it cannot prevent recurrent infection, or exogenous reinfection, but that it may explain the milder symptoms and more localised form of the disease in such cases. Similarly the role of secretory (IgA) antibody in tears and saliva is not understood; it has been claimed to be important in the



eye (Centifanto and Kaufman, 1970) although Douglas and Couch (1970) failed to relate the presence of virus in saliva to decreased IgA levels.

More emphasis has been placed recently on the role of cell-mediated immunity in herpes virus infection. Wilton, Ivanyi and Lehner (1972) studied (i) patients with primary herpetic stomatitis or recurrent herpes labialis, (ii) healthy individuals with a history of herpes labialis, and (iii) a group of normal subjects. Peripheral blood lymphocytes from the patients were tested by the lymphocyte transformation, cytotoxicity, and macrophage inhibition tests; the results showed that lymphocytes from patients with primary and recurrent infections are sensitised to type 1 herpes simplex virus and can transform with this antigen. A similar conclusion has been reached by Ennis (1973a) from studies with rabbit lymphocytes; he also showed that sensitised lymphocytes can differentiate between type 1 and type 2 antigens. Wilton et al. (1972) found that, compared with the control group, patients with herpetic infections showed a dissociation between lymphocyte transforming ability and complement fixing titres. Further comparison between the two groups revealed that lymphocytes from the herpetic patients had a reduced macrophage migration inhibition response when stimulated with virus antigen, and they also failed to give a positive cytotoxicity response. These differences were not found with another antigen and the authors suggest that patients show a deficiency in the production of lymphokines, although no difference was found between the primary and recurrent groups by any of the tests.

Experimental studies with mice have shown that spleen cells from immune animals will reduce the size of herpes simplex virus plaques in

cell culture monolayers (Ennis, 1973a) and that this effect can be enhanced by the addition of antibody. Similar results were obtained by Lodmell et al. (1973).

Studies in vivo (Ennis, 1973b) with mice challenged intracerebrally with herpes simplex virus showed that the transfer of syngeneic, sensitised spleen cells conferred a significant degree of protection to the recipient animals. This effect did not appear to be related to antibody or interferon production, although low levels of the latter were detected in some animals after transfer of sensitised spleen cells. The release of lymphotoxin by the sensitised cells was tested for, but no destruction of target cells was found. The importance of immunological mechanisms had been emphasised earlier by the experimental work of Good and Campbell (1948) in rabbits that had recovered from infection after intramuscular inoculation of virus. If the animals were sensitised to egg albumen before virus inoculation, they could be made to develop encephalomyelitis up to 3 months later, when anaphylactic shock was induced with egg albumen. Schmidt and Rasmussen (1960) found that intramuscular adrenaline was effective in inducing encephalitis in latently infected rabbits; in the same system cortisone and sustained fever were ineffective. Reactivation of corneal infection in rabbits has also been demonstrated with adrenaline (Laibson and Kibrick, 1966).

In man, emotional stress has often been effective in producing a recurrence (Juel-Jensen and MacCallum, 1972), although the most efficient method is elevation of body temperature in response to another infection or to artificial fever therapy (Keddie, Rees and Epstein, 1941). By these methods, as many as 80 per cent. of patients

developed herpes simplex at some site. Both these factors of stress and pyrexia could act via the release of adrenaline, an effective inducer, at least in the rabbit. Hormonal changes, and the rise in body temperature that occurs during the second half of the menstrual cycle could account for the precipitation of recurrences just before menstruation. Ultraviolet irradiation is another potent inducer, although whether this and the other factors described act via the same route is unknown. Indeed, as cell-mediated immune responses are of importance, it is possible that the various inducing agents act on these systems rather than directly on the virus-infected cell: an attack on either could upset the status quo and lead to overt infection.

#### Epidemiology

During primary infection, antibody appears in the blood, and after some fluctuation persists for life, presumably reflecting the persistence of the virus in host tissues. Sero-epidemiological studies therefore give an estimate of the number of individuals who have been infected at any age. Burnet and Williams (1939) in a small survey, established the importance of socio-economic conditions in determining the prevalence of infection: 51 of 55 (98 per cent.) hospital patients had antibody to the virus, whereas only 10 of 27 (37 per cent.) university graduates were positive. This effect of social background had been noted earlier by Andrewes and Carmichael (1930) and has been confirmed on many occasions since, in the U.S.A. (Kibrick and Gooding, 1965), and England (MacCallum, 1959). The survey of Buddingh et al. (1953) among a predominantly negro population in New Orleans has been widely cited to illustrate the epidemiology of herpes virus infections.

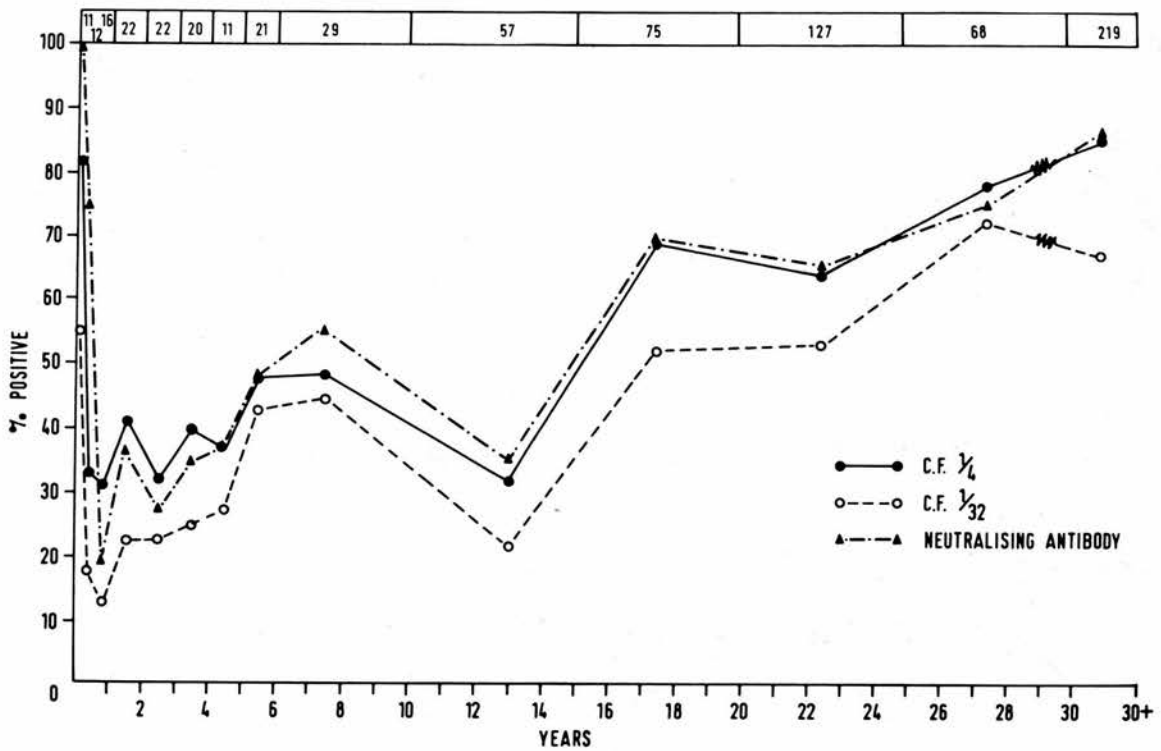
These workers found antibody in almost 80 per cent. of the newborn; this decreased to about 40 per cent. between 1 and 2 years, but in the 3 - 14 year group 85 per cent. were positive, and this level was maintained thereafter. The rise in the number of patients with antibody corresponds to the age distribution of primary herpetic infection, which results in serological conversion.

Also in 1953, Holzel et al. reported an antibody survey from Manchester, and showed the same general trend as the New Orleans study. Sixty per cent. of sera from children aged 4 to 15 years were positive and this rose to 80 per cent. over the age of 16 years. From similar serological studies Yoshino et al. (1962) suggested that they had found a change in the age distribution of antibody in Japan compared with the results of a previous survey. For this reason, Smith, Peutherer and MacCallum (1967) studied a population in South-East Scotland for the presence of antibody by both complement-fixing and neutralising antibody techniques, and compared the results with several other published studies, including the Manchester survey of Holzel et al. (1953). When the Edinburgh results were analysed according to the age groups used by Buddingh et al. (1953) the most important difference was seen in the 3 - 14 year group, of whom only 40 per cent. had antibody to the virus, whereas 85 per cent. were positive in New Orleans. Comparison with the Manchester survey showed the same trend in children between 4 and 16 years: 35 per cent. from Edinburgh had antibody compared with 60 per cent. in Manchester, although the percentage positive was similar in older age groups.

Taken together, these surveys suggest that the age at which serological conversion occurs, or the age distribution of primary

herpetic infection, varies in different populations, and has changed in recent years. The Edinburgh results of 1965 are comparable to those of Yoshino et al.(1962), and changes noted between the British surveys of 1953 and 1965 suggest that improvements in housing and social conditions in general have delayed primary infection, and that this should be reflected in the detection of primary infections in older children and adolescents. The numbers of sera tested in the Edinburgh survey allowed an analysis of the results in smaller age groups; this showed the overall trend as described, but also that there were relatively fewer children with neutralising antibody in the 10 - 14-year-olds (35 per cent.) than in the 6 - 9-year group (55 per cent.). This decrease was followed by an increase in the number of positives so that in the 15 - 19 and 20 - 24-year-olds, 69 and 65 per cent. respectively were positive (Fig. 1). An obvious criticism would be that the numbers studied were too small; in the 10 - 14, 15 - 19 and 20 - 24 year age groups discussed 57, 75 and 127 sera were examined, numbers which compare favourably with those of other published surveys.

The difference in antibody prevalence between 10 - 14 and 15 - 19 years implied that adolescents and young adults were undergoing primary infections. The mode of transfer could be by kissing and other close personal contact, as exemplified by infectious mononucleosis, or by sexual intercourse. The definition of the two antigenic types of herpes simplex virus and the association of type 2 strains with genital tract infection (Dowdle et al., 1967; Schneeweis, 1967) led to serological studies with both type 1 and 2 antigens. The results with type 1 antigen confirmed earlier studies; antibody



**FIGURE 1.** The age distribution of neutralising and complement fixing antibodies in the Edinburgh 1965 survey.

The numbers in boxes across the top of the figure are the numbers of sera tested in each age group.

CF 1/4 and CF 1/32 are dilutions of the standard complement fixing antigen preparation.

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assumed to indicate type 2 infection was associated with adolescents, young adults and older ages suggesting that this was acquired in response to the sexually acquired strains (Rawls et al., 1968b). The antigen used in the survey of Smith et al., (1967) has been shown to be type 1, and the antibody response to type 2 virus has been shown to produce cross-reacting antibody (Nahmias et al., 1969b); thus genital type 2 infection in the susceptible age groups mentioned above in Edinburgh would be detected by the techniques used in the survey.

The 65 per cent. of 10 - 14-year-olds without antibody in Edinburgh should be susceptible to either type 1 or 2 infection and it was considered worthwhile to search for evidence of primary herpetic infection in older children and young adults, either with type 1 or type 2 virus, and to examine the claim that genital infections are almost exclusively due to type 2 strains, a conclusion based on studies of predominantly negro patients in the U.S.A. with a known high background of type 1 infection.

## INFECTION AT DIFFERENT CLINICAL SITES

The features of infection at each of the main sites are described in the following sections. In most instances only a brief account is given of the history of the development of knowledge. However, the review of the older literature is more extensive in the sections dealing with oral and genital tract infections as many of the findings reported in these publications are relevant to the present study.

### Infections of the mouth

Acute herpetic gingivostomatitis is characterised by the sudden onset of fever, malaise and loss of appetite; intraoral lesions appear within 1-2 days. Initially these are vesicular, but they rapidly break down to form 6-8 ulcers 1-2 mm in diameter. The commonest sites involved are the tongue, lips (inner surface) and the buccal mucous membrane; the gums are often inflamed and bleed easily. Salivation is increased and the child may complain of pain or may refuse to eat. Submandibular and submental lymphadenopathy is frequently present (Dodd et al., 1938; Knox, 1967). The aetiology of this condition was uncertain for many years, after the isolation of herpes simplex virus had established the viral aetiology of herpetic keratitis, herpes febrilis and herpes simplex: Dodd et al. (1938) investigated twelve patients referred to a hospital out-patient department during the period September, 1936 to April, 1937, and isolated virus on the rabbit cornea. No serological testing was undertaken, but on clinical grounds the authors considered that stomatitis was a form of primary infection.



Proof of this conclusion was provided by Burnet and Williams (1939) who confirmed the isolation of virus from 6 cases and also showed that neutralising antibodies were present in convalescent sera whereas acute phase sera were free of any neutralising ability.

Confirmation that stomatitis could be the presentation of primary herpes virus infection was provided by Scott, Steigman and Convey (1941) who studied 22 patients and performed virus isolation and antibody tests. An aetiological role for herpes simplex virus in other forms of intraoral ulceration has not been established (Dodd and Ruchman, 1950; Blank et al., 1950), and while Vincent's organisms can be found in association with herpetic lesions, Reade (1961) asserted that the lesions of acute necrotising gingivitis and herpetic gingivostomatitis could be distinguished clinically.

Herpetic gingivostomatitis is a disease of young children between the ages of 1 and 4 years: Scott et al. (1941) found that 70 per cent. of their patients were under 6 years of age, and 40 per cent. were less than 3 years. Dodd et al. (1938) recorded a similar age distribution, although both studies included children aged 12 and 14 years. In addition, Scott et al. (1941) made the diagnosis in an adult aged 24 years, and speculated that the adult disease of herpetic fever (Youmans, 1932; Long, 1933) might be identical to the stomatitis they were describing in children. More recent studies by Farmer (1956) and Juel-Jensen and MacCallum (1972) have confirmed that primary infection can occur in the adult.

#### The spread of infection

The studies discussed above showed that the virus is present in the lesions and saliva of patients undergoing primary infection, and

also in the vesicle fluid and exudates of recurrent lesions such as the common herpes febrilis. Dodd et al. (1938) and Scott et al. (1941) reported that family outbreaks could occur and that some of these were associated with labial herpes in a parent. The familial spread of infection was also suggested by Burnet and Williams (1939) and has been confirmed on many occasions (Reade, 1961; Knox, 1967) the incubation period being estimated at 3-10 days. In special conditions with large numbers of susceptible children in close contact, the disease may assume epidemic proportions. Outbreaks have been described in an orphanage (Anderson and Hamilton, 1949) when clusters of cases were seen at intervals of a few months. During the year of study, 29 serological conversions were found among a total population of 51 children - all were associated with recognisable clinical episodes, except in 9 instances. In another study of orphanage children, Hale et al. (1963) recorded that within a period of 1 month, 77 per cent. (of 13 children) suffered from a febrile disease with stomatitis, although the severity of symptoms varied, and in 3 cases consisted of only a mild fever of short duration associated with a single oral ulcer. This high rate of clinical detection of primary infection is in contrast to most estimates of the high rate of subclinical infection: this has been estimated to be over 80 per cent. (Scott, 1954) based on the known high proportion of the population with antibody, and the relative rarity of stomatitis in outpatient departments. The orphanage incidents referred to above suggest that closer scrutiny of children might reveal a few lesions in many "subclinical" cases, although from observations made in general practice Knox (1967) suggested that many more cases of herpetic stomatitis may

be seen than realised, as a diagnosis of teething or upper-respiratory tract infection is often applied to these children.

Children recovering from primary infection can excrete the virus in saliva for 15 - 42 days after clinical recovery (Buddingh et al., 1953). These same workers were able to isolate the virus from the saliva of 20 per cent. of asymptomatic negro children and from 2.5 per cent. of asymptomatic adults. On this basis, spread of the virus in families and populations would be easy to envisage, but these high carriage rates have never been repeated, and a more recent estimate based on the examination of 13,500 specimens, two-thirds from children, only showed an isolation rate of 0.75 per cent. (Herrmann, 1967). None of these patients had detectable lip or oral lesions at the time the specimens were collected.

Recently Douglas and Couch (1970) investigated 10 patients known to have antibody to herpes simplex virus by collecting saliva and parotid secretions thrice weekly for 5 months. This study showed that virus can be isolated from saliva in the absence of detectable lesions in the mouth, although half of the isolations were made at the onset or during a recurrent infection of the lips. Over the study period, 8 of the 10 patients were positive for virus, and 7 of these experienced at least 1 recurrence. Parotid secretion samples were all negative, strongly suggesting that the gland is not the site of chronic infection, an observation in agreement with the much earlier report of Levaditi et al. (1921) who decided that the buccal mucosa must be the site of infection. The location of the virus has not been identified.

All virus strains from the oral cavity have been found to belong to antigenic type 1, except for 3 strains reported by Nahmias

and Roizman (1973b) and one strain isolated by Kaufman and Rawls (1972). All four were from adult patients and the practice of fellatio was postulated to be the method of transfer from the genitalia to the mouth.

#### Recurrent oral disease

Although recurrences of herpes facialis are clearly defined clinically, an exact description of the site involved is necessary to distinguish between recurrent herpes labialis or febrilis and intraoral lesions which may be extensions of labial ulcers. Despite this problem, intraoral infection may present in patients with a history of recurrent disease at another site. Six of the patients with oral lesions described by Farmer (1956) gave a history of cold sores, and in a 3-year follow up, 10 of 31 patients were diagnosed as having a vesicular lesion or ulcer in the mouth although virus isolation did not appear to have been attempted. This high recurrence rate has not been demonstrated in any other study and most authors either doubt that it occurs (Scott, 1954; Dudgeon, 1970) or state that it may occur (Juel-Jensen and MacCallum, 1972). The possibility of intermittent salivary excretion of virus in adults (Buddingh et al., 1953) makes interpretation difficult, but Weathers and Griffin (1970) claim to have distinguished recurrent intraoral herpes of the hard palate from recurrent aphthous ulceration and describe 51 patients with similar features to the case of Muller (1968). Although no virus isolation or antibody tests have been performed, it is possible that the vesicular lesions which follow a variety of dental procedures could be recurrent in type. Griffin (1965) and Southam, Colley and Clarke (1968) have reported two such cases; the diagnosis was made by examination of smears from the lesions.

Although the clinical entity of herpetic stomatitis has been

known for many years, no explanation for the relative absence of intraoral recurrences has been offered, and although infection of the trigeminal ganglion could explain the localisation of recurrent herpes labialis this could equally involve the mouth, since it is postulated that the virus may ascend sensory fibres during primary infection, and there is no known reason to prevent its descent. This process could occur to an unknown site to explain the reported isolation of the virus from asymptomatic patients. On the other hand, if virus is shed into the mouth from some site, there must be some barrier to prevent reimplantation through breaks in the buccal mucous membrane.

#### Association with infection at other sites

From the earliest studies the spread of virus from the oral cavity to other sites has been recorded: in some the virus apparently was transferred in saliva to the lips, face and fingers (Dodd et al., 1938; Burnet and Williams, 1939). Subsequent studies have confirmed these observations (Farmer, 1956) and extended the areas involved to the genital tract (Nahmias et al., 1968a). The fingers are favoured sites of infection with virus from the mouth, especially in nail-biters (Davies and Longson, 1970). Disseminated infection is most frequent in the neonatal period, but may follow primary infection at any age, especially in those suffering from severe malnutrition. The route of spread in such cases is probably via the blood from which virus has been recovered during a primary infection (Ruchman and Dodd, 1950). The severe effects associated with primary disseminated infection in malnourished children have been documented by Hansen (1961) and Kipps et al. (1967); the disease resembles the neonatal form with evidence

of infection in all organs. Flewett, Parker and Philip (1969) and Juel-Jensen and MacCallum (1972) have described young adults with generalised disease, in whom oral lesions were present, although hepatitis and meningeal signs developed. One of the patients reported by Juel-Jensen and MacCallum had a negative Paul-Bunnell test, but small numbers of atypical mononuclear cells were detected in peripheral blood. Two of the adult patients reported by Davies and Longson (1970) also showed these cells in their blood.

Immunologically deficient patients may show evidence of widespread infection: although this is usually a reactivation of a latent infection, the mouth is involved, and virus may be present in the oesophagus, ileum and lungs. This may not be due to dissemination, but result from reactivation of widespread latent infection (Montgomerie et al., 1969).

Herpes simplex virus has been implicated as a cause of pharyngitis and tonsillitis (Evans and Dick, 1964), because of its isolation from young adults with pharyngitis. Such reports are difficult to interpret due to the reported isolation of virus from the mouths of asymptomatic carriers.

#### Infections of the skin

The skin is the commonest site of recurrent herpes simplex, the lesion consisting of groups of thin-walled vesicles on an erythematous base. The skin of the face and the mucocutaneous junction is most frequently involved although any site may be affected. Various names are used to describe the typical eruptions of herpes simplex, depending on the site and the precipitating factors: the terms herpes labialis,

herpes facialis, herpes febrilis, cold sores or fever blisters are popular. The vesicles in these conditions are usually localised and not associated with the distribution of a sensory nerve, although a zosteriform distribution has been recorded (Lynch et al., 1945; Slavin and Ferguson, 1950).

Prodromal sensations of tingling or itching are frequently present before the vesicles appear; systemic upset and local adeno-pathy are minimal in the localised recurrent form, although a febrile illness such as pneumonia (Sylvest, 1952), malaria or artificial fever therapy may be the precipitating factor (Warren, Carpenter and Boak, 1940). Other factors are mechanical trauma, as during administration of an anaesthetic (Humphrey and McClelland, 1944), ultraviolet irradiation, menstruation and emotional disturbance. In any individual a particular site may be favoured, although the exact location of the lesions does vary slightly (Juel-Jensen and MacCallum, 1972). The lips and the skin around the mouth and other areas of the face are often involved during overt primary herpetic stomatitis (Knox, 1967), and a latent infection of the skin or the sensory ganglia could be established. Farmer (1956) followed 36 adult patients with stomatitis over a 3 year period, and found that 21 experienced 3 or 4 recurrences on or around the lips. This frequency is in agreement with observations in children (Buddingh et al., 1953). Knox (1967) made the clinical diagnosis of herpes facialis in 25 of 50 children within 2 years of the primary oral infection. The alternative explanation for recurrent infections around the mouth is reinfection by virus excreted in the saliva from a chronic infection of the salivary glands or some other site.

Auto-infection at a distant site during a primary oral infection has been mentioned earlier, and in this way infection could be established at almost any skin site. A similar route of infection can also occur during recurrences when other body sites may be involved, often as the result of some minor abrasion or trauma to allow entry of the virus. Indeed transfer to another skin site has been performed to try to reduce the frequency of recurrences and has succeeded in establishing recurrent infection at the inoculated sites (Stalder and Zurukzoglu, 1936). Infection with type 2 virus strains has been claimed to occur in the genital tract despite previous type 1 infection, and the two types may be present simultaneously (Terni and Roizman, 1970; Nahmias, Naib and Josey, 1971d).

The epithelial cells of the skin have not been excluded completely as the site of latency, but attempts to recover virus from the skin either directly or following cultivation of cells have been unsuccessful (Rustigian et al., 1966); skin excision and grafting experiments have produced equivocal results (Juel-Jensen and MacCallum, 1972).

Viruses isolated from lip, face and many other skin sites have proved to be antigenic type 1, whereas most strains from the buttocks, thighs and perineal region are type 2. The hand and fingers have been found to be infected with either type of virus in equal proportion; the type 2 strains are believed to be acquired through skin abrasions during sexual contact with an infected partner (Dowdle et al., 1967). Type 1 infections of the fingers may occur during a primary stomatitis; among nurses, doctors and dentists, the virus can be acquired from infected secretions of patients. Stern et al. (1959) described



herpetic whitlows in nurses working in neurological units, the virus coming from the secretions of patients with tracheostomies. Similar lesions have been noted in dentists and other members of the medical profession (Hambrick, Cox and Senior, 1962; Juel-Jensen and MacCallum, 1972), a reflection of the fact that 60 - 70 per cent. of nurses and medical students have no detectable antibody to herpes simplex virus (Smith et al., 1967). Herpetic whitlows are painful and virus may spread from the initial site; systemic disturbance, fever and lymphadenopathy are common as would be expected in a primary infection. The lesions begin to abate after 10 days, and healing is complete by 3 weeks in the absence of secondary bacterial infection (Stern et al., 1959). A history of local trauma such as a needle prick is not always given, and virus may enter through damaged skin and cuticle at the base of the nail, to cause a herpetic paronychia.

In children and adults with primary dermal herpes, the lesions may be few in number and scattered rather than grouped as in the recurrent form, although quite extensive involvement of the skin of a region may be seen as in the herpetic napkin rash. The extreme form of generalised skin involvement is eczema herpeticum a variety of Kaposi's varicelliform eruption. This is a complication of eczema in a child when the affected skin is colonised by virus that can spread to other non-eczematous areas (Dudgeon, 1970). These infected patients are a danger to other eczema sufferers; Brain (1954) reported 5 cases in a dermatology ward following the admission of a child with a recurrence of the disease. In addition to the 5 patients three nurses developed lesions on the forearms. As at other sites, corticosteroids are a severe hazard to the patient, beneficial though they may be to

the treatment of the underlying eczema.

Signs of systemic involvement may develop, and death may ensue (Brain, 1954) as a result of adrenal necrosis, dehydration or bacterial infection. Immunosuppressed patients (Montgomerie et al., 1969), or those undergoing treatment for leukaemia (Muller, Herrmann and Winkelmann, 1972) are liable to suffer from a chronic form of recurrent disease, and this may proceed to the disseminated form involving mouth, pharynx, oesophagus and other organs.

Skin infections have been treated with idoxuridine (5-iodo-2'-deoxyuridine), but results have varied in different trials. A major problem is the insolubility of the compound: penetration may be increased by the use of dimethylsulphoxide (Juel-Jensen and MacCallum, 1972).

#### Infections of the eye

The eye may be involved in either a primary or a recurrent infection (Gallardo, 1943). In the primary infection, a follicular conjunctivitis with regional adenitis may present in association with stomatitis (Jones et al., 1957), although extension to involve the cornea occurs in about two-thirds of patients (Jones, 1959). Jones also found that patients undergoing primary infection fell into two age groups, one comprising children under 10 years of age, and a second including adolescents and young adults aged 15 - 30 years. The first group corresponds to that described for primary infection at other sites especially of the mouth. However, the majority of patients attending outpatient departments suffer from a recurrent form of the disease, in which conjunctivitis is less prominent and various forms of keratitis or deeper involvement are prominent (Gundersen, 1936; Norn, 1970).

The proportion of primary ocular disease is estimated to be 4.5 - 7 per cent. of all cases.

The most frequent form of keratitis is the simple dendritic ulcer, which may form by the coalescence of several superficial punctate erosions, and can involve a large part of the cornea as a geographic or amoeboid ulcer. The latter form frequently follows the use of corticosteroids (Jones, 1959; Lebensohn, 1971). Uncomplicated keratitis usually resolves in 2 - 3 weeks, although if stromal reaction occurs, the condition may persist for many weeks. Varying degrees of stromal involvement have been recorded (Thygeson and Kimura, 1957), ranging from a disciform keratitis to hypopyon keratitis; iridocyclitis may complicate either. Residual scarring or corneal opacity has been found to occur in up to 85 per cent. of cases, and this may increase with successive attacks (Norn, 1970). Indeed herpes simplex virus may constitute a serious risk to sight in the affected eye either by frequent recurrence or following rupture of the cornea, a serious complication of the deep forms of keratitis (Thygeson and Kimura, 1957).

A uniform finding is that patients with dendritic ulceration of the cornea are in the 30 - 50 year age range (Norn, 1970), although the same report suggests that recurrences are more frequent if the first involvement of the eye occurs between 20 and 30 years of age, when 7 or 8 recurrences may ensue as opposed to an overall expectation of 3-4 attacks. Gundersen (1936) found an average of 2.1 recurrences in his patients. The time between episodes may vary widely and Norn (1970) quotes intervals of 1 to 47 years, although Kaufman et al. (1968) state that patients who have experienced more than one attack

have a 43 per cent. chance of a further recurrence within 2 years.

Eye infection in children may occur by extension of herpes facialis during eczema herpeticum (Dudgeon, 1970), and both Gundersen (1936) and Norn (1970) found that in adults an associated herpes labialis or facialis was either present or the patient gave a history of such an episode in 37.4 per cent. (Gundersen, 1936) or 61 per cent. (Norn, 1970) of cases. The finding by Gundersen that 62.1 per cent. of patients had a simultaneous or recent upper respiratory tract infection suggests that these illnesses could have provoked a recurrence of herpes labialis or perhaps virus excretion in saliva or tears.

The location of the virus in chronic recurring herpes has been much discussed. To explain the frequency of recurrence, and the isolation of virus from the tears of patients without signs of infection (Hanna, Jawetz and Coleman, 1957; Kaufman, Brown and Ellison, 1967) Kaufman and colleagues have investigated the conjunctival and lacrimal glands as possible sites of chronic infection. The number of patients examined was small: 3 of 4 conjunctival specimens and 2 of 4 lacrymal glands from known sufferers were found to harbour the virus, whilst 3 of 7 conjunctivae and 4 of 7 glands were positive from individuals with no history of eye infection. This is an interesting observation and could explain virus isolation from the tears of healthy individuals and a reported isolation from tears after enucleation of an eye and recurrences of infection in corneal grafts (Kaufman et al., 1968; Norn, 1970).

A chronic infection of the lacrimal gland would lead to frequent shedding of virus and hence reinfection of the cornea. Auto-inoculation is well established in other parts of the body even in the

presence of antibody and could occur in the avascular cornea. To allow virus entry, minor trauma of the epithelium might be necessary, and this could be provided by the frequently reported upper respiratory tract infection or the finding that up to 10 per cent. of patients report some form of minor injury before an attack. This association with trauma is the only explanation that has been proposed to explain the preponderance of males with this disease.

The failure of idoxuridine to reduce the relapse rate (Kaufman et al., 1968) could also be due to the chronic infection of the lacrimal gland, which is not affected by drugs applied to the cornea.

Debate has also centred around the role of virus multiplication and invasion of deeper tissues, and hypersensitivity reactions, as explanations of stromal keratitis and iridocyclitis. Evidence has been reported to support both concepts and there is no reason why both mechanisms should not operate. Hypersensitivity was proposed by Jones (1959) to explain stromal reaction occurring after epithelial healing had taken place, and Swyers, Lausch and Kaufman (1967) have confirmed that the guinea pig eye can show corneal oedema due to lymphocyte infiltration when immunised animals are challenged with virus free infected cell culture supernates.

The source of the antigen to provoke this response could be virus that has invaded deeper corneal layers. Attempts to isolate from such sites have not been successful, but Dawson et al. (1968) demonstrated herpes virus particles in 4 of 5 eyes enucleated from chronic sufferers. Large numbers of particles were seen among the collagen lamellae of the cornea. In 4 eyes, viral capsids were present in cell nuclei; this indicates that virus multiplication had taken

place, and therefore viral antigens including those incorporated to cell membranes would be produced at these levels. As far as could be ascertained, the cells involved were fibroblasts or keratocytes. Virus can even penetrate to the anterior chamber, as shown by Kaufman, Kanai and Ellison (1971) in patients with herpetic iritis.

Neonatal infection is a severe disease and most organs may be infected, including the eye. The majority are caused by type 2 strains of virus; ocular infections at all other ages are due to type 1 viruses.

Various forms of treatment have been developed and applied to eye infections; the DNA inhibitors idoxuridine and cytosine arabinoside can bring about healing of lesions. The use of corticosteroids is strongly contra-indicated in simple herpetic conjunctivitis and keratitis as the lesions take longer to heal and spread of the virus may be encouraged.

#### Infections of the genital tract

Nahmias and Dowdle (1968) reviewed the history of the clinical awareness of genital herpetic infection, and attribute the first description to Astruc in 1736; Juel-Jensen and MacCallum (1972) and Nahmias and Dowdle describe the development of knowledge up to the first quarter of this century. Lipschütz (1921; 1932) maintained that genital herpetic infection was aetiologically distinct from herpes febrilis; he based this view on a consideration of the clinical presentation, and laboratory studies of virus isolates.

Little attention was paid to herpes genitalis for many years, although Slavin and Gavett (1946 a and b) described primary herpetic

vulvovaginitis in 3 patients; in one case this followed a penile infection in the husband, and as discussed later the virus isolated from this patient appeared to differ from a virus isolated some years earlier from a lip infection. They concluded from antibody studies that the husband's infection was of long duration, and that transmission to the wife was related to a recurrence. Interestingly, Slavin and Gavett comment that the concept of the venereal nature of herpes progeneralis had attracted little attention, a theme which had also been taken up by Charlitt (1940) in describing a patient and his consort in whom there was clear indication of transfer of infection from the male to his sexual partner. Both these reports established again that while herpetic infection of the penis was quite common, unequivocal evidence of infection of the female genitalia had been presented on only a few occasions in this century, despite its description in the 19th century. This was mentioned also by Lazar (1955) in his discussion of three patients with virologically proven primary herpetic vulvovaginitis. One patient was a child of 4 years who presented with urinary retention and ulceration of the vulva 8 days after a recurrent episode of herpetic whitlow in her mother: this case was similar to that of a child described earlier by Krugman (1952).

Lazar (1955) also gave details of two young adult patients who presented with oedema and ulceration of the labia, and a vesicular eruption spreading to the thighs; in one the cervix was involved. Tender enlargement of the inguinal glands was present and there was a febrile response. It was noted that opposing surfaces of the labia were involved, suggesting direct transfer between skin surfaces in

close contact, and that vesicles on moist skin areas broke down rapidly to form acutely tender coalescing ulcers. Both these patients had sexual intercourse 8 and 9 days before the onset of symptoms with the same man who was a known sufferer from recurrent infection of the penis. Lazar investigated these cases fully and established by antibody studies that each female patient was undergoing a primary infection and that the source patient was suffering from a recurrent infection. Virus was isolated on the rabbit cornea, and passed subsequently to the chorioallantoic membrane where typical herpetic pocks were produced after 48 hr. If incubation had been continued the size of the pocks might have been sufficient to show that the viruses from the two older patients were associated with larger lesions than that from the child.

The clinical picture described, and the demonstration of venereal transmission are typical of this disease, although it was seldom recognised. Indeed the idea that the genital tract might be infected in childhood and hence become the site of recurrences in later life was accepted by some workers for many years (Hutfield, Wasley and Gray, 1967; Dickie, 1969). Recurrent episodes involving the genital tract are common, and are not associated with the same degree of systemic upset and lymphadenopathy as noted with primary infections. Lazar (1955) stated that both of his patients suffered from monthly localised recurrences for one year after the primary infections, an observation in agreement with many old observations reviewed by Hutfield (1968). That herpes genitalis was a sexually transmitted disease was indicated by further reports: Dooley, Jones and Pearson (1957) and Diddle, Williamson and Gardiner (1963) described a total of 8 patients with primary and recurrent infections, and noted cervical



involvement in two patients.

Herpetic genital infection in the male usually presents as a vesicular eruption on the shaft of the penis, or an ulcerated area on the glans or coronal sulcus; the ulcerated lesions could be mistaken for other venereal conditions such as syphilis. The frequency of penile herpes among United States army personnel in Japan was studied by Barile et al. (1962) who found that it accounted for one-third of all patients with penile lesions in this population. Despite this frequency the first virologically established case of primary infection in the male was only reported in 1959 by Duxbury and Lawrence, who described a patient with systemic upset and vesicular lesions developing 7 days after intercourse.

In recent years, the availability and development of laboratory techniques has allowed a more rapid and precise diagnosis to be made. Isolation studies now rely on inoculation of cell cultures, and the recognition of the characteristic cytopathic effect. However, as discussed later, older laboratory procedures such as the inoculation of the chorioallantoic membrane are still relevant in that distinction between virus strains can be made by this procedure (Parker and Banatvala, 1967). Antigenic analysis of virus isolates is now possible (Schneweis, 1962a; Pauls and Dowdle, 1967; Rawls et al., 1968a), and these same techniques may be applied to the analysis of the antibody type of patients' sera: apart from these refined techniques, standard complement fixation and neutralising antibody tests allow the separation of infections into primary and recurrent categories.

One other method of detection has come into use in recent years due to the development of routine screening of exfoliated cells from

the cervix uteri (Papanicolau smears). This method depends on the cytological recognition of changes characteristic of herpes simplex virus (Blank et al., 1951) and Varga and Browell (1960) described the presence of intranuclear inclusions and multinucleate giant cells in smears. No virological confirmation was undertaken, and the authors appeared to be looking for cytomegalovirus, but the published micrographs appear to be those of herpes simplex virus infection, although a recent report (Diosi, Babusceac and David, 1973) of the isolation and cellular changes associated with cytomegalovirus infection of the cervix suggests that both viruses can cause similar cellular changes. Cytological findings were confirmed by virus isolation in 1 patient reported by Stern and Longo (1963). A more extensive study of herpetic changes was published by Naib, Nahmias and Josey (1966), and a follow-up study by Nahmias et al. (1967) established that these findings were associated with the presence of the virus provided isolation was attempted within 10 days of the cytological diagnosis. The interpretation of the records of cervical screening clinics has allowed examination of much larger populations of women than could otherwise have been attempted, and hence an estimate of the prevalence of infection can be made (Wolinska and Melamed, 1970).

The application of all these methods has resulted in clarification of knowledge of the clinical manifestations of infection, confirmed the venereal mode of spread, the association with other venereal infections, and emphasised that the virus is widespread. As will be discussed, great interest has resulted from the finding of an association between genital herpetic infection and carcinoma of the cervix.

The description of the two antigenic types and the isolation of

type 2 strains from the genital tract of both male and female patients has been of considerable importance in the development of many of these ideas. The serological and biological features of the two types are discussed in a later section.

#### Clinical presentation

As at all sites, infection may be classified as primary or recurrent on the basis of the serum antibody titre; this classification can be applied to most infections at other sites, but the existence of two types of virus poses a problem with regard to genital infection. Type 1 infection is widespread and the virus is usually acquired early in life, whereas type 2 strains are transmitted by sexual contact, and therefore are found in the sexually active age group. The presence of antibody may modify the response of the patient, so that, in the presence of antibody to type 1 virus, an initial type 2 infection is localised and resembles a typical recurrence at other sites (Kaufman *et al.*, 1973). Once established, type 2 infection can recur as at any other site (Sharlitt, 1940; Lazar, 1955). Some of the clinical features of genital herpetic infection have been mentioned. In the male, crops of vesicles or ulcers may be seen on the glans and shaft of the penis: in a few instances these may be situated in the urethral meatus and symptoms of urethritis may be present. Coutts (1948) described two cases with meatal and endourethral lesions, and Tyler (1957) a case with recurrence of urethritis and herpetiform penile lesions. Nasemann and Nagai (1960) reported 3 cases with involvement of the glans and floor of the meatus. Virological confirmation was available in these patients and examination of urethral smears revealed intranuclear inclusions and giant cells.

The suggestion that the male genitourinary tract could harbour the virus asymptotically was made by Jeansson and Molin (1970, 1971) in studies of the prevalence of herpetic infection in venereal disease clinic patients and healthy controls. They isolated virus from 5.4 per cent. of the male patients with venereal diseases and from none of 133 healthy controls. Of interest is the comment that only 3 of the 130 males investigated had lesions suggesting herpes infection. Nahmias et al. (1969c) found that 0.54 per cent. of male venereal disease patients suffered from herpes infection, and quoted results from Germany (Unna) in the 19th century that were in good agreement with their own findings. A very high isolation rate was reported by Centifanto et al. (1972) who isolated the virus from 15 per cent. of specimens from the genitourinary tracts of asymptomatic patients.

In the infected female, extensive involvement of the genital tract is now recognised to be normal; lesions of the labia, introitus, vagina, clitoris, perineum, perianal region, and skin of buttocks and thigh are readily detected, and the cervix is frequently involved. The studies discussed above recorded that the cervix occasionally showed erosions or ulcerated areas. Vesicular lesions on an erythematous base are preceded by local irritation and pain. The lesions are more widespread in primary infections in contrast to recurrent episodes in which the vesicles are usually grouped together. In moist skin areas, the vesicles quickly rupture and produce ulcers that may coalesce and involve extensive areas of the labia: contact spread to adjacent skin areas is common (Hutfield, 1968; Josey, Nahmias and Naib, 1972). The asymptomatic nature of cervical infection has been emphasised by Nahmias and coworkers and reviewed (Ng, Reagan and Yen, 1970; Josey

et al., 1972). However, on rare occasions an acute necrotic cervicitis may be found; in some this is so severe, and the deposition of fibrin so great, that it is mistaken for an ulcerating tumour (Stein and Siciliana, 1966; Willcox, 1968). Lesions on the cervix may present as several small ulcers, a single large, deep ulcer, or as a diffuse inflammation (Poste, Hawkins and Thomlinson, 1972); the virus has been isolated from the apparently normal cervix (Ng et al., 1970). There is a well-established association between menstruation and recurrences (Hutfield, 1968; Poste et al., 1972) and in many cases the eruption precedes menstruation. Analysis of the data from cytological screening services has also shown that smears taken in the post-ovulatory phase of the menstrual cycle more frequently show evidence of herpetic infection than from other stages (Josey, Nahmias and Naib, 1968). Pregnancy is another condition that apparently increases the frequency of viral reactivation and the duration of virus excretion (Nahmias et al., 1971c). Between 1964 and 1971 these authors apparently diagnosed genital infection in 436 patients of whom 183 were pregnant. Earlier Ng et al. (1970) had reported that in a 5-year period, 106 of their 256 patients with herpes virus infection were pregnant. In the study of Nahmias et al. (1971c), primary infections in pregnancy were also described, and these were associated with virus excretion of up to 100 days, compared with a maximum of 40 days in proven recurrent episodes.

These observations suggest that hormonal changes are important in reactivating infection or rendering the cervix more susceptible to inoculation (Poste et al., 1972). However, Wolinska and Melamed (1970) failed to relate the use of oral contraceptives incorporating hormones

of this type to an increased frequency of genital infection.

As a result of the widespread use of cytological screening for the detection of carcinoma of the cervix, evidence is available of the prevalence of infection in this population. Smears suggestive of herpetic infection were found in 0.16 per cent. (Naib et al., 1966), 0.1 per cent. (Kleger et al., 1968), 0.03 per cent. (An, 1969) 0.09 per cent. (Wolinska and Melamed, 1970) and 0.48 per cent. (Nahmias et al., 1969c), of the populations examined. Jeansson and Molin (1970) examined 396 female patients not known to be suffering from herpetic infection and isolated the virus from 0.5 per cent.

The frequency of clinical infection has also been studied, and various estimates published. Kleger et al. (1968) diagnosed herpetic infection in 1.6 per cent. of 494 female patients; Nahmias et al. (1969c) found that virologically confirmed infection accounted for 5.7 per cent. of all their referrals. Jeansson and Molin (1971) reported that 8 per cent. of their patients suffered from herpes virus infections.

The development of methods to classify sera as containing type 1, type 2 or intermediate antibodies has allowed epidemiological studies to be performed, and the results have been used to justify the specificity of the antibody typing methods (Nahmias et al., 1970b; Rawls et al., 1968b). Apart from the difference in specificity of the microneutralisation and kinetic neutralisation tests used a major problem has been the finding of large numbers of patients with intermediate-type antibodies. The age distribution of type 1 and 2 reacting antibodies has been described in different age and social groups: Nahmias et al. (1970b) only detected intermediate and type 2 reacting antibodies in persons over 14 years of age. Rawls, Tompkins and

Melnick (1969) made similar observations, and recorded that 54 per cent. of prostitutes tested had evidence of type 2 infection; Nahmias et al. (1970c) could diagnose previous type 2 infection in only 1 of 35 nuns tested. Josey et al., (1972) concluded that the prevalence of type 2 antibodies is greatest in people from a poor socio-economic background, a finding already established by Rawls et al. (1971).

The early reports of transfer of infection to sexual contacts has also been further documented. Rawls et al. (1971) detected infection in 14 of 18 sex contacts of males with genital herpes, and Nahmias et al. (1969c) cite evidence of transmission from male to female and vice versa. Kaufman et al. (1973) have confirmed these observations.

#### Association with other sexually transmitted diseases

An association between herpetic infection and other infections of the genital tract has been recorded by many authors. Josey et al. (1972) in reviewing this subject, listed established associations with chancroid, syphilis, genital warts, trichomoniasis and pediculosis pubis. Concurrent gonorrhoea and herpes simplex infection have been discussed in three reports; Beilby et al. (1968) studied 209 patients and found that 7 of 8 patients with gonorrhoea also had herpes virus infection. Two further reports, Jeansson and Molin (1971) and Nahmias et al. (1973) have indicated that the link between the two infections is not as close as this, in that they isolated the virus from equal numbers of patients with and without confirmed gonorrhoea. These papers suggest that gonorrhoea may precipitate recurrences of herpetic infection, but that simultaneous infection is more usual; this is further evidence of the venereal transmission of the virus.

Infection of the foetus and neonate

MacCallum (1959) reviewed the frequency of generalised herpes simplex in the neonatal period and found 23 cases documented in the literature; he concluded that infected attendants were the most likely source of virus. By 1965, Witzleben and Driscoll had found 43 reports. The case that they described occurred in a child delivered by caesarian section 12 hr after rupture of the membranes; the father had a history of herpes progeneralis, and the mother suffered a febrile illness 1 month before delivery. The child developed symptoms on the sixth day after birth. The authors considered that the infant had been infected transplacentally but the infected cervix would seem to be the most likely source of virus, a point made by Wheeler and Huffines (1965) who thought that once the membranes had ruptured it was difficult to prevent foetal infection and that caesarian section should be considered, especially if the maternal infection could be proved to be primary. This recommendation had also been made by MacCallum and Partridge (1968) and by Nahmias et al. (1971c). This last study of 283 pregnant patients is by far the largest published, and analyses the effects of maternal genital infection at different stages on the outcome of the pregnancy. These workers reported that the frequency of genital herpes is at least doubled in pregnancy. They estimated that there was a 10 per cent. risk of foetal involvement, although this could be as high as 40 per cent. when the virus was present during birth, or at any stage if the maternal infection was primary. There was an increased abortion rate if infection occurred in the first 20 weeks of pregnancy, and an increased prematurity rate if infection was detected later. An earlier study from the same group (Nahmias, Alford and Korones, 1970a)



reviewed 148 cases and found that although two-thirds of infants suffered from a disseminated infection with a 95 per cent. mortality, the remaining third had localised infections of the skin, eye, or central nervous system. The mortality rate was 22 per cent. in this group, and the survivors showed varying degrees of defect. At this time, Nahmias et al. (1970a) found that 80 per cent. of neonatal infections were due to type 2 strains, and it was thought that the type 1 infections that did occur resulted from transplacental infection or were acquired in the postnatal period. Maternal antibody transferred across the placenta did not prevent infection of the child. The most recent figures from Nahmias and Roizman (1973b) show that 27 per cent. of neonatal infections are due to type 1 virus.

#### Infections of the nervous system

The main forms of involvement of the central nervous system are meningitis and meningo-encephalitis.

#### Meningitis

A definitive diagnosis of this form of aseptic meningitis is difficult as virus is rarely isolated from the cerebrospinal fluid, and serological studies to demonstrate a rising antibody titre are usually necessary. The serological evidence may be difficult to interpret, as the antibody response could be due to a reactivation of a latent infection of any site due to the febrile meningitis (Olson et al., 1967).

Virus was first isolated from the CSF in 1940 by Armstrong (1943). Subsequent studies have shown that herpes simplex virus is not a common cause of meningitis. Olson et al. (1967) in a retrospective study of 49 patients with proven or suspected infection of the

CNS found 10 cases of herpetic meningitis diagnosed on serological responses. The patients were mainly young adults and this has been quoted by Craig and Nahmias (1973) in support of their claim that benign aseptic meningitis is associated with type 2 strains of virus. Earlier observations may also support this hypothesis, as Janbon, Chaptal and Labraque-Bordenave (1942) described two patients with meningitis in association with herpetic lesions of the genitalia. A 21-year-old soldier was seen with meningitis 1 week after a genital infection, and the meningitis recurred 14 months later. Virus isolation was not attempted, but isolation from the CSF appeared to be successful in a second case, a 34-year-old woman with a 4-year history of herpes in association with menstruation. In this patient, meningitis developed during menstruation, unaccompanied by skin lesions and a specimen of CSF produced encephalitis in rabbits. The patient recovered, and after a few months the pattern of recurrences with menstruation was restored. These 2 histories also raise the possibility of recurrent meningeal infection, and support other observations suggesting that latency and reactivation can occur in the central nervous system. Fever therapy is an efficient stimulus to herpes virus, and Warren et al. (1940) noted signs of involvement of the nervous system during such procedures. A number of other examples are reviewed by Kibrick and Gooding (1965). The association of genital infections and type 2 strains with meningitis has also been indicated by the reports of Terni et al. (1971) and Skoldenberg (1972) of concurrent meningitis and herpes progeneralis. On the basis of these reports and their own isolation of type 2 virus from the buffy coat of two patients with meningitis, Craig and Nahmias (1973) have proposed that type 2 virus

is the usual cause of meningitis in adolescents and adults and that it reaches the meninges via the blood, perhaps carried in lymphocytes as the virus can grow in these cells (Nahmias, Kibrick and Rosan, 1964). Confirmation of these observations and investigation of the value of culturing the buffy coat would be of interest.

### Encephalitis

Herpes simplex virus was first isolated from the brain of a 4-week-old child by Smith, Lennette and Reames (1941). The association of herpes virus with an acute form of localised encephalitis was not established for some years (van Bogaert, Radermecker and Devos, 1955; Harland, Adams and McSeveney, 1967). Pathologically, the virus infects the cerebral cortex, often unilaterally, affecting the medial temporal and orbital regions most frequently. Areas of necrosis and softening develop, accompanied by focal haemorrhages and a mild inflammatory response. Perivascular cuffing is present, and inclusions can be seen in oligodendroglia and nerve cells (Kibrick and Gooding, 1965). Electron-microscopical examination has confirmed the presence of herpes virus particles (Harland et al., 1967) and infected cells can be detected by immunofluorescence (Juel-Jensen and MacCallum, 1972). Serological investigation can also be undertaken in patients with encephalitis, although as in meningitis interpretation of fourfold or greater rises in titres may be very difficult, since infection with the varicella-zoster virus can cause cross-reacting antibody responses to herpes simplex antigens in a significant proportion of varicella (48 per cent.) and zoster (26 per cent.) cases (Ross, Subak-Sharpe and Ferry, 1965).

The age distribution of patients with encephalitis shows that infection may occur at any age, although the disease is most frequent

in young children or adolescents and young adults (Olson et al., 1967). Involvement of the brain in the congenital form of disseminated infection has been mentioned earlier, and the viruses recovered from these patients may be either type 1 or type 2. Similar involvement may be met with in the disseminated infections that develop in malnourished children and eczema sufferers. The virus is type 1 and the outcome is usually fatal.

In older patients, a poor prognosis has also been found (Miller and Ross, 1968; Juel-Jensen and MacCallum, 1972). For this reason the use of antiviral drugs such as idoxuridine and cytosine arabinoside must be considered; diagnosis is best attempted by examination of brain biopsy material from the temporal lobe (MacCallum, Potter and Edwards, 1964).

Johnston, Olson and Buescher (1968) concluded that herpes simplex virus was the commonest cause of sporadic fatal encephalitis in the U.S.A. and Miller and Ross (1968) found it to be the agent most frequently incriminated in the West of Scotland. Juel-Jensen and MacCallum (1972) have reviewed the clinical findings, and these may be summarised as fever, headache, personality changes, fits and a decreasing level of consciousness: in some cases, localising signs are present, in others a diffuse infection is present. The course may be rapidly downhill, with death within a few days or months in over half the patients, although the death rate varies between series, and has been reported as high as 70 per cent. (Olson et al., 1967). In those who survive, residual neurological defect is common, and the extent and nature of this will depend on the site of neuronal damage. Craig and Nahmias (1973) suggest on the basis of their experience of antigenic

typing of virus isolates that encephalitis in older children and adults is associated with type 1 virus, perhaps by spread during a primary gingivostomatitis. Reactivated infection may occur in some cases (Kibrick and Gooding, 1965; Leider et al., 1965), and it has been suggested (Longson, 1970) that this could account for the different age distribution of primary herpetic infection and encephalitis. However, as discussed in earlier sections, primary infections have been recorded in adults.

THE MORPHOLOGY AND COMPOSITION OF HERPES SIMPLEX VIRUS

Virus structure

All members of the herpes virus group have the same appearance when negatively stained and visualised in the electron microscope. Early studies by Wildy, Russell and Horne (1960) established that herpes simplex virus consists of a core structure containing the viral deoxyribonucleic acid (DNA) with a diameter of approximately 75 nm enclosed within the protein capsid: this icosahedral structure consists of 162 capsomeres 9-10 nm by 12-13.5 nm of which 150 are hexagonal and 12 pentagonal in cross-section, each with a central canal. Surrounding the entire structure is an envelope with the appearance of a trilaminar membrane; the overall diameter of the virion is variable due to the flexibility of this membrane, and lies within the range 145-200 nm. Examination of ultra-thin sections indicates that the envelope is closely applied to the capsid and that there is a central dense core or "nucleoid" of 35 nm diameter (Epstein, 1962a). Although this description of the basic structure has not been modified, knowledge of the organisation of the core has been extended by the work of Roizman and his colleagues, who have examined ultra-thin sections of viruses and interpreted them to indicate that the DNA takes the shape of a "doughnut" with a protein rod occupying the central hole (Furlong, Swift and Roizman, 1972). The same group claim to have demonstrated the presence of a "fibrilous material" surrounding and closely applied to the capsid: this layer has been named the "tegument" (Nahmias and Roizman, 1973a).

### Virus composition

The DNA of herpes simplex virus is double-stranded and originally was estimated to have a molecular weight of  $50-60 \times 10^6$  (Russell and Crawford, 1963). Subsequent observations have revised this figure to  $99 \pm 5 \times 10^6$  daltons (Becker, Dym and Sarov, 1968; Kieff, Bachenheimer and Roizman, 1971) and in addition a small but reproducible difference in guanine and cytosine (G + C) content between type 1 and type 2 strains has been found (Goodheart, Plummer and Waner, 1968); type 1 strains have a G + C base composition of 67 - 68 moles per cent., whereas this is 69 - 70 moles per cent. for type 2 viruses.

The envelope is acquired from the inner lamella of the nuclear membrane (Darlington and Moss, 1968) as the newly matured capsids migrate out from the nucleus. As a result, some of the components of the envelope reflect this origin; Epstein and Holt (1963) demonstrated adenosine triphosphatase activity in extracellular virus and Watson and Wildy (1963) reported clumping of enveloped particles after exposure to an antiserum raised against uninfected host cells. Cell membranes are substantially altered during the virus growth cycle (Nii et al., 1968b; Nii, 1971) and at least 11 virus-specified glycoproteins are incorporated to all cell membranes including the nuclear membrane (Heine, Spear and Roizman, 1972) and are also present in virions.

The specific virus antigens of type 1 and 2 strains are located in the membrane or immediately below it (Hampar, Miyamoto and Martos, 1971) and apart from their serological reactivity may be related to the different cytopathogenic properties of virus strains. Both common and type specific antigens appear to be present in virions, and reaction of antisera with either may result in neutralisation of infectivity (Sim

and Watson, 1973).

The function of the membrane in virus infectivity has been much debated. Both naked and enveloped particles have been claimed to be infectious (Watson, Wildy and Russell, 1964) and this finding was supported by Spring and Roizman (1968) who found that virus extracted from nuclei was infectious although this could be the result of enveloped virus present in the convoluted nuclear membrane. Other reports (Smith, 1964; Olshevsky and Becker, 1970) have claimed that the infectivity of a virus suspension declines as the proportion of enveloped particles diminishes. The conclusion is that both particles are infectious, although the presence of the envelope enhances infectivity; as this is the form in which virus is released from cells, it would appear to be the important form in natural transmission between cells and hosts. Type 1 and 2 strains appear to differ in the proportions of enveloped particles detected in the cytoplasm of infected cells; type 2 strains give low yields of infectious virus, and this might be associated with a high proportion of unenveloped particles as with varicella-zoster virus (Cook and Stevens, 1968; 1970).



THE GROWTH CYCLE OF HERPES SIMPLEX VIRUS

Stages of adsorption, penetration and uncoating

Initial attachment of herpes simplex virus to a cell may be by electrostatic attraction; in support of this claim is the effect of the negatively-charged sulphated polysaccharide heparin, and dextran sulphate on the adsorption of virus (Nahmias, Kibrick and Bernfield, 1964). In the presence of this substance, virus adsorption is markedly reduced and even if it is added after virus attachment, virus may be released from cell surfaces (Hochberg and Becker, 1968). Comparison of the effect of heparin on type 1 and 2 virus strains (Plummer, Waner and Bowling, 1968; Hutton, Ewert and French, 1973) showed that type 2 strains were more sensitive than type 1.

There is disagreement regarding the mechanism of entry of the herpes virus nucleocapsid to cells. According to one report (Morgan, Rose and Mednis, 1968) the envelope of the virion fuses with the cell membrane, and the capsid is released into the cell cytoplasm. On the other hand, Dales and Silverberg (1969) claimed that entry was always by pinocytosis, and argued that the observations of Morgan et al. (1968) were attributable to damaged virions produced by the use of an ultrasonic disintegrator in the production of virus stocks.

Specific antibody can block virus attachment, and may even prevent penetration of adsorbed virus. The sensitivity of this method of distinguishing virus strains is discussed in a later section of this thesis.

### Viral ribonucleic acid synthesis

After entering a cell, the DNA and an associated protein component are released from the capsid - either by lysosomal enzymes discharged into the pinocytotic vesicle, or by some unknown mechanism, and the released DNA and protein enter the nucleus. No virion-associated polymerase enzyme activities have been detected, and in support of this observation, Lando and Ryhiner (1969) reported that extracted viral DNA is infectious. It follows therefore that initial transcription of the viral DNA must depend on host cell DNA dependent RNA polymerases. The transcription of viral DNA has been reviewed by Roizman and Frenkel (1973), who have shown that 50 per cent. of the viral DNA is transcribed, and that the synthesis of ribonucleic acid (RNA) is controlled in two ways. Firstly, study of the time sequence of synthesis shows that types 1 and 2 differ in the extent of early transcription; 2.1 per cent. of type 2 DNA is transcribed within 2 hr whereas 44 per cent. of type 1 DNA is transcribed at this time, even in the presence of cycloheximide (Frenkel et al., 1973). The proteins specified by this RNA include at least 20 structural proteins and in this respect the growth of herpes simplex virus differs from the typical description of a bacteriophage growth cycle in which structural proteins are made after DNA synthesis, and are termed "late proteins". A second control of DNA transcription has been described by Roizman and his group, who found that the RNA pool could be divided into abundant and scarce classes and that the abundant classes could be distinguished by the presence of up to 160 adenosine residues attached to the 3-prime end of the RNA molecule (Silverstein et al., 1973).

### Viral protein synthesis

Virus specified proteins are divisible into structural and non-structural classes, and 42 new proteins have been detected in infected cells (Nahmias and Roizman, 1973a). Twenty-seven of these are components of the virion; of the remaining fifteen so far detected, only a few have been characterised to any extent. Most convincing evidence has been reported to establish that the thymidine kinase found in infected cells is virus-specified (Klemperer et al., 1967). The DNA dependent DNA polymerase, a DNA exonuclease, and the enzyme deoxycytidine monophosphate kinase also seem to be virus specified (Keir and Gold, 1963; Hay et al., 1971b). Differences in heat stability and immunological specificity have been shown between the thymidine kinase (Thouless and Skinner, 1971) and the exonuclease (Hay, Moss and Halliburton, 1971a) enzymes of type 1 and type 2 viruses.

Both structural and non-structural viral proteins are synthesised in the cytoplasm on host ribosomes, and then migrate into the nucleus to participate in viral DNA replication and capsid formation; some are incorporated into cell membranes. The transfer of newly synthesised proteins from the cytoplasm to the nucleus has been established by radioactive pulse and chase experiments (Olshevsky, Levitt and Becker, 1967) and immunofluorescence studies (Ross, Watson and Wildy, 1968). The flow of proteins into the nucleus depends on the presence of arginine in the medium: various reports have established that completion of the growth cycle can be prevented by starving the cells of arginine (Becker, Olshevsky and Levitt, 1967; Inglis, 1968). A similar failure of transfer may account for the absence of nuclear membrane reduplication in arginine-deprived cells (Mark and Kaplan,

1972).

The complexity of the structure of the herpes simplex virus has already been described. Knowledge is still lacking of the exact association of group and type reacting antigens with specific proteins. Nahmias and Roizman (1973a) record a personal communication from D. H. Watson claiming that glycoproteins numbers 7 and 8 of Roizman and colleagues are type specific. An earlier report (Watson, 1969) described the separation of a protein, "band II", from infected cells: later it was shown that both type 1 and type 2 infected cells (Sim and Watson, 1973) contained this antigen which can be separated by electrophoresis and detected by immunoprecipitation tests. A further report established that rabbit antisera specific to the Band II antigen of type 1 virus could neutralise both type 1 and type 2 viruses (Watson and Wildy, 1969; Sim and Watson, 1973) and it must therefore be a shared antigen. Although reaction between this antigen and antiserum results in neutralisation of virus infectivity, evidence reported by Wildy (1972) indicates that the antigen is not part of the envelope. This could imply that it is one of the "internal" surface antigens described by Hampar et al. (1971), perhaps part of the tegument of Nahmias and Roizman (1973a).

#### Viral deoxyribonucleic acid synthesis

A virus-specific DNA dependent DNA polymerase is produced early in infection (Keir and Gold, 1963). The exact mechanism of DNA replication is not known, but studies by Kieff et al., (1971) suggest that the DNA of herpes simplex virus has single-strand breaks. DNA synthesis is believed to originate at several points along the molecule,

the new fragments being united finally as they are enclosed in capsids. An excess of viral DNA is found in infected nuclei, especially with type 2 strains (Halliburton, Hill and Russell, 1971).

#### Assembly and release of virus

Electron microscopical observations of infected cells revealed that nuclear particles were not enveloped, and that cytoplasmic particles were usually surrounded by an envelope (Morgan et al., 1954 and 1959; Falke, Siegert and Vogell, 1959).

Within the nucleus, virion cores with a single "membrane" can be seen either as empty or DNA-containing full capsids (Nii, Morgan and Rose, 1968a): these have a diameter corresponding to the capsid. If DNA synthesis is inhibited by hydroxyurea, empty cores accumulate in the nucleus and are poorly released (Nii et al., 1968c). Occasionally, large crystalline arrays of capsids can be found (Melnick, Rabin and Jenson, 1968; Nii and Ono, 1971). Envelopment of nuclear capsids has been the subject of debate. Early workers (Morgan et al., 1959) claimed that the envelope was acquired from "extraneous host material" as the capsids passed into the cytoplasm. However, Epstein (1962b) found some virus particles acquiring an envelope by budding into cytoplasmic vesicles or even from the cell membrane. Later studies (Darlington and Moss, 1968) with three herpes virus, including herpes simplex virus, demonstrated that the inner lamella of the nuclear membrane was the usual site of envelopment. It is possible that the extensive reduplication of the nuclear membrane with the formation of intranuclear vacuoles and intracytoplasmic processes (Epstein, 1962b; Nii et al., 1968b) has led to some confusion as to the origin of the

membranes involved in envelopment. Alternatively, although the nuclear membrane is the normal site of envelopment it can occur at any cell membrane (Schwartz and Roizman, 1969b).

To reach the cell surface, enveloped virus has to cross the cell cytoplasm, and is usually found in vesicles derived from the outer layer of the nuclear membrane (Morgan et al., 1959) or from the rough-surfaced endoplasmic reticulum (Schwartz and Roizman, 1969a); Nii (1971) found both forms of egress to operate. Type 1 and 2 strains of herpes simplex virus differ in the proportion of enveloped particles found in the cytoplasm (Schwartz and Roizman, 1969b) and Figueroa and Rawls (1969) record that only 6.8 per cent. of type 2 virus is enveloped compared with 38 per cent. for type 1.

Electron microscopical examination of infected cells has shown that type 2 strains of virus produce microtubules or filaments of unknown composition (Couch and Nahmias, 1969; Schwartz and Roizman, 1969b).

One-step growth studies in BHK21 cells indicate that under optimal conditions, the laboratory strain HF has completed a replication cycle within 9-10 hr, the majority of the progeny virions being assembled from the 6th hour onwards (Russell et al., 1964). As with all animal viruses, an excess of non-infectious particles is produced during infection; particle:infectivity ratios are within the range 10:1 to 100:1 for type 1 viruses and 500:1 to 1000:1 for type 2 strains (Nahmias and Roizman, 1973a).

Various authors have noted the lower yields of type 2 virus from cell cultures; this difference is consistently about  $10^2$  plaque forming units of final concentration of virus (Plummer et al., 1968;

Figueroa and Rawls, 1969).

Effects of virus infection on cell membranes

Electron microscopical studies (Epstein, 1962b; Nii et al., 1968b) show that dramatic changes occur in the nuclear membrane. Considerable thickening and folding of the membrane is seen, a virus specified function that occurs even if viral DNA synthesis is interrupted (Nii et al., 1968c). The infected cell membrane contains new antigenic determinants and these can be detected by immune lysis (Roane and Roizman, 1964; Smith et al., 1972); mixed haemadsorption to antibody coated cells (Watkins, 1964); immunofluorescence (O'Dea and Dineen, 1957; Nahmias et al., 1971b) and immune electron-microscopy with ferritin-conjugated antibody (Nii et al., 1968b).

Biochemical analysis of infected cells has established that new glycoproteins are incorporated into all cell membranes (Olshevsky and Becker, 1970; Heine et al., 1972; Spear and Roizman, 1972): the same 11 glycoproteins are present in the virus envelope and in all cell membranes. As discussed earlier, these include two type-specific proteins, and this would account for the ability of immunofluorescent studies with unfixed cells to distinguish between type 1 and type 2 strains of virus (Nahmias et al., 1971 a and b; Geder and Skinner, 1971).

The agglutinability of infected cells by concanavalin A (Tevethia et al., 1972) may be related to the incorporation of new glycoproteins to the cell membrane, although the effect is not confined to herpes simplex virus and has been demonstrated with other viruses (Becht, Rott, and Klenk, 1972).

Cell membrane changes are the most probable explanation of the

altered behaviour of cells, in particular the ability of infected cells to form syncytia. The type-specific glycoproteins could be important in determining the different cytopathic effects shown by type 1 and 2 strains of virus, especially the cell fusing property of type 2 strains (Schneeweis, 1962b; Munk and Donner, 1963).

#### Effects on infected cells

Biochemical studies of infected cells have revealed that herpetic infection results in a number of changes in host cell metabolism. Early studies with the syncytial strain HF (Stoker and Newton, 1959) showed that mitosis was inhibited by infection: other studies with non-syncytial strains established that a stimulation of cell division occurred and that cell counts increased after infection (Gray, Tokumaru and Scott, 1958). The most intensively studied virus strains are both syncytial, the HF strain of Flexner and Amoss (1925b) and the MP strain of Hoggan and Roizman (1959a). MP virus infection results in the interruption of host DNA synthesis (Roizman, 1969). Other reports have shown that chromosome breaks occur (Hampar and Ellison, 1961), perhaps due to the action of the virus-specified exonuclease; breaks are random and occur before viral DNA synthesis commences and are not at sites of viral DNA synthesis (Waubke, Hausen and Henle, 1968). Host-cell RNA synthesis is also interrupted, both in the production of ribosomal precursor RNA and in its maturation (Roizman *et al.*, 1970a).

The events of the growth cycle lead to the development of the histological intranuclear lesion as recorded by Scott *et al.* (1953). The earliest change is the break-up of the nucleolus at 2 hr, and a





fine granular appearance of the nucleus develops at 6-7 hr post-infection. These changes are followed by the appearance of infectious virus and the development of the intranuclear inclusion body as described by Lipschütz (1921) and Cowdry (1934). The appearance of this type A inclusion, in particular the halo that surrounds the central eosinophilic body has been claimed to depend on the type of histological fixative used (Barski and Robineaux, 1959); fully developed inclusions appear late in infection at about 14-16 hr (Scott et al., 1953).

#### Growth of virus and detection of viral antigens

Herpes simplex virus can infect a wide range of cells of different species and undergo the productive cycle described above. Primary rabbit kidney (Sosa-Martinez, Gutierrez-Villegas and Sosa, 1955) and BHK 21 cells (MacPherson and Stoker, 1962) are sensitive, although HEp2, and HeLa cells can also support growth as well as human amnion and human embryonic cell cultures (Scott, McLeod and Tokumaru, 1961). Chick embryo fibroblast cultures are sensitive (Stulberg and Schapira, 1953; Scott et al., 1961); some isolates cannot infect these cells, and this ability has been used to classify strains (Lowry, Melnick and Rawls, 1971).

Viral cytopathic effects are discussed later, as well as the variation of plaque morphology in various cell culture systems. Cytological changes have been used extensively in the diagnosis of infection (Blank et al., 1951), a method which has returned to favour in the diagnosis of herpetic infection of the female genital tract by examination of exfoliated cervical cells.

Complement fixation tests have been widely used in the diagnosis of herpes virus infection, from the time of the early studies of Bedson and Bland (1929) and Brain (1932). The test was applied extensively by Hayward (1949) and Dudgeon (1950) using egg-grown antigens. Brown (1953) attempted to analyse the antigens of the virus and concluded that three activities could be detected in infected allantoic fluids. An antigen participating in neutralisation was present; this was associated with virus particles and could be removed by ultracentrifugation. In the absence of this antigen, there was no change in the other 2 properties of skin reactivity and complement fixation which were designated soluble antigens. The skin reactivity antigen was found to be more heat stable than the complement fixing antigen. No attempt was made to analyse these soluble antigens, and precise information as to their nature is lacking, although a more recent study (Martin, Palmer and Kissling, 1972) in which viral capsid, envelope and soluble antigens were compared, indicated that the soluble antigen did not appear to consist of capsid precursor molecules. The clearest definition of the production of complement fixing antigen (CF antigen) is that of Gold, Wildy and Watson (1963): this study confirmed that the antigen did not sediment with virus particles, and established that it was detectable in cells during the eclipse phase before infectious virus was produced. This finding was confirmed by Russell et al. (1964). It appears that the CF antigen is a complex of early virus-specified proteins and Russell et al. (1964) noted that viral DNA polymerase activity increased at about the same time as the antigen. Diagnostic CF antigens are made by lysing infected cells, and ultracentrifuging to remove cell debris and

approximately 90 per cent. of virus infectivity (Dr. Isabel Smith, personal communication).

Further interest has been stimulated by the finding of Tarro and Sabin (1970, 1973) that a type-specific CF antigen is present in cells soon after infection. The exact time of detection of this unstable antigen varies for different host cells: in guinea pig kidney cultures it is at maximum titre at 3 hr post inoculation, while in HEp2 and rabbit kidney cells, incubation for 24 hr is necessary. This is a soluble antigen and whilst the specificity of this early viral protein(s) is surprising, it is in agreement with a tentative conclusion of Frenkel et al. (1973). Analysis of the homology between type 1 and type 2 abundant species of viral RNA suggested that there should be greater differences between non-structural than structural proteins of type 1 and 2 strains. These observations are relevant to the problem of classifying sera as type 1 or 2 specific in epidemiological studies of the association of genital type 2 virus infection and carcinoma of the cervix.

#### Alternative growth cycles

As discussed earlier, herpes simplex virus infections are known to recur in an individual, despite the presence of virus-neutralising antibody, and this has led to investigation of virus-cell interactions in which virus persists or does not give rise to infectious progeny.

#### Conditions affecting productive infection

Early studies (Stoker, 1958; Wheeler and Canby, 1959) established that virus could persist and spread in cell cultures even

if specific antibody was added to the extracellular fluid. The reports of Hoggan and Roizman (1959a) and Wheeler (1960) showed that a persistent infection could be established in the presence of antibody and that virus could survive several subcultures.

The effects of the temperature of incubation and the pH of the medium on the production of virus were investigated by Farnham and Newton (1959), Hoggan and Roizman (1959b) and later by Waddell and Sigel (1966); an acid pH reduces yields of virus and penetration of the virus is reduced if virus-cell mixtures are incubated at temperatures below 37°C (Farnham and Newton, 1959) although virus yields were greatest at 32°C, perhaps related to the greater thermal stability of the virus at this temperature. Hoggan and Roizman (1959b) showed that virus yields from infected cells were reduced to 1/7 at 37°C and 1/20 at 39°C compared with incubation at 35°C.

The greater sensitivity of type 2 strains to incubation at temperatures greater than 34-35°C has led to the development of a temperature marker test. Longson (1971) incubated cultures at 34.5°C, 39°C and 40°C; the least affected were two laboratory strains, followed by type A and type B strains in increasing order of sensitivity. Type B strains of this worker appear to correspond to type 2 of other workers. A similar test has also been proposed by Ratcliffe (1971).

Cell-dependent differences in the production of infectious virus have also been found when cultures are incubated at temperatures greater than 37°C (Crouch and Rapp, 1972). The yield of type 2 virus at 39°C was found to be reduced by 2 log<sub>10</sub> in rabbit kidney cells compared with 33°C, and no virus was produced from hamster embryo fibroblasts at 39°C, although viral antigens were detected in the cells.

An inhibitor of DNA synthesis, cytosine arabinoside, was used by O'Neill, Goldberg and Rapp (1972) to inhibit a type 2 virus. Removal of the inhibitor up to 22 days after infection allowed virus to replicate, although a delay of 5-6 days was necessary, and very few cells were involved in virus production. A reversible inhibition can also be produced by arginine deprivation of infected cells (Inglis, 1968) or incubation of some strains at 40°C (Yoshino, Taniguchi and Takeuchi, 1968).

#### Temperature-sensitive mutants

A special category of interrupted growth cycle is shown by the temperature-sensitive mutants of herpes simplex virus induced by bromo-deoxyuridine treatment of both type 1 and 2 viruses. These mutants will grow at 32°C but not at 38°C; viral replication may be interrupted at any stage and by means of complementation studies, genetic linkage maps can be deduced (Brown, Ritchie and Subak-Sharpe, 1973). Complementation and recombination have also been demonstrated between type 1 and 2 strains (Timbury and Subak-Sharpe, 1973).

#### Persistent infections in vitro

Persistent infections may be established in the presence of antibody (Hampar and Copeland, 1965). Some cytopathic effect can usually be seen, but virus does not spread throughout the cell sheet: infected cells fall off the glass, non-infected cells grow in and the cycle is repeated (Hampar and Burroughs, 1969). This state may be maintained for some months and Hampar and Keehn (1967) have shown that the virus isolated from such a culture after many passages is

different antigenically from the original strain. This model has obvious relevance to the study of recurrent infections in man.

#### Herpes simplex virus-induced cell transformation

As an association between carcinoma of the cervix and type 2 herpes virus infection has been postulated, it would be expected that laboratory evidence of cell-transforming ability could be obtained. A major problem has been that herpes simplex virus types 1 and 2 usually undergo a productive cycle resulting in cell death: under these circumstances transformation cannot be detected. To overcome this difficulty, the virus can be inactivated before inoculation; two methods of inactivation have been reported, using ultraviolet irradiation (Duff and Rapp, 1971) and the chemical 2,12 dimethylbenz-(a)anthracene (Docherty, O'Neill and Rapp, 1971). The use of a carcinogenic chemical seems questionable as it is possible that cell transformation could be attributed to residual amounts of this substance in the inactivated virus suspension. Using chemically inactivated virus, Rapp and his colleagues have developed transformed hamster embryo fibroblast lines, one of which lacks a single chromosome and is resistant to infection with type 2 virus, although type 1 virus can grow normally in this cell line. The mechanism of resistance is believed to be at the stage of uncoating or DNA synthesis (Docherty, Mäntyjärvi and Rapp, 1972). No evidence of persistent herpes virus could be found, and it was concluded that the chromosome deletion could have resulted from the known ability of the virus to cause chromosome breaks.

Transformed cell lines produced by infection with ultraviolet

inactivated type 2 virus (Duff and Rapp, 1971) are oncogenic in newborn hamsters and both the original transformed cells and tumour derived cells have been shown to contain herpes virus antigens, although no infectious virus could be recovered. Recently, cell transformation has been reported with inactivated type 1 virus (Duff and Rapp, 1973). No evidence of an oncornavirus was found in the type 2 transformed cells.

ANTIGENIC ANALYSIS OF HERPES SIMPLEX VIRUS

Neutralisation tests

The clear division of isolates of herpes simplex into two antigenic types was only established between 1962 and 1968 and the association of antigenic type and site of infection in 1967. Before this time a number of studies had examined the relationship between strains and had reached varying conclusions. Laboratory strains of virus have played a prominent part in these studies; HF virus has been used on a number of occasions, often to compare with a few freshly isolated strains (Burnet and Lush, 1939b; Jawetz, Coleman and Merrill, 1955). The use of standard strains has obvious advantages in allowing comparison of results from different laboratories, but these strains have been passaged many times in various hosts, and selection of mutants and contamination with another virus are possible. Cross-contamination seems the most likely explanation for the findings concerning laboratory strains HF and HPF as reported by Wheeler (1960; 1964). The syncytial strain HPF was found to be different antigenically from the parental HF strain, a result confirmed later by Dowdle et al. (1967), who found that the HF strain of Wheeler was antigenic type 2, whereas HF virus from 3 other sources was type 1. Such a major discrepancy has been recorded on another occasion, with strain 1166 of Armstrong (1943). This was originally reported as being similar to strain HF, a finding which was not substantiated by Florman and Trader (1947). Later reports from Chu and Warren (1960) and Schneeweis (1967) also failed to show that 1166 was similar to HF; the most obvious conclusion is that contamination occurred early in the



passage of this virus, as subsequent studies have shown that the majority of virus strains recovered from nervous tissue are antigenic type 1 (Nahmias and Roizman, 1973b).

The reports of Shubladze and Chi-Hsiang (1959) and Plummer (1964) also suggest that laboratory contamination is a real hazard. The Russian work compared seven strains of herpes simplex virus by neutralisation tests in mice and found that these were divisible into 3 groups related to 3 reference strains L2, US and K. All 3 prototype strains were tested by Plummer (1964) who used a refined kinetic neutralisation method to show that L2 and US were different. However, strain K, reputedly isolated from a lip lesion was not herpes simplex virus, but appeared to be a virus related to Japanese B encephalitis.

The early difficulties in showing that more than one antigenic type of virus existed were related to the use of relatively insensitive methods for the neutralisation tests, and the examination of only a limited number of strains. In reporting the isolation of virus 1166 from cerebrospinal fluid, Armstrong (1943) compared this virus with HF and showed that the 2 viruses were similar in mouse neutralisation tests. Kilbourne and Horsfall (1951a) also used HF as a reference strain in cross-neutralisation tests with 2 strains isolated from the pharynx: they found no difference between the 3 strains. Ashe and Scherp (1963) employed a sensitive kinetic neutralisation test to compare 14 virus isolates, comprising 10 from the lip and 4 from the pharynx. Antigenic differences were found and the strains were divided into 4 groups; no account was taken of the ability of herpes simplex virus to infect other skin sites, the eye, the central nervous system or the genital tract. Using a similar

technique, Plummer (1964) was able to divide a series of strains from various laboratories into two groups, thus confirming the earlier reports of Schneweis (1962a and b) who was the first to produce clear evidence based on neutralisation, complement fixation and biological markers that two types of virus existed. Unfortunately, the type 2 viruses were laboratory strains originally isolated from the arm and thigh and the association between type 2 strains and the genital tract was not realised. Analysis of the cytopathogenic effect of isolates had shown that the syncytial forms were isolated from the genital tract (Munk and Donner, 1963) but it was not until 1967 that the reports of Dowdle et al. and Schneweis established that antigenic type 2 strains were recovered from the genital tract or associated areas of skin.

An alternative method of comparing strains was employed by Florman and Trader (1947); animals were immunised with one strain and then challenged with other strains. Resistance to infection, or protection, was taken to indicate antigenic identity. Only 4 strains were included in this study and no differences were found, although it might be difficult to detect minor antigenic differences by this method as it is established that large doses of virus have to be inoculated to render an animal immune (Urbain and Schaefer, 1929), whilst in man it has been argued that previous infection with type 1 virus does not protect against reinfection (Nahmias and Dowdle, 1968).

The papers of Slavin and Gavett (1946a and b) show that the limitations of methods of assaying virus need not have prevented the discovery of the two virus types. The first paper reported the isolation of two strains of virus from 3 patients with herpetic

vulvovaginitis and the second presented the results of an antigenic analysis of these strains. With convalescent rabbit antisera, and a constant-serum neutralisation test assayed in mice by intracerebral inoculation, strain HF was compared with the two new isolates: one was found to be similar to HF, while the other was different. This observation of the recovery of an antigenically distinct strain from the genital tract was not followed up until 1967.

Apart from the discrepancies mentioned above with HF and 1166 virus strains, other studies involving laboratory virus have shown good agreement in the allocated types. Strains PET, MAX and KER isolated originally by Jawetz et al. (1955), have been examined by Schneweis (1962a) and Nahmias and Dowdle (1968). Both groups found that strain PET was antigenic type 1 and KER was type 2: MAX, which was found to differ from 10 other strains by Jawetz et al. (1955), fell into Schneweis' (1962a) type 2. The Russian strains L2 and US (Shubladze and Chi-Hsiang, 1959) were reported as belonging to different groups, a finding confirmed by both Plummer (1964) and Dowdle et al. (1967). Finally, strain MS of Gudnadottir, Helgadottir and Bjarnason (1964) isolated from the brain of a patient with multiple sclerosis was classified as type 2 by both Plummer (1964) and Dowdle et al. (1967).

The major papers in the appreciation of the typing of herpes simplex strains were produced by Nahmias and his associates. The cumbersome nature of the kinetic neutralisation test and the difficulty of applying this to a wide range of viruses from many sites was overcome by adapting a quantal neutralisation test (St. Groth, 1961) to microtitre plates (Pauls and Dowdle, 1967). This procedure allowed

a suitable number of observations to be made for reliable TCID<sub>50</sub> and neutralising antibody titre calculations, applying the formula of Reed and Muench (1938). With rabbit antisera prepared according to the same schedule as Plummer (1964), Pauls and Dowdle (1967) found that cross-micro-quantal neutralisation tests with strains L2, US, MS and a strain VR3 and the corresponding antisera grouped VR3 and L2 as type 1 and US and MS as type 2. Dowdle et al. (1967) applied the micro-quantal technique to 91 virus strains and confirmed the division into 2 types. Seven strains from Ashe and Scherp (1963) were placed in type 1 - despite the findings of the original paper that by neutralisation kinetic tests these same strains belonged to 4 groups: this must be a reflection of the sensitivity of the method and the nature of the antisera used.

The major conclusions were that a large number of isolates (Dowdle et al., 1967) could be divided into two groups and that there was a good correlation between the type and the site from which the virus had been isolated. Type 1 strains were recovered from the mouth, lip, skin and CSF, whereas type 2 strains were from the genital tract, including the cervix, and associated areas of skin of the perianal region, thighs and buttocks. Rawls et al. (1968a) established that 4 strains isolated from 198 smegma samples were distinct from other isolates by kinetic neutralisation tests. A further study from Plummer and colleagues (Plummer et al., 1970) found that 11 oral isolates were type 1 and 29 genital and anal strains were type 2.

#### Complement fixation tests

The studies of Hayward (1949) established that infected egg

fluids (amniotic, allantoic) or chorioallantoic membranes were suitable antigens for complement fixation tests. Antigens prepared in eggs from 4 freshly isolated strains were compared in cross-complement fixation tests with guinea pig antisera and found to be identical. Womack and Hunt (1954) also applied this technique to the study of 6 isolates. No information is given as to the source of these isolates, but two, including HF were laboratory strains. The antisera were produced in rabbits by corneal inoculation of egg-adapted virus. The antigens were prepared in eggs and single antigen dilutions were used in the complement fixation tests. Serum titres were in the range 4 to 64, although most were only 8 or 16. Calculation of ratios between homologous and heterologous titres showed that the strains were not identical, but no clear groupings were apparent.

The studies of Schneeweis (1962b) yield more convincing evidence that complement fixation tests could separate strains. Chessboard titrations between virus antigens and rabbit antisera were presented as histograms and the areas of complement fixation calculated. The results were in good agreement with the precise neutralisation studies reported (Schneeweis, 1962a).

Martin et al. (1972) studied the reaction between the virion capsid and envelope components and the soluble antigens released from cells infected with strains VR3 and MS. Capsid antigens were found to be highly cross-reactive, whereas the results with the other two preparations suggested that the homologous titres were greater than heterologous. As there are type-specific membrane glycoproteins it is possible that this approach could be useful, although further extraction

and purification of the specific components might be necessary. Obviously the need to prepare these antigens makes this a difficult method to apply to the routine typing of virus isolates, but it could be of value in the study of the antibody content of human sera.

#### Other methods of typing strains

##### Immunofluorescence

Immunofluorescence techniques with rabbit antisera were developed by Nahmias et al. (1969a; 1971a and b). Cross-reactions were avoided by selecting appropriate test dilutions of the reference antisera and by absorption of sera with heterologous infected cells. Type-specific antigens can be prepared by treating infected cells with undiluted heterologous antiserum; Geder and Skinner (1971) studied both unfixed and acetone-fixed preparations and showed specificity of reaction by both methods. Although fluorescent staining of fixed cells gave 4-to 8-fold higher titres, the membrane fluorescence tests with unfixed cells showed a greater degree of specificity.

##### Passive haemagglutination

Passive haemagglutination was demonstrated by Felton and Scott (1958) with HF virus adsorbed to tanned sheep red blood cells. This method was investigated by Fuccillo et al. (1970) and Schneweis and Nahmias (1971) who showed that type-specific reactions could be obtained by absorbing extracts of type 1 and type 2 infected cells to tannic acid treated sheep red blood cells. However, there are difficulties in standardising the amount of protein adsorbed to the cells.

### Immunodiffusion

Immunodiffusion studies have played a part in many reports (Mäntyjärvi and Arvilommi, 1964; Tokumaru, 1965; Watson et al., 1966) and varying numbers of precipitin lines have been found in tests with infected cells, ranging from 3 or 4 to 11 respectively for the above publications. There are many problems with this method but it has been claimed that type-specific precipitation lines can be detected, and that cross-absorption tests confirm the specificity of these lines (Schneweis and Nahmias, 1971).

A variation of this precipitation method was developed by Jeansson (1972) who prepared antigens from small numbers of infected cells and reacted them with absorbed antisera by immunoelectrophoresis. Type-specific reactions were obtained, and this would be a most useful typing method if the absorbed sera are available.

### Cytopathic effects in cell cultures

Many reports have confirmed that herpes simplex virus can cause cell fusion in addition to cell rounding and proliferation (Scherer and Syverton, 1954; Gray et al., 1958). The ability to form syncytia is seen most markedly with laboratory strains of virus such as HFEM (Stoker, 1958), MP (Hoggan and Roizman, 1959a), HPF (Wheeler, 1960), GC (Scott et al., 1961) and GC (Nii and Kamahora, 1961). At the time of study all these strains had been maintained in cultures for varying lengths of time and it has been established that these syncytium-forming strains are genetically stable (Hoggan and Roizman, 1959a; Schneweis, 1962b). The cell fusion seen in cultures infected with these viruses could arise from the rapid fusion of cells due to

the inoculation of large numbers of infectious or inactivated virus particles per cell (Nii and Kamahora, 1961; Tokumaru, 1968) when the mechanism may be similar to that of the cell fusion brought about by inactivated Sendai virus (Harris and Watkins, 1965). With smaller inocula, syncytia are detectable within 24-48 hr (Hoggan and Roizman, 1959a; Barski and Robineaux, 1959); these appear to develop by the recruitment of neighbouring cells until a very large multinucleated cell is formed (Stoker, 1958; Barski and Robineaux, 1959).

The direct cell-to-cell transfer implied by this method of syncytium formation is supported by the observations that several of these syncytial strains were isolated from infected cultures in the presence of specific antibody (Hoggan and Roizman, 1959a; Wheeler, 1960).

Apart from these syncytial strains, plaque purification and limiting dilution methods have allowed the isolation of apparently stable strains that cause cell rounding accompanied by cell proliferation (Gray et al., 1958; Hoggan and Roizman, 1959a, and Wheeler, 1964). A similar type of strain has been derived from HF virus by Hinze and Walker (1961). Most of the derived strains could not be distinguished from the parental viruses by sensitive neutralisation tests.

In addition to these two types of cytopathic effect, several reports have indicated that intermediate forms may exist. The original report of Gray et al. (1958) included the description of an effect consisting of loose aggregates of round cells, with no cell proliferation, sometimes accompanied by cell fusion; a similar form has been reported by Nii and Kamahora (1961), Schneweis (1962b), Munk and Donner (1963) and Ejercito, Kieff and Roizman (1968). The last three



studies established that a round-cell, non-proliferative cytopathic effect was characteristic of strains isolated from the genital tract, and strains can be classified by this means.

#### Plaque morphology

Plaque production with strains of herpes simplex virus was first reported by Kaplan (1957) with rabbit kidney cells and an agar overlay. Farnham (1958) showed that an antiserum-containing overlay gave reliable plaque titrations, and also that if the infected cultures were examined by the third day, no additions to the medium were necessary; later, secondary plaques began to appear as the virus spread throughout the cell sheet. A rapid and convenient method of plaque production with herpes simplex virus was described by Russell (1962); in this procedure, cells are infected in suspension, with agitation, transferred to petri dishes and an overlay medium containing carboxymethyl cellulose (methocel) is added.

Rapp (1963) investigated variants of a laboratory strain and noted that large and small plaque forms could be detected under methyl cellulose. Munk and Donner (1963) gave a clear account of variation in plaque size under agar with virus strains isolated from various sites, and established that large plaques were associated with syncytium-producing strains. Smith, Rodriguez and McKee (1971) were able to clone large and small plaque variants of both type 1 and 2 virus strains under an agarose overlay, infecting monolayer cultures directly with material from patients' lesions. After 3 single-plaque passages large and small plaque strains were isolated: these variants were not associated with syncytium formation. The large plaque character did not breed true after 20 passages in cell culture; at this time a

greater range of plaque size was observed with both type 1 and 2 strains, and small numbers of giant cells were seen with type 2 virus. The usefulness of plaque morphology as a typing procedure for virus isolates is uncertain as there does not seem to be agreement as to plaque size or the associated type of cytopathic effect.

#### Plaque formation in chick embryo fibroblast cultures

The ability of a virus to grow in chick embryo fibroblast (CEF) cultures has been used to type strains. Schneeweis (1962b) compared the growth of 10 virus strains in a range of cell cultures, including CEF, and found that all strains could produce a cytopathic effect: eight of the strains belonged to his antigenic type 1 group. Figueroa and Rawls (1969), working with 9 relatively recent isolates passaged for an unspecified number of times, found that the 5 oral strains all failed to produce plaques in CEF, although careful examination of plates inoculated with 1000 plaque forming units of virus (determined in rabbit kidney cells) showed some evidence of cytopathic effect. Amstey and Balduzzi (1970) used this method to classify 15 isolates from genital infections. The reliability of the method was investigated more fully by Lowry et al. (1971) who found that formation of plaques with a diameter of 2.3 mm was a reliable marker of type 2 strains, provided low passage virus was used. Even with freshly isolated virus, a few small plaques with a diameter of 1.3 mm could be seen with type 1 strains, although the number of plaques produced was only a small proportion of the plaque-forming ability as assayed in rabbit kidney monolayers, the proportion ranging from  $1:10^2$  to  $1:10^6$ . It was noted that passage of the type 1 strains in rabbit kidney or human embryonic lung cultures resulted in an increase in the

number of plaques produced when the virus stocks were tested on CEF cultures, until after 25 passages 12-33 per cent. of the stock could produce plaques in both rabbit kidney and CEF cells. Plaque purification of type 1 virus in CEF cultures gave rise to clones that reliably plaqued in CEF with an efficiency of 18-75 per cent. of the rabbit kidney count; these strains were designated  $ch^+$  strains as opposed to  $ch^-$  strains that could not produce plaques. The  $ch^+$  type 1 strains were found to be type 1 by antigenic analysis, thermal stability, proportion of enveloped particles, and pock size on the chorioallantoic membrane. The observation is made that  $ch^-$  strains could not produce pocks on the chorioallantoic membrane. No quantitative data are given of the plaque and pock counts of wild type 1 strains: a considerable difference would be expected if  $ch^+$  variants form only a small proportion of the population.

Although the  $ch^+$  strains were not separable from the parental wild type virus by the classification features listed above, they were found to differ in their sensitivity to the DNA inhibitor 9- $\beta$ -D-arabino-furanosyladenine (ara-A). 5-iodo-2'-deoxyuridine has been shown to inhibit type 1 strains to a greater extent than type 2 strains (Nahmias and Dowdle, 1968) and this was confirmed by Lowry et al. (1971) for wild type virus; both  $ch^+$  and  $ch^-$  strains behaved as type 1 in this respect. Ara-A was found to be most effective against  $ch^-$  strains whereas  $ch^+$  and type 2 strains were relatively resistant. The corresponding sensitivity of wild type 1 strains was not recorded. This differential effect of ara-A was interpreted to indicate a difference in the enzymes of DNA synthesis of  $ch^+$  strains, compared to the wild and  $ch^-$  types.

Growth on the chorioallantoic membrane of the developing chick embryo

The earliest description of the growth of herpes simplex virus on the chorioallantoic membrane (CAM) was published by Dawson (1933) who also reported the histological changes associated with pock production. He used the HF strain of virus, propagated in rabbits, and inoculated the membranes of 12-14-day-old eggs with emulsified infected brain or brain fragments. Well-defined greyish opacities were detectable at 36 hr post-inoculation, when some lesions were more than 1 mm in diameter. Dawson also noted considerable variation in the effects of the virus on the embryo and the type of lesions produced. He recorded that some embryos died, especially if there was heavy involvement of the CAM, whereas others apparently hatched as healthy chicks. Similar observations were made by Shaffer and Enders (1939). Dawson (1933) noted that in some eggs lesions developed, then regressed and healed completely; one healed egg membrane was reinfected successfully. Anderson (1940) found that 9-19-day-old embryos could be infected, although older embryos did not show gross lesions. Shaffer and Enders (1939) working with a neurotropic strain of virus found that 7-9-day-old embryos did not develop pocks, but that 12-15-day-old embryos developed pocks of 0.5-1.0 mm diameter by 48 hr; these increased in size, and the central areas appeared necrotic by 72-96 hr. At 120 hr the lesions were 1.5 - 2.0 mm in diameter, but by 6-7 days, the lesions were more circumscribed and frequently appeared to resorb, a finding in agreement with the observations of Dawson (1933). Shaffer and Enders (1939) noted that the lesions on the CAM of 15-16-day-old embryos showed the same sequence of events, but the lesions developed more rapidly compared with younger embryos. No explanation of the

regression of the lesions is known, but since the chick hatches at about 21 days immunological competence will develop during the last week of incubation. These early reports were based on studies with laboratory strains HF and a neurotropic strain of unspecified origin: they must have been passaged many times, a procedure with a marked effect on the rate of development, the size of the pocks and their histology (Anderson, 1940; Coriell, Blank and Scott, 1949).

Histologically, Dawson described a spectrum of effects; at one extreme, the lesions showed a polymorphonuclear leukocyte infiltration beneath the CAM ectoderm, with marked hyperplasia of the ectoderm and a minimal degree of necrosis. Similar descriptions were given by Anderson (1940), Shaffer and Enders (1939) and Coriell et al. (1949). The virus strains tested by the last authors were all from sites usually associated with small pock producing viruses (or type 1 strains) and the histological findings are similar to those given by Nahmias et al. (1968b) as typical of oral or type 1 strains.

Dawson (1933) described lesions in which necrosis was a prominent feature; this was not associated with cellular hyperplasia. Shaffer and Enders (1939) described invasion of mesodermal tissues; Anderson (1940) made a similar observation and showed that if virus HF was propagated in the mouse brain and then transferred to the egg, the extent of ectodermal necrosis and mesodermal invasion increased with passage number, especially from the 25th passage. The necrotic, invasive lesion is associated with increased pock diameter, a fact amply confirmed by Nahmias et al. (1968b) who associated this type of pock with antigenic type 2 strains of virus.

Apart from laboratory strains, variation in pock diameter has

been reported by Schneeweis (1962b) and Hamar (1964), but an association between pock size and the clinical site was not established until 1967 by Parker and Banatvala although these authors quote an earlier personal communication from Barton (1965).

Nahmias et al. (1968b) inoculated virus isolates to the CAM of 10-12-day-old embryos and measured pock diameters after 3-4 days. They found that the virus strains could be divided into two groups; one produced pocks with an average diameter of less than 0.5 mm and another pocks larger than 0.5 mm. Greater differences can be demonstrated if incubation is extended to 7 days; Parker and Banatvala (1967) noted that the pocks of strains isolated from the genital tract continue to increase in diameter to an unspecified size, although Nahmias and Dowdle (1968) quote that this was 3-5 mm. The lesions become necrotic, whereas the pocks of oral strains are still less than 1.0 mm in diameter and show no necrosis. Sequential studies of the development of established type 1 pocks do not seem to have been published, although earlier results may indicate the possible course of events. Shaffer and Enders (1939) examined infected membranes at intervals up to 7 days and found histologically that the CAM lesions at 24-48 hr were characterised by extensive proliferation of ectodermal cells, but invasion of the mesoderm soon followed. At 4 days, fibroblastic invasion of the lesions was marked, and eventually by 7 days the lesions had almost disappeared or healed completely. A growth curve performed over the same period indicated that maximum virus titres were present at 96 hr, and declined steeply over the next 3 days. Taniguchi (1966) examined this problem in greater detail, and showed that the drop in virus titre between days 2-3 could be reduced by egg

passage. Cell proliferation in the ectodermal layer continued for a day or two longer despite the decreased virus production. The proliferating cells could not be shown to contain herpes simplex virus antigens by complement fixation or immunofluorescent studies, although dispersal of the cells with trypsin led to the reappearance of the virus. All the strains examined were probably antigenic type 1, and it is interesting that regression might explain the small diameter of type 1 pocks noted after 7 days incubation. At 3-4 days, Nahmias et al. (1968b) have recorded that type 2 pocks showed a marked infiltration of monocytes and polymorphs, whereas type 1 lesions contain only a mild monocyte infiltrate. As a measure of the extent of virus growth in these lesions, intranuclear inclusions and giant cells are common in type 2 lesions, but are rare in those of type 1. At this same time Shaffer and Enders (1939) noted that the strain they examined seldom produced inclusions; this observation suggests that it was a type 1 strain. On the other hand, Coriell et al. (1949) record that their strains, apparently all small pock or type 1 strains, produced homogeneous intranuclear inclusions in many ectodermal cells.

The recorded differences in the ability of type 1 and 2 strains to grow in chick embryo fibroblast culture are relevant to this problem (Figueroa and Rawls, 1969; Lowry et al., 1971). In addition, Nahmias et al. (1968b) record that 9 of 105 strains examined failed to grow on the CAM; four of these were type 1 and the remaining 5 were antigenic type 2 strains from genital sites of infection. Failure of growth of type 2 strains on chick embryo fibroblasts was not described by Lowry et al. (1971).

Chick embryo fibroblasts are of mesodermal origin, but the

failure of fresh type 1 isolates to complete replication in such cells cannot entirely explain the differences between type 1 and 2, in particular the cessation of growth or even the regression of the pocks of type 1 strains.

It is possible that the reported ability of inactivated type 1 virus (Duff and Rapp, 1973) to transform rodent embryonic cells could explain the ectodermal hyperplasia, which is reported to continue after virus growth declines (Taniguchi, 1966). In some manner, type 1 virus is inhibited from completing a productive cycle, and the stimulatory effect is revealed.

Whatever the explanation of the differential growth of the two types of virus, this appears to be a reliable and simple method of typing fresh isolates. To enhance the differences in size and appearance of the pocks, the prolonged incubation period of 7 days described by Parker and Banatvala (1967) appears most suitable.

#### Thermal stability

The study of Wallis, Ver and Melnick (1968) concerned the thermal stability of cell-free herpes simplex virus: the main conclusion was that the herpes virus was least stable in isotonic salt solution.

To explain the finding that type 2 strains gave lower yields of infectious virus from infected cultures, Plummer et al. (1968) compared the survival of the two types in serum-free medium and found that type 2 strains were more rapidly inactivated at 37°C. Ejercito et al. (1968) also examined both virus types, but failed to detect differences between the two types.

Caution would appear to be necessary in interpreting the



results of thermal inactivation studies.

#### Production of filaments within infected cells

In addition to the differences in the relative numbers of fully enveloped particles in the cytoplasm of type-2-infected cells, Schwartz and Roizman (1969b) noted that the nuclei contained numerous filaments or tubules. Similar structures had been observed earlier by Murphy, Harrison and Whitfield (1967) when they examined ultra-thin sections of mouse brain 3 days after intra-cerebral inoculation with the Rex strain of herpes simplex virus. Tubules were of two sizes: those with a diameter of 100 nm were associated with capsids and probably represented elongated capsomere assemblies. The smaller tubules had a diameter of 14 nm, and when cut in cross-section the spacing of adjacent filaments was 27 nm. The virus strain Rex was isolated from a skin infection and has been shown subsequently to belong to antigenic type 2.

Examination of choric-allantoic membranes infected with a known type 2 strain revealed almost identical filaments or microtubules (Couch and Nahmias, 1969) although in this instance, they were not confined to the nucleus. These observations have been confirmed (Dr. Isabel W. Smith, personal communication).

#### Pathogenicity in laboratory animals

Since the first reported isolation of herpes simplex virus on the rabbit cornea (Grüter, 1920), a considerable body of information has accumulated concerning the ability of the virus to infect a wide range of animal species. Rabbits, mice, rats, hamsters, guinea pigs,

cats, dogs and some primate species have all been shown to be susceptible to infection by a variety of routes, whereas sheep, chickens, pigeons and toads have been reported to be resistant to infection. To review these studies is outside the scope of this work, but Nahmias and Dowdle (1968) have provided a very complete summary.

Of greater relevance are some recent observations comparing the pathogenicity of type 1 and 2 strains for rabbits and mice. Plummer and Hackett (1966) studied the ability of two laboratory viruses, L2 and MS, to cause paralysis and encephalitis in rabbits and mice inoculated at different sites. These two viruses belong to types 1 and 2 respectively, and MS virus was found to cause an increased frequency of paralysis and encephalitis after inoculation to the leg. Alford, Snider and Stubbs (1967) investigated an isolate from a child infected soon after birth and showed that it was 100 times more virulent for mice than strains recovered from lip and eye infections. Nahmias et al. (1967) also reported a greater neurovirulence of genital strains after infection of the mouse cervix. Plummer et al. (1968) extended the study to newly isolated viruses, and found that type 2 strains were consistently more virulent than type 1 strains, although the latter could produce neurological involvement if sufficiently large inocula were administered. However, a subsequent publication (Plummer et al., 1970) noted that some virus strains of intermediate virulence had been found. Figueroa and Rawls (1969) concluded that 4 type-2 strains were on average eight times more virulent for 3-week-old mice than 4 type-1 strains when parallel titrations of virus suspensions were performed on rabbit kidney cell monolayers and by intra-cerebral inoculation. A recent study by Maasab and McFarland (1973) records that by adapting

viruses to grow at 25°C, there was a considerable reduction in neurovirulence in mice as assayed by intracerebral inoculation.



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SUMMARY OF ANTIGENIC AND BIOLOGICAL DIFFERENCES  
BETWEEN STRAINS OF HERPES SIMPLEX VIRUS

A considerable number of different characters have been described to distinguish type 1 and 2 strains of herpes simplex virus. As discussed, the reliability of some of these markers has not been fully established. The following list (Table 1) is derived largely from the published reports of Nahmias and Dowdle (1968), Figueroa and Rawls (1969) and Nahmias and Roizman (1973a). The differences are listed individually, but some may be related, especially the groups related to cytopathic effect, plaque size, and pock type; the yield of virus from infected cultures, thermal stability, proportion of enveloped particles, and particle to infectivity ratios, may form another set.

TABLE 1

Summary of features that can distinguish type 1 and type 2 strains of herpes simplex virus

Property	Antigenic type	
	Type 1	Type 2
1. Site of infection in man	non-genital	genital
2. Pathogenicity in rabbits or mice	less neurotropic	more neurotropic
3. Pock size on chorio-allantoic membrane	small	large
4. Histology of pock	ectodermal involvement with cell proliferation	all layers involved giant cells common
5. Chick embryo cells	very few plaques	plaques
6. Cytopathic effect in various cell types	rounding of cells	syncytia usually present
7. Plaque size	small	large
8. Virus yields from infected cultures	good	1 per cent. of type 1 yield
9. Ratio of particle to plaque-forming units	10 - 100:1	500 - 1000:1
10. Per cent. enveloped virus	30 - 40	5 - 10
11. Thermal stability	good	poor
12. Filaments in infected cells	absent	present
13. Incubation of cultures at 40°C	growth	reduced growth
14. Sensitivity to sulphated polyanions	less resistant	more resistant
15. Sensitivity to adenine arabinoside	less resistant	more resistant
16. Sensitivity to idoxuridine	less resistant	more resistant
17. Homology of viral DNAs	about 50 per cent.	

TABLE 1 contd.

18. Repetitive sequences of viral DNA	not present	present
19. Guanine + cytosine content of DNA	67 moles/100 ml	69 moles/100 ml
20. Type-specific glycoproteins	present	present
21. Thymidine kinase and perhaps other enzymes	specific	specific



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MATERIALS AND METHODS

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CELL CULTURES

BHK 21 cells (MacPherson and Stoker, 1962) were grown in either Eagle's MEM (Wellcome Reagents Ltd.) or Eagle's-based diploid cell medium (Grand Island Biological Corporation) containing per 100 ml: calf serum 10 ml; tryptose phosphate broth (Oxoid) 10 ml; 1.4 per cent. sodium bicarbonate solution 3 ml; penicillin  $2 \times 10^4$  units and streptomycin 0.02 g. Stock cultures were produced in 1.0-litre Roux flasks seeded with  $8-10 \times 10^6$  cells and harvested after 3 days. Cells were detached from the glass by decanting the medium, rinsing with Dulbecco's salt solution, and incubating at room temperature with 10 ml 0.1 per cent. trypsin for 2 min; in turn, the trypsin was decanted and incubation continued at  $37^\circ\text{C}$  until the cells began to detach from the glass, when they were pipetted into suspension in 10 ml fresh growth medium; a viable cell count was performed on a sample with trypan blue as a vital stain and cells were dispensed into fresh cultures. For virus inoculation or neutralisation tests, cells were cultured in "Pyrex" (15 cm x 1.2 cm) or neutral glass (10 cm x 1.2 cm) tubes seeded with  $10^5$  cells in 1 ml medium per tube. After static incubation overnight, tubes were rolled (1 revolution per 3 min.) at  $37^\circ\text{C}$ .

RK13 cells were grown in 199 medium (Wellcome Reagents Ltd. or Glaxo Laboratories Ltd.) containing per 100 ml: inactivated calf serum 10 ml; 1.4 per cent sodium bicarbonate 3 ml; penicillin  $2 \times 10^4$  units and streptomycin 0.02 g. Stock cultures were produced as described above and removed from the glass with 0.1 per cent. trypsin solution containing 0.02 per cent. versene.



### Cell preservation

Samples of both cell types were stored at  $-65^{\circ}\text{C}$  throughout the period of study in the appropriate growth medium containing 10 per cent. glycerol.

### Checks for contamination with mycoplasmas

Both cell types were checked for contamination with Mycoplasma species by Dr Sheila Stewart, Edinburgh City Hospital, and reported to be free of such organisms.

### Specimen transport medium

Skim milk (0.1 per cent.) or Hanks' balanced salt solution pH 7.2, which contained penicillin  $2 \times 10^4$  units and streptomycin 0.02 g per 100 ml, was used as transport medium for specimens with the exception of the eye specimens mentioned below: 2 ml volumes were dispensed in 6 ml glass vials (bijou bottles). Swabs on wooden applicator sticks were broken off into a bottle; scrapings were shaken into the medium from the collecting instrument.

The skim milk transport medium was used for the majority of specimens during the period of this study. In the later stages, however, unnoticed contamination of some outdated stocks held in outpatient departments led to a change to the Hanks' based transport medium.

If cell cultures for isolation studies were not available, specimens were stored at  $-65^{\circ}\text{C}$  until inoculated.

PATIENTS

Specimens were submitted from patients suffering from mouth, eye, skin and genital tract infections. The total time span of the studies reported was from 1964 to 1971, but specimens were not collected from all sites of infection throughout this time. The sources of specimens and the duration of the study periods are as follows.

Patients suffering from eye infections

Specimens were submitted from 93 patients attending the two Ophthalmology Units of the Royal Infirmary, Edinburgh. The great majority were collected during the years 1965-1966, from patients with corneal dendritic ulceration suspected to be herpetic in origin.

Patients suffering from oral infections

During the period of study, specimens from 201 patients were examined: these were obtained from the following sources:-

(i) Young children living in a new council housing estate and attending a general practice on the North side of Edinburgh during 1965-1967. The children were diagnosed as suffering from herpetic stomatitis and have been described elsewhere (Knox, 1967).

(ii) Children and adults attending the Periodontal Department of the Edinburgh Dental Hospital during the period 1967-1971. They were diagnosed as suffering from herpetic gingivostomatitis.

(iii) Patients from the South of Fife attending the Infectious Diseases Hospital, Cameron Bridge: The Royal Infirmary, Western General

and Leith Hospitals, Edinburgh and Edinburgh general practitioners: these specimens were received throughout the period of study.

Patients suffering from skin infections

Included in this category are patients diagnosed as suffering from herpes labialis, herpes facialis, infection of the skin of the limbs and trunk, and herpetic whitlow. 125 specimens were examined; the patients attended the hospitals listed above, and in addition some were seen in the Dermatology Department and the Nurses Sick Room, Royal Infirmary of Edinburgh.

Patients suffering from genital tract infections

Specimens were collected from 170 patients attending or referred to the Sexually Transmitted Diseases Clinics of the Royal Infirmary. These patients suffered from suspected herpetic infection or ulceration of the skin or mucous membranes of the genitalia.

**This study lasted from 1967 - 1971.**

### VIRUS ISOLATION

Initially, virus isolations were made in RK13 cells but later BHK21 cells were substituted. To isolate virus, 2 monolayer cultures in tubes were each inoculated with 0.2 ml of the specimen. The tubes were incubated at 35°C in a roller apparatus and the tubes examined daily for signs of cytopathic effect (CPE); this was usually evident within a few days, but occasionally passage to fresh monolayers was necessary. Identification was by neutralisation of approximately 100 TCID<sub>50</sub> of virus with sera provided by the Standards Laboratory, Central Public Health Laboratory, Colindale, or by a rabbit hyperimmune serum produced in Edinburgh as described below. Before passage, and neutralisation, viruses were harvested and released from infected cells by freezing to - 65°C and thawing before use. Virus isolates were stored at this temperature in growth medium in small glass bottles (Johnson and Jorgensen Ltd). In the early stages of the study, the technique described by Peutherer and Smith (1966) was applied to the isolation of virus from specimens collected from eye infections. In this method, the specimen of corneal scrapings or conjunctival swab, was collected directly into 1 ml growth medium containing antibiotics at the usual concentration, 1 ml medium containing 200,000 cells was added to the specimen, and after shaking for 20 min. at 37°C, the mixture was dispensed in 1-ml amounts to 2 tubes, incubated stationary overnight then rolled.

### Stock virus suspensions

Stock virus suspensions for infectivity and neutralisation tests, plaque morphology, electron microscopical examination and thermal

stability studies were grown in BHK 21 cells by passing the virus through a well-grown tube culture of cells, and after freezing and thawing, inoculating to an almost confluent cell sheet in a hexagonal Pyrex baby feeding bottle (250 ml). Virus was adsorbed to the drained cell sheet for 3-4 hr when the medium was replaced. CPE was usually complete at 24 hr and the virus was harvested as described and inoculated to a 1.0-litre Roux flask of cells. The infected cells were harvested into 10 ml of the existing growth medium after 24 hr by rolling off with glass beads, sonicated at full power for 5 min. in an MSE ultrasonic disintegrator and stored at  $-65^{\circ}\text{C}$  in small volumes.

DILUTION AND TITRE EXPRESSION CONVENTIONS

Throughout this thesis the following conventions are used:

- a) a 100-fold dilution is represented by the dilution factor  $10^2$ .
- b) a TCID<sub>50</sub> calculated by the method of Reed and Muench (1938) is expressed in the same way e.g. if the 50 per cent. end-point dilution of a virus suspension is  $10^{4.6}$  then the titre is  $10^{4.6}$  TCID<sub>50</sub>.
- c) serum neutralising antibody 50 per cent. end-points are expressed in the same way.
- d) both in neutralisation and complement fixation tests, the serum titre is expressed as the initial serum dilution used in the test, irrespective of any subsequent dilution resulting from addition of virus or complement or inoculation to the indicator system.

A more detailed explanation of the dilution and titre conventions is given in Appendix A.

ANTIGENIC TYPING OF VIRUS ISOLATES

Cells: BHK21 cells were used for all antigenic typing studies. Cells were used either on the same day they were removed from the stock culture bottles or after storing in growth medium for up to 5 days at 4°C.

A concentration of  $5 \times 10^5$  cells per ml was found to produce suitable cell sheets when inoculated in 0.02 ml-volumes to the micro-plates described below.

Plates: Either "Linbro" ISFB96, or "Cooke Microtiter" rigid polystyrene plates with 96 flat bottomed wells were used (Fig. 6). To remove toxic factors, plates were immersed in absolute alcohol for at least 30 min.; soaked in Hanks' salt solution for at least 30 min., rinsed in sterile, glass distilled water, shaken and dried at a distance of 12 inches from an ultraviolet tube in an inoculating hood. When dry, the plates were covered with metal lids and stored in the hood. The lids were cut to size from sheet aluminium, wrapped in Kraft paper and were sterilised in the hot air oven. Plates covered with lids could be stored for several weeks without contamination arising.

Droppers: Standard dropping pipettes were prepared immediately before use; they were drawn from lengths of sterile glass tubing and calibrated with a standard wire gauge to deliver 0.02 ml per drop.

Infectivity titration

The method for infectivity and neutralising antibody titrations is based on that of Pauls and Dowdle (1967). Tenfold dilutions of virus suspensions were prepared in growth medium in 6 ml screw-topped glass

vials. A 0.2-ml volume of stock virus suspension was added to 1.8 ml medium, and 0.2 ml of this dilution added to a further 1.8 ml growth medium with a fresh pipette. Dilutions from  $10^1$  to  $10^8$  were made: 0.02 ml of growth medium and 0.02 ml of cell suspension were added to each well of a sterilised plate and finally 0.02 ml of virus dilution was inoculated. Each of the virus dilutions was inoculated to 10 wells. The plates were sealed with overlapping strips of 1 inch Sellotape and incubated at  $35^{\circ}\text{C}$  for 3 days when each well was examined for the presence of CPE with a Leitz inverted microscope. A well showing any recognisable degree of CPE was scored as positive; 50 per cent. end-point dilutions and TCID<sub>50</sub> values were calculated from these observations by the method of Reed and Muench (1938).

#### Neutralisation test

Rabbit antisera were produced against reference type 1 and type 2 strains as described below. A 10-fold dilution of each serum was made in growth medium (0.2 ml serum + 1.8 ml medium) and inactivated at  $56^{\circ}\text{C}$  for 30 min. A series of doubling dilutions of each serum was made in the range 10 to 1280 in 0.9 ml growth medium in 6 ml glass bottles. A 0.1-ml volume of a virus dilution known to contain approximately  $10^3$  TCID<sub>50</sub>/0.02 ml was added to each serum dilution. The virus-serum mixtures were incubated at room temperature for 30 min, and then inoculated to plates: each well received 0.02 ml cell suspension and 0.04 ml virus-serum mixture. The plates were sealed, incubated and read as described above. Serum 50 per cent. end-point titres were calculated by the method of Reed and Muench (1938). An infectivity titration of the virus under test was



performed at the same time.

#### Calculation of pN values

Using the serum 50 per cent. end-point titre, a neutralising potency or pN value was calculated for each virus:serum neutralisation reaction according to the following formula, adopting the dilution conventions described earlier. The formula used was that of Pauls and Dowdle (1967), who derived it from St. Groth (1961).

The derivation of the formula is given in Appendix B; substituting the test volumes described, the formula reduces to:-

$$pN = \log (c - 1.16 - a) + t + 0.23$$

where  $c = \log_{10}$  (TCID<sub>50</sub> of virus control titration)

$a = \log_{10}$  (virus dilution used in neutralisation reaction)

$t = \log_{10}$  (serum 50 per cent. end-point dilution)

A pN1 value was obtained for each virus with a type 1 reference serum and a pN2 value with a type 2 serum by substituting the 3 values determined for the virus stock titre, virus test dilution and serum 50 per cent. end-point in the basic formula given above.

In some instances, a serum did not neutralise a virus; all such end-points were taken to be the least dilution tested (10) and a pN value calculated on this basis. The serum titre and pN value are listed as less than or equal to ( $\leq$ ) or greater than or equal to ( $\geq$ ) for the pN difference (pN1 - pN2).

#### Production of typing sera

##### Virus strains

HF virus was taken as a representative type 1 strain and MS as

a type 2 strain. Each was inoculated to the chorioallantoic membranes of developing chick embryos and after 3 days incubation, the membranes were removed and washed in saline; separate discrete pocks were then excised, washed in saline and placed in 1 ml of 0.1 per cent. skim milk. After freezing and thawing these preparations were inoculated to RK13 cell cultures in tubes. When the CPE was complete, the contents of the tube were frozen and thawed and inoculated to a hexagonal baby feeding bottle culture of RK13 cells. The cells were harvested to 10 ml when the CPE was complete, frozen and thawed and 1.0-litre Roux flask cultures of RK13 cells inoculated. After adsorption for 4 hr at 35°C, the cell sheets were washed twice with Dulbecco's salt solution and medium 199 added; in some preparations this contained 5 per cent. of the rabbit's own serum, but in other instances no serum was added. When CPE was complete (24-48 hr) the cells were brought into suspension with glass beads in 30 - 40 ml medium per Roux bottle. Each virus stock was titrated in microplates as described above.

Similar preparations of 1657 virus and an uninfected cell control antigen were produced.

#### Immunisation schedule

Three ml samples of the RK13 propagated virus either used neat or diluted in Hanks' salt solution were inoculated intramuscularly and intraperitoneally to rabbits of the department stock. Three weeks after the first inoculations the same procedure was repeated. One week after this the rabbits were bled out by cardiac puncture and the separated serum stored at - 30°C.

Virus strains HF, MS, 1657 and control uninfected cells were

inoculated to produce antisera.

Antigenic typing of unknown strains

Viruses were grown in 4 tube cultures of BHK cells and when the CPE was almost complete the contents of the tubes were frozen and thawed and pooled. A preliminary infectivity titration of this stock determined the dilution that contained 1000 TCID<sub>50</sub>/0.02 ml.

Reference serum dilutions were made and the test completed as described.

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## OTHER SEROLOGICAL TESTS

### Tube neutralisation test

This antibody assay was performed according to the method of Peutherer and Smith (1966). Doubling dilutions of inactivated serum were prepared in skim milk (0.4 ml) in 6 ml glass vials and 0.4 ml of a virus suspension added; this contained 100 TCID<sub>50</sub>/0.1 ml. Neutralisation was for 1 hr at 4°C when 3.2 ml of BHK21 cell suspension (containing 800,000 cells) was added, shaken for 20 min at 37°C and 1-ml volumes inoculated to each of 4 tubes. After static incubation overnight the tubes were rolled until the virus control tubes showed complete CPE when all the tubes were read. The serum titre was the highest dilution capable of reducing the viral CPE to 50 per cent. of the virus control CPE in at least 50 per cent. of the inoculated tubes.

### Complement fixing antibody titration

The method used follows that described in Smith et al. (1967).

The rabbit antisera produced as described above were titrated in full chessboard tests against viral antigen preparations.

### Viral complement fixing antigens

Antigens were grown in BHK21 cells in 1.0-litre Roux flasks. Cell sheets showing almost complete CPE were harvested to 5 ml distilled water per Roux flask, sonicated at full power for 5 min. in an MSE disintegrator, and ultracentrifuged at 32,600 G for 30 min. in a Spince model L ultracentrifuge in the SW39 or SW25 head. Antigen<sup>s</sup>

were made in this way for viruses HF, MS and 1657 and uninfected cells. The supernate was used as antigen, at dilutions varying from 4 to 128. Dilutions of inactivated serum were prepared in microtitre plates with standard droppers and loops (Cooke Engineering) with a volume of 0.025 ml. Complement (Wellcome Reagents Ltd) was used at a strength of 2HD50 as determined by a preliminary complement titration in the presence of the highest concentration of antigen (4). Fixation was overnight at 4°C, 2 per cent. sensitised sheep red blood cells added and the plates incubated with shaking for 30 min. at 37°C. Only wells showing 50 per cent. or more surviving red cells were scored as positive.

Antigens for use with human sera were prepared from infected RK13 cells. Early in the study titrations were performed in WHO perspex trays, with a unit volume of 0.1 ml. Serum dilutions were prepared in glass tubes before transfer to the plate. The test procedure was identical to that described above.

#### Neutralisation kinetic test

Virus assay Confluent BHK monolayers were established in unscratched tubes (Pyrex). Ten-fold dilutions of virus suspensions were made in skim milk and 0.1 ml- or 0.2 ml-volumes inoculated to 2 drained tubes per dilution. In some experiments the cell sheets were washed with Dulbecco's solution after adsorption of virus at either 4°C or 37°C. The medium was replaced and tubes were incubated at 37°C for varying times; in some experiments the tubes were rolled and in others they were kept stationary. The variations were introduced to establish the best conditions for the assay. The tubes were read by strong

oblique illumination against a black background with a binocular plate microscope. The objective magnification was 1.25x, eye piece 7x, giving an 8.75x total magnification. This gave a good depth of focus and field of view. Individual foci of infection were visible as bright clumps of cells or as small plaques. Titres were expressed as focus forming units per ml (f.f.u. per ml).

Neutralisation test The method was based on that of Nakano, Gelfand and Cole (1963). Dilutions of rabbit antisera were prepared in skim milk in sterile Wasserman tubes with metal caps. Serum dilutions and virus stock suspensions containing  $10^4 - 10^5$  focus forming units per ml were warmed to  $37^\circ\text{C}$  in a water bath, and equal volumes (0.4 ml) mixed. A virus control tube containing 0.4 ml virus suspension and 0.4 ml skim milk was prepared. 0.1-ml volumes were withdrawn with a 0.1 ml pipette from the virus control tube at time zero and at 7 min.: the virus-serum mixtures were sampled after 7 min. The samples were transferred immediately into 9.9 ml of cold Dulbecco's solution supplemented with 5 per cent. calf serum: these and a further 10-fold dilution were assayed by the method described. Each dilution was inoculated to 4 tubes.

Neutralisation rate constants (K values) were calculated from the formula

$$K = \frac{D}{t} 2.3 \log \frac{V_0}{V_t}$$

where D = dilution factor of the serum

t = time of sampling

$V_0$  = initial virus concentration\*

$V_t$  = virus concentration at time t

\*The virus control titre at time t was substituted for  $V_0$ .

### Serum fractionation

Sucrose solutions of 10, 20, 30 and 40 per cent. concentration (w/v) were made in phosphate-buffered saline pH 7.2. Gradients were prepared in 5 ml liner tubes for the Spinco SW50 head by pipetting 1.25 ml of the 40 per cent. sucrose solution into each liner, and then successively carefully layering 1.15 ml of the 30, 20 and 10 per cent. solutions on top of the previous solution, taking care to preserve the menisci. The gradients were then allowed to equilibrate overnight in a vibration-free refrigerator.

To fractionate a serum a 2-fold dilution was centrifuged at 1,000 G for 10 min. in a bench centrifuge and the clarified serum layered on top of the gradient - again preserving the meniscus with the top of the gradient. A bacteriological stab inoculating wire with the tip bent at a right angle was used to break the meniscus with the top of the gradient. The gradients were spun at 135,000 G for 16 hours in a Spinco model L ultracentrifuge.

A 0.5-ml. fraction was removed from the top of the gradient with a 1 ml tuberculin syringe and a needle with the tip bent at right angles with the bevel down. Fraction 1 was from the top of the gradient and the successive fractions were numbered 2, 3, 4 etc.

Fractionation of a serum known to contain the Paul-Bunnell heterophile agglutinin established that this antibody was located in fractions 7 and 8. Three gradients were spun for each serum. Fractions 4 and 5 were pooled and called IgG antibody: fractions 7 and 8 were taken as the IgM component of the serum. The samples were tested for antibody by complement fixation and neutralisation both as

collected and after three-fold concentration. Removal of water was achieved with polyethylene glycol; the samples were held in 1.25 cm dialysis tubing and dialysed overnight at 4°C against Dulbecco's solution after concentration.



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TUB SIZE



## BIOLOGICAL TYPING OF VIRUS ISOLATES

### Pock production

The chorioallantoic membranes of 10-12-day-old embryonated eggs were dropped by standard technique (Beveridge and Burnet, 1946). Shells were wiped with alcohol before the shell membrane was pierced. A 0.2-ml volume of virus diluted in skim milk was inoculated by syringe (1 ml); the opening in the shell was sealed with Sellotape, and the eggs were incubated at 37°C in a humidified atmosphere for 7 days. The eggs were chilled at 4°C for 1-2 hr, the membranes excised and fixed in formal-saline in petri dishes. The diameter of at least 6 discrete pocks was measured with a vernier hand lens held just above the membrane.

### Electron microscopy

Monolayer cultures of BHK cells in baby feeding bottles or 100 ml medical flats were inoculated with virus and incubated at 37°C. CPE was complete within 48 hr and the cells were removed with glass beads into Dulbecco's solution, centrifuged, fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in araldite, according to the method of Glauert (1965). An LKB1 microtome was used to cut ultrathin sections which were stained with uranyl acetate and lead citrate and viewed in an AEI EM6 electron microscope.

### Cytopathic Effect

Tube cultures of BHK21 cells were inoculated with sufficient virus to give several foci of infection per tube; the cultures were

examined unstained with the light microscope at a magnification of 30 - 50 times and the CPE recorded.

#### Plaque production

Plaques were produced under methocel by the method of Russell (1962). Ten-fold virus dilutions were prepared in skim milk and 0.2-ml samples added to  $3.5 \times 10^6$  BHK21 cells suspended in 1.8 ml Eagle's growth medium. After shaking for 20 min. at  $37^{\circ}\text{C}$ , the total volume of cell suspension and virus was transferred to a glass petri dish containing methocel overlay medium (Eagle's medium as described for cell culture, but containing methocel to give a final concentration of 0.75 per cent. in the inoculated dish). Petri dishes were incubated for 3 days at  $35^{\circ}\text{C}$  in an incubator flushed with 5 per cent.  $\text{CO}_2$  in air, the overlay sucked off and the cell sheet fixed and stained with 0.1 per cent. (w/v) methyl violet in formal-saline.

#### Thermal stability

Stock virus suspensions were prepared in BHK cells grown in 1.0-litre Roux flasks; ultrasonicated for 5 min. at full power in an MSE disintegrator and stored at  $-65^{\circ}\text{C}$ . Stock titres were within the range  $10^5 - 10^7$  focus forming units per ml.

One ml of virus suspension was added to 9 ml Eagle's growth medium supplemented as described, in a screw-topped universal container. Incubation was continued for 6 hr at  $4^{\circ}\text{C}$  in a refrigerator or at  $37^{\circ}\text{C}$  in a water bath when a 0.5 ml-sample was removed and diluted in 4.5 ml skim milk. This and further dilutions were titrated as described.



# Eden Grove Board

RESULTS

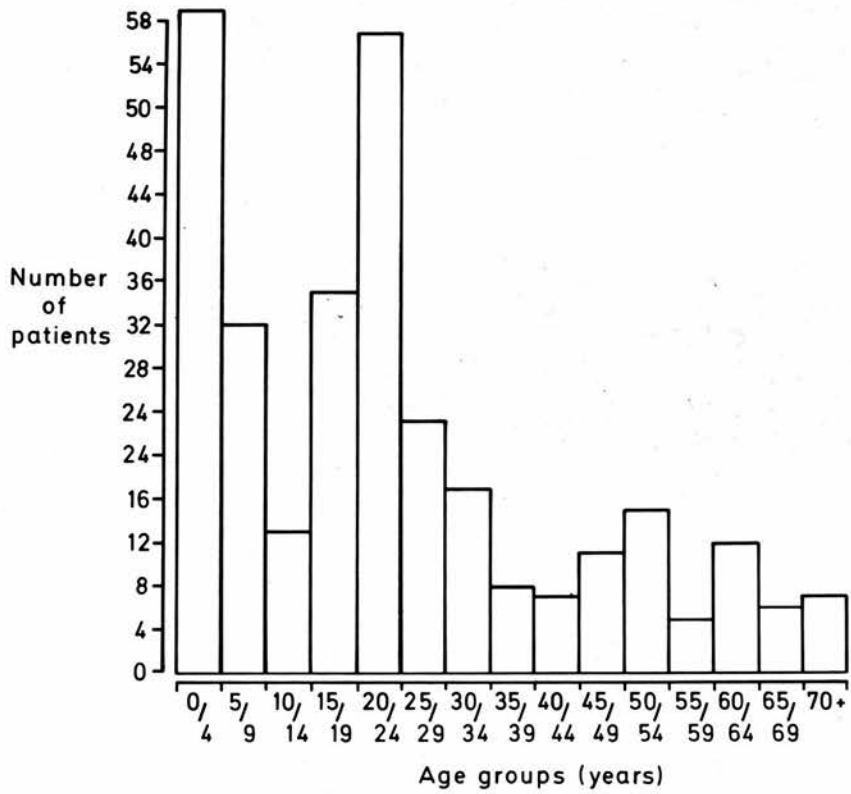
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### VIRUS ISOLATION STUDIES

Specimens from 589 patients were examined for herpes simplex virus during the period of study from 1964 to 1971. The patients were diagnosed as suffering from herpetic infections of the mouth, eye, skin or genital tract. In all, the virus was isolated from 307 patients, giving an isolation rate of 52 per cent. It is difficult to calculate a valid isolation rate from these data (see Discussion). The age distribution of the patients suffering from infections of the different sites is given in Table 2 and Fig. 2; although virus was isolated from patients of all ages, most strains were recovered from young children and young adults.

In the following sections, the recovery of virus from each of the major clinical sites of infection is described. The classification of sites of virus isolation is straightforward when considering such defined anatomical regions as the eye, or finger, but boundaries have to be defined when infections of the mouth, skin and genital tract are considered. As has been mentioned in the Introduction, the mouth is the commonest clinical site for primary herpetic infection - acute gingivostomatitis - and this may be associated with the development of vesicles on the lips and on the skin of the face around the mouth, on the chin, nose or cheek. However, it is claimed that recurrent intra-oral infection is rare, and by far the commonest site of recurrent infection is the external aspect of the lips, herpes labialis, and around the mouth - herpes febrilis or herpes facialis. Because they are apparently different clinical entities distinguishable by serological studies and by the severity of the illness, infections

**FIGURE 2.** Age distribution of 307 patients from whom herpes simplex virus was isolated.



of the lip have been separated from intra-oral infections, although the ages of presentation do coincide.

When considering the subdivision of infections of the skin and genital tract problems also arise. In addition to herpes labialis, herpes of the face is classified as a subdivision of the skin group. No attempt has been made to separate viruses isolated from the skin of the region between waist and knee although there are reasons for considering these sites as different from the rest of the skin as the virus type associated with this region is the same as that for the genital tract (Dowdle et al., 1967). The reason for not dividing skin infections on this basis is that only 4 isolations were made from this region.

Classification of infections as genital is straightforward in most instances but there may be confusion in female patients. In this study, external genital sites include any superficial infections of the labia and do not include lesions around the anus. This is anatomically correct although infections at such sites, or closely related areas of skin, are considered the province of the venereologist.

As the nursing profession was the main source of patients with whitlows they were considered as separate from infections of the hand, arm and skin of the trunk.

Thus the four main sites of infection considered are mouth, eye, genital tract and skin which includes finger, lip, face and skin of trunk and limbs.

#### Virus isolation

In the great majority of cases a viral cytopathic effect was

TABLE 2

Age distribution and site of infection of source patients and result of virus isolation studies of all specimens submitted for herpes simplex virus isolation

Age Group (years)	Number of specimens positive or negative for virus from the stated site of infection							
	Mouth		Skin		Eye		Genital Tract	
	Pos*	Neg <sup>+</sup>	Pos	Neg	Pos	Neg	Pos	Neg
0 - 4	47	12	12	25	0	0	0	0
5 - 9	21	8	9	7	2	0	0	0
10 - 14	8	5	5	6	0	1	0	0
15 - 19	16	26	6	8	5	0	8	23
20 - 24	15	18	13	6	4	0	25	35
25 - 29	3	5	2	4	3	6	15	14
30 - 34	2	3	2	2	4	2	9	12
35 - 39	2	1	1	2	2	1	3	6
40 - 44	0	2	0	0	5	2	2	3
45 - 49	0	2	3	1	6	2	2	5
50 - 54	1	1	0	2	14	4	0	3
55 - 59	1	0	1	1	3	3	0	3
60 - 64	0	1	3	0	9	4	0	0
65 - 69	0	0	0	0	4	1	2	0
70 - 74	0	1	1	1	3	0	0	0
75 - 79	0	0	0	1	2	0	0	0
80 +	0	0	0	1	1	0	0	0
<b>Totals</b>	116	85	58	67	67	26	66	104

\* Pos = herpes simplex virus isolated

+ Neg = no virus isolated

detectable within 48-72 hr incubation. The characteristic CPE consisted of rounded translucent cells with occasional ballooned cells and as discussed later syncytium formation was detectable with some strains of virus and could be used as a typing procedure. The cell substrate, rate of appearance and the features mentioned made the diagnosis almost certain, but the identity of all isolates was confirmed by neutralisation with specific antiserum.

Eden Grove

Bond

PUB SIZE

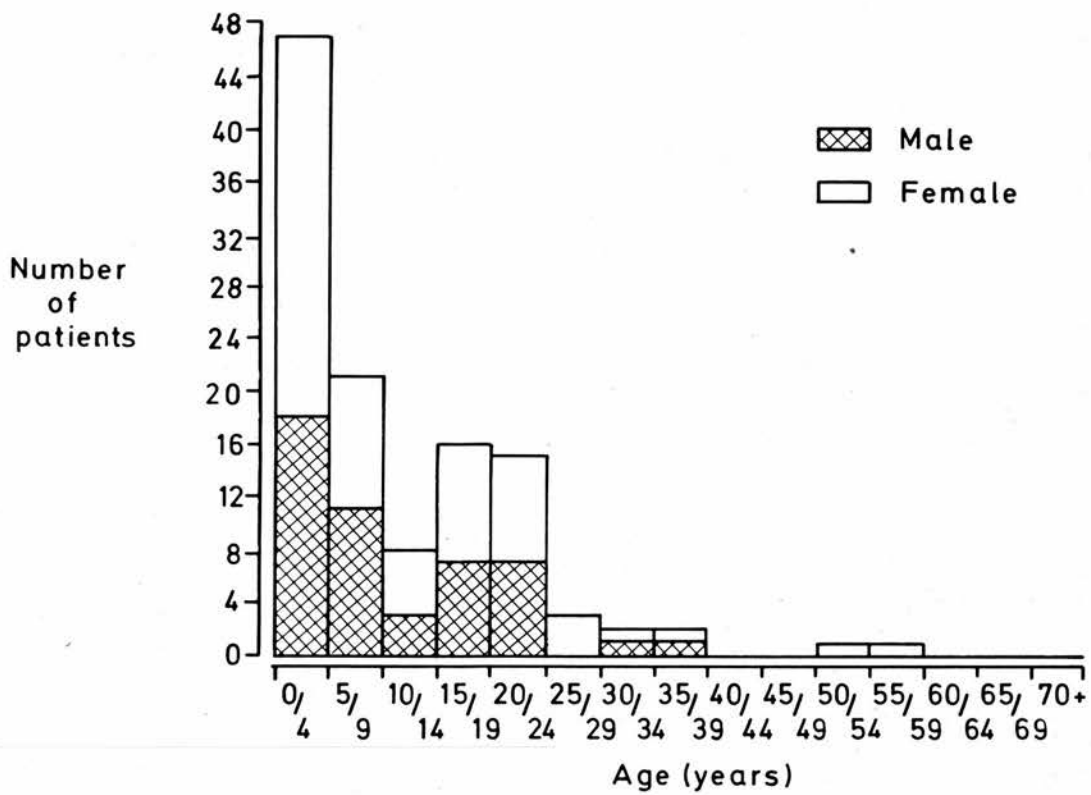


### Oral Infections

Herpes simplex virus was isolated from swabs or scrapings collected from the mouths of 116 of the 201 patients examined giving an isolation rate of 57.7 per cent. (Table 2). The age distribution of the patients is shown in Fig. 3. Most patients (47) were less than 4 years-old, of this group only three patients were aged under 1 year and the rest were evenly divided between ages 1 and 2 years. Virus was isolated from 21 patients aged 5 to 9 years and from only 8 patients aged between 10 and 14 years. More patients were seen in the next two age groups up to 24 years; analysis of the 15 - 19 year age group showed that there were only a few isolations from 15- and 16-year-olds. There were only 9 patients older than 25 years, but the virus was isolated from the intraoral lesions of a 54- and a 55-year-old patient.

Table 2 shows that there were more failures to isolate the virus from oral secretions in the young adult age group than among the younger patients. The isolation rate from children was 77 per cent. and only 41 per cent. for ages 15 - 24 years. Information about the duration of symptoms was scanty, but most isolations were made within one week of the onset of the illness.

The ratio of male:female patients was 48:68 or 1:1.43, and the age distribution of male and female patients is shown in Fig. 3. There was a shortage of boys among the 1- and 2-year-olds; no reason is known for this finding apart from the small size of the sample. This feature could also explain the failure to detect a significant seasonal variation (Table 3).



**FIGURE 3.** Oral infections: age and sex distribution of patients from whom herpes simplex virus was isolated.

Serological studies were performed by the complement fixation and neutralising antibody techniques described; applying the criteria of serological conversion, a negative acute-phase serum, or the ratio of complement fixing to neutralising antibody titres (Smith and Peutherer, unpublished results), 41 patients were found to be undergoing a primary infection at the time of virus isolation. Fifteen of the virus isolations from children under 10 years of age were from patients with serologically confirmed primary infections; of the remaining 6 patients examined, single serum specimens contained antibody. Over the age of 15 years, 26 primary infections were diagnosed among 36 patients tested. These patients were evenly distributed over the age group 17 - 24 years, and included two patients aged 27, one of 28, one of 34 and one of 54 years. The greater number of proven primary infections in the young adult group reflects the greater willingness of clinical colleagues to venepuncture older patients. Among the 10 patients who could not be shown to be undergoing primary infection, single sera from 3 patients contained antibody and another 3 patients showed a significant antibody response. Four others showed no change in titre, and on this evidence, these 4 patients could be suffering from an intra-oral recurrence of infection, but there was no report of lesions within the mouth.

In three patients in this study virus isolation from the mouth was associated with another clinical condition. These patients were (i) a five-year-old girl with no clinical evidence of stomatitis but probable coxsackie B2 meningitis, (ii) an eighteen-year-old girl with pericarditis and (iii) a thirty-six-year-old man suffering from Behçet's syndrome. Another patient, a twenty-seven-year-old girl admitted to

TABLE 3

Site of infection and month of isolation of 307 strains of  
herpes simplex virus

Month	Number of isolations made from the stated site of infection							Totals
	Mouth	Lip	Face	Skin	Finger	Eye	Genital Tract	
January	10	2	1	0	1	4	4	22
February	6	2	0	4	1	7	3	23
March	7	0	4	0	0	13	3	27
April	13	0	1	0	1	9	7	31
May	9	0	3	1	2	7	4	26
June	13	0	2	1	1	7	6	30
July	5	0	0	2	0	7	6	20
August	7	1	0	1	0	2	6	17
September	10	2	1	0	2	3	15	33
October	15	3	3	0	0	2	2	25
November	13	6	1	0	0	2	6	28
December	8	3	1	1	4	4	4	25
Totals	116	19	17	10	12	67	66	307

hospital with diabetic keto-acidosis was found to have vesicles on the throat; antibody studies showed that this was a primary infection. One other patient, aged thirty-seven years, apparently suffered from intra-oral infection following a dental extraction, and paired sera showed a rise in antibody titre.

The isolation of virus from young adults is of some interest as this form of infection is not so well recognised as the childhood type. The younger patients were mainly from an Edinburgh general practice whereas the older group were attending the Periodontal Department of the Edinburgh Dental Hospital. Clinically, the disease presented as a stomatitis with vesicles and ulcers in the mouth; in the younger patients involvement of the lips, chin and face was a regular feature.

No information was available concerning the socio-economic background of the older patients.

The age structure of the general practice from which most of the young children came has been described (Knox, 1967); the population density was high with a bias to the younger ages in that 1/3 of the population was under 12 years of age. Housing standards were good, this practice being centred on a large new council estate, although patients with disease were more often seen in large families. In a number of instances, infection could be shown to spread among siblings or playmates. Two of the older patients aged 17 and 24 years gave a history of contact with a boyfriend who suffered from cold sores shortly before the herpetic stomatitis developed.

### Skin Infections

One hundred and twenty-five specimens were examined from patients with infections of lip, finger, face and skin. Fifty-eight strains of virus were isolated, an overall isolation rate of 46.5 per cent. (Table 2). The patients were young children or young adults, although infection was detected over a wide age range up to 74 years: most isolations were from specimens collected within 7 days of the onset of symptoms. The overall ratio of male:female patients was 1:1.2 (Fig. 4A); females were in the majority in the 15 - 24 years age group (2.2:1), due to the inclusion of the nurses with finger infections. Table 4 shows the results subdivided by sites of infection.

#### Lip and facial skin infections

The isolation rates were 51 per cent. for lip and 61 per cent. for facial lesions; failure to isolate virus was commonest in children under 4 years of age. The ratio of male to female patients for the labial group was 1.1:1 and for the face infections 1.4:1. No obvious seasonal distribution of facial infections was found (Table 3). Although the numbers are small, the lip infections occurred mainly in the autumn and winter months.

Serum specimens were not available from all the herpes labialis patients and serological conversion was noted in only one case; this was a patient who had undergone renal transplantation, although reactivation and not primary infection is usually believed to occur. Of the other 8 patients investigated 6 were shown to have recurrent infections.

TABLE 4

Ages and clinical sites of infection of patients with herpetic lip, face, skin and finger infections

Age Group (years)	Number of isolations made from the stated site of infection									
	Lip		Face		Skin		Finger		Total	
	Pos*	Neg <sup>+</sup>	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
0 - 4	4	9	4	5	1	9	3	2	12	25
5 - 9	2	3	3	1	3	1	1	2	9	7
10 - 14	2	1	1	1	1	4	1	0	5	6
15 - 19	2	2	1	3	2	3	1	0	6	8
20 - 24	2	0	3	0	2	6	6	0	13	6
25 - 29	0	1	2	1	0	1	0	1	2	4
30 - 34	2	1	0	0	0	1	0	0	2	2
35 - 39	1	1	0	0	0	1	0	0	1	2
40 - 44	0	0	0	0	0	0	0	0	0	0
45 - 49	1	0	2	0	0	1	0	0	3	1
50 - 54	0	0	0	0	0	2	0	0	0	2
55 - 59	1	0	0	0	0	1	0	0	1	1
60 - 64	1	0	1	0	1	0	0	0	3	0
65 - 69	0	0	0	0	0	0	0	0	0	0
70 +	1	0	0	0	0	3	0	0	1	3
<b>Total</b>	<b>19</b>	<b>18</b>	<b>17</b>	<b>11</b>	<b>10</b>	<b>33</b>	<b>12</b>	<b>5</b>	<b>58</b>	<b>67</b>

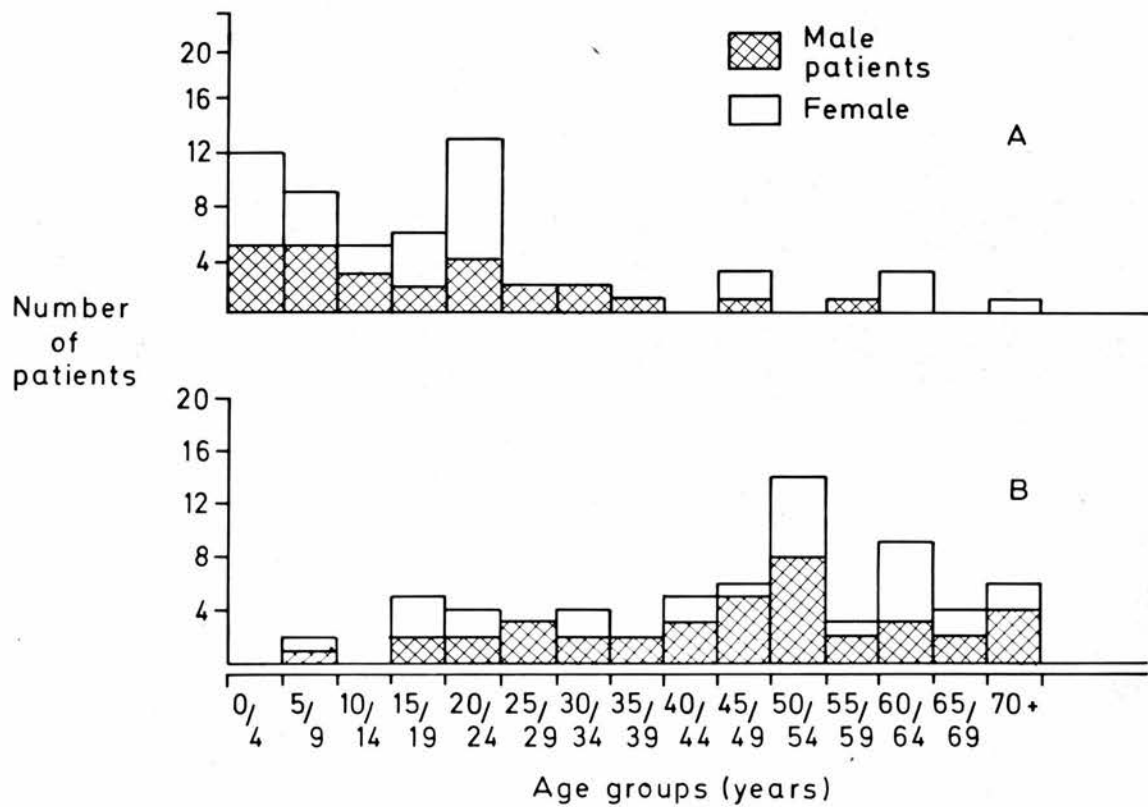
\* Pos = herpes simplex virus isolated

+ Neg = no virus isolated

**FIGURE 4.** Age and sex distribution of patients from whom herpes simplex virus was isolated.

**A** = patients with skin infections

**B** = patients with eye infections





Specimens were available from 9 herpes facialis patients and of these, 6 had titres suggesting recurrent infection; two were probably primary in nature in that no antibody was detected in single acute phase sera and one other patient, a 20-year-old male with a diagnosis of zoster, showed a serological conversion during the illness. Included in the group are (i) a 23-year-old female patient who developed herpes labialis during the treatment of primary syphilis; antibody studies indicated that this was of a recurrent type, (ii) a 21-year-old male patient who was suffering from hepato-renal failure following excessive exposure to lead in paint; during hospital treatment, a typical crop of recurrent herpetic vesicles developed on his cheek, (iii) a 45-year-old male who developed herpes labialis following renal transplant; (iv) a 47-year-old female with von Bickerstaff's benign mesencephalitis who suffered from a localised eruption on the cheek while in hospital; serologically this was recurrent in type.

#### Skin infections

The sites included in this group are the hand, limbs, neck, lower lumbar region, buttock and perianal skin. Only 10 virus isolations were made and no clear indication of age or seasonal distribution can be obtained. The isolation rate of 23 per cent., however, was the lowest recorded in this study, and was contributed to by a large number of negative specimens in the 0 - 4 years age group; this may reflect a greater diagnostic problem with vesicular skin eruptions in young children. True herpes simplex could be confused with a number of other non-herpetic skin diseases. The duration of the illnesses when the viruses were isolated was not different from any other site. Serological studies indicated that 3 of the patients,

a five-year-old girl, a 12-year-old boy and a girl of 21 years were experiencing a primary infection; the remaining 5 patients examined gave results in keeping with a recurrent type of infection.

Two young patients aged 5 and 12 years had scattered lesions on the trunk and limbs; both were primary infections, and the 5-year-old boy had stomatitis at the same time. Two of the patients had more localised lesions of the neck and buttock, and an 18-year-old girl developed perianal vesicles after a septic abortion. The patient with recurrent infection of the buttock was a nurse, and she had experienced several recurrences of a linear eruption associated with menstruation when the virus was isolated. Another patient, an 18-year-old girl, gave a history of herpes labialis two weeks before vesicular lesions developed around a cut on the dorsum of the hand; no blood samples were available.

#### Infection of fingers (herpetic whitlow)

Isolations from finger lesions were made either from young patients less than 3 years of age or from young adults aged 20 - 24 years. Twelve of the 17 specimens examined were positive, the majority of negative specimens coming from young children.

Among the 15 - 24-year-old patients there were 7 nurses working in the Royal Infirmary and Western General Hospitals, Edinburgh. The inclusion of the nurses accounts for the ratio of 3 male to 9 female patients in the whitlow group. In 6 of the 7 patients tested there was serological evidence that the infection was primary in nature; this was true of 5 of the 6 nurses tested. No samples of blood were collected from the children, although in one instance there was clinical evidence of stomatitis. One patient in

this group was seen eighteen months after the initial episode with a recurrence of infection at the same site.

### Eye Infections

During the two years of the study, 93 patients with eye infections were examined for the presence of herpes simplex virus: a total of 67 were found to be positive giving an isolation rate of 72 per cent. In this group the clinical entity is well defined and more information was available concerning the duration of symptoms, clinical severity at isolation, and in most cases both corneal scrapings and a conjunctival swab were submitted for examination.

Both conjunctival swabs and corneal scrapings were collected from the anaesthetised eye. Swabs had wooden sticks and were broken off into transport medium; cellular material was scraped from the edge of ulcers with an orange stick and this was broken off into transport medium.

The results of the isolation studies are shown in Table 5. Eighty-four pairs of specimens were examined, 42 were both positive, and 26 were both negative for virus. The finding that in 14 instances the virus was isolated from corneal scrapings but not from the conjunctiva suggests that the corneal scrape is the specimen of choice in the attempted virological diagnosis of dendritic corneal ulceration.

The age distribution of all patients, irrespective of whether this was a first or subsequent attack is shown in Fig. 4B. Fourteen of the 67 (21 per cent.), were aged between 50 - 54 years, and 36 (54 per cent.) were aged 45 - 69 years. Corneal infection had persisted for 2-3 weeks in more than a quarter of the patients before attendance at the Eye Outpatient Department.

The clinical severity of the corneal or dendritic ulceration

TABLE 5

Results of virus isolation from conjunctival swabs and corneal scrapings

Specimens examined and result	Number in category
Both swab and scrape positive for virus	42
Both swab and scrape negative for virus	26
Swab negative for virus Scrape positive for virus	14
Swab positive for virus Scrape negative for virus	2
Scrape positive for virus No swab submitted	9

at the time of attempted isolation was assessed on a grading I, II, III and IV by the examining ophthalmologist. Grade I cases showed the smallest lesions, with not more than two branches to the ulcer. Grade IV lesions were large with many fronds on the ulcer outline. As shown in Table 6, of the 46 patients for whom information was available, 33 were classified as grade I or II. The number of patients is small, but the more severe grades II and III were associated with a higher isolation rate of virus. There was no obvious association of clinical grade with age, although the 2 youngest patients in the study, aged 8 and 9 years, were placed in clinical grade IV.

Serum specimens were available from all but 6 patients. Antibody was detected in all the early sera, although some infections were of 2-3 weeks duration by the time the blood sample was withdrawn. However, on the basis of the complement fixing and neutralising antibody titres all sera were found to be of recurrent type, including the two young patients described above. Paired serum samples were available from 12 patients: eleven of these showed no change in antibody titre. One patient, a 17-year-old girl with a primary ocular infection showed a significant rise in both complement fixing and neutralising antibody titres, although the relative antibody titres indicated that this was not a true primary infection; the first serum sample was collected 10 days after the onset of infection, and both types of antibody were already present.

Information was scanty about the number of previous attacks, but 15 patients were said to have had previous episodes of infection, and 4 of these had experienced several recurrences. Five patients

TABLE 6

Clinical grading at time of attempted isolation of herpes simplex  
virus from patients with eye infections

Clinical grade	Number positive for virus	Number negative for virus	per cent. positive
I	15	8	65
II	18	5	78
III	8	2	80
IV	5	2	71
Totals	46	17	73

Grades I - IV allocated clinically depending on the size and number of branches of the ulcer.

Cases allocated to group I were the least severe, with not more than 2 branches of the ulcer.

Grade IV lesions were the largest, with many branches.

were definitely stated to be suffering from their first eye involvement, although from the serological evidence they had all been infected with herpes simplex virus prior to this. These incidents suggest, as does the age of the patients in general, that corneal infection may be acquired during a recurrence of infection at another site, perhaps aided by minor trauma to the eye. In only 3 instances in this study was there a record of concurrent cutaneous herpes simplex: two of these patients, the 8-year-old girl and a 22-year-old male had herpes facialis near the orbit at the time of diagnosis. The third patient, a 54-year-old female had recently been vaccinated, and suffered from herpes labialis at the same time as the dendritic ulcer.

A history of previous trauma was elicited in only one patient, a 53-year-old male, but this was complicated by the administration of corticosteroids after the injury. Another two patients had been treated with steroids before the diagnosis was made. Seven patients suffered from upper respiratory tract infections - "colds" or "flu" - before the ulcer developed. Perhaps reflecting this association is the finding that in both years of the study, most cases (43 per cent.) occurred during the months of February, March and April.

The overall male:female ratio was 1.39:1 and dividing this into patients under 40 years and over 40 years gave ratios of 1.5:1 and 1.35:1 respectively. Virologically confirmed corneal infection constituted 0.1 per cent. of all attendances at the eye department clinics during the study period.

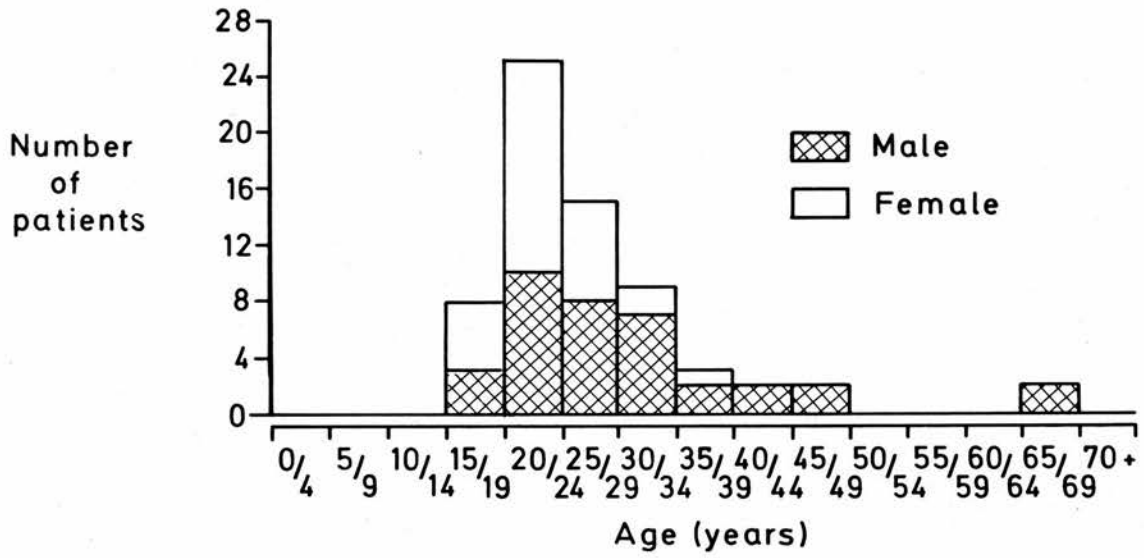


### Genital Tract Infections

Specimens from male and female patients with suspected herpetic infections were collected over a 5-year period. The patients were seen by the staff of the Sexually Transmitted Diseases Department of the Royal Infirmary of Edinburgh. A total of 170 patients were examined, and 66 strains of virus were isolated, an overall isolation rate of 38.8 per cent. The success of isolation was equal for male and female patients. Of the 24 patients with adequate histories, 10 or 42 per cent. had symptoms for more than 1 week before coming to the clinic. The age distribution of the patients is shown in Table 2, and those from whom virus was isolated are represented in Fig. 5. Female patients were aged from 15 to 49 years with a mean of 24 years, while the male patients were aged from 17 to 68 years with a mean of 30 years. The patients from whom virus was isolated show the same trend in that the mean ages of the female and male patients were 24 and 31 years respectively.

Virus was isolated from vesicular or ulcerated lesions of the skin or mucous membranes of the genital tract. With two exceptions, all isolations from male patients were from lesions of the penis, the glans being the site most frequently involved. The exceptions were (i) a patient with lesions on the scrotum and (ii) a 67-year-old male with suspected cytomegalovirus infection; herpes simplex virus was isolated from a urine sample.

The majority of virus isolations from female patients were from superficial external lesions of the labia or closely related areas: however, virus was also isolated from the cervix uteri. Cervical material was collected during routine vaginal examination by swabbing



**FIGURE 5.** Genital tract infections: age and sex distribution of patients from whom herpes simplex virus was isolated.

or scraping the external cervical region with a wooden spatula and placing the swab or scrapings in virology transport medium. Table 7 lists the patients from whom cervical and other specimens were received, and the outcome of virus isolation attempts.

Specimens from the cervix and another site in the genital tract were collected on the same day from 5 patients: virus was isolated from all specimens in 4 instances and from neither in the other case. In one other instance, herpes simplex virus was isolated from the cervix and from an ulcer on the mons pubis 15 months later.

Specimens from the cervix alone were received from 10 patients, and virus was isolated from 2. In 7 instances, no lesions of the lower genital tract were apparent, and a diagnosis of cervicitis or cervical erosion was recorded: no virus was isolated from any of these specimens.

As detailed in Table 8, virus was isolated on the same day from 2 separate specimens from 4 female patients with vulval infections: one of these patients had concurrent infection of the labia and inner aspect of thigh.

In 8 instances (5 female and 3 male patients) virus was cultured at intervals ranging from 1 day to 3 years 9 months after the initial isolation. In 7 cases the repeat isolation was from the same or similar clinical site, and in one female patient already described, isolation from the cervix was followed by isolation from an ulcer of the mons pubis 15 months later. Virus was isolated from 3 attacks of labial infection in a 22-year-old patient at intervals of 6 weeks and 5 weeks respectively after the initial episode; serological studies indicated that the first isolation was associated with a primary

TABLE 7

Clinical features of patients from whom specimens were  
collected from cervix uteri

Patient	Age (years)	Specimen number	Virus Isolation	Sites from which specimens collected
G4	22	6653	+	cervix
		6654	+	labium
G29	24	9290	+	introitus
		9291	+	cervix
G23	21	9007	+	cervix
		9008	+	ulcer at Bartholin's gland
G30	24	9793	+	cervix
		9794	+	vulva
		9795	+	perianal ulcer
G8	26	5423	+	cervix
		8003	+	ulcer of mons pubis 15 months later
G20	18	9541	+	cervix
G32	25	7637	+	cervix
G75	29	7546	-	cervix
		7547	-	healing lesion of introitus
G67	26	5483	-	cervix; cervicitis
G68	29	5516	-	cervix
G69	30	5472	-	cervix; cervical erosion
G70	20	5648	-	cervix; acute cervicitis
G71	22	6319	-	cervix; acute cervicitis
G72	18	6919	-	cervix; acute cervicitis; recurrent oral ulcers
G73	31	7807	-	cervix; cervicitis
G74	20	8182	-	cervix; ulceration of labium

+ = herpes simplex virus isolated

- = no virus isolated

TABLE 8

Patients from whom herpes simplex virus isolated: (a) from separate specimens collected on the same day and (b) at successive recurrences of infection

Patient	Sex	Age (years)	Specimen number	Date of specimen collection	Interval between isolations	Sites of infection
G7	F	24	5285 5286	22/5/68 "	0	vulva* "
G3	F	22	6587 6588	9/ 1/69 "	0	vulva* "
G26	F	23	8681 8682	11/ 3/70 "	0	labia* "
G35	F	26	9431 9434	16/ 2/71 "	0	labia* thigh
G39	F	38	7289 7365	31/ 3/69 10/ 4/69	10 days	vulva* "
G24	F	22	8730 8740 8771	11/ 3/70 28/ 4/70 12/ 6/70	6 weeks 5 weeks	labia* " "
G31	F	24	9810 0003	4/ 8/71 16/11/71	3 months	labia* "
G8	F	26	5423 8003	26/ 6/68 18/ 9/69	15 months	cervix mons pubis
G19	F	17	8790 0184	30/ 6/70 4/ 2/72	18 months	vulva* "
G61	M	36	9663 9692	2/ 6/71 3/ 6/71	1 day	penis "
G11	M	21	5179 5456	23/ 4/68 2/ 7/68	10 weeks	penis "
G62	M	40	4159 9864	12/12/67 6/ 9/71	3 years, 9 months	penis "

\* Virus isolation was not attempted from the cervix of any of these patients.

infection. The two patients from whom repeat isolations were made after one and 10 days were still suffering from the original clinical episode. A repeat isolation from a 24-year-old female patient followed 3 months after primary infection. In another case, a 21-year-old male, virus was re-isolated 10 weeks after the first episode, when there was still no antibody detectable in serum by either complement fixation or neutralisation tests. In the 3 other cases where serological evidence was available, the recurrences were associated with steady antibody levels or a recurrent pattern of antibody titres.

Serological studies showed that 13 of the 42 patients from whom paired sera were available had undergone serological conversion; the virus isolation therefore was related to a primary infection. There were four female and nine male patients; they were aged between 18 and 25 years, with the exception of a 39-year-old male patient.

Analysis of the month of presentation showed that 15 isolations were made from specimens collected in September. This is more than twice as many as in any other month and represents almost 23 per cent. of the total isolations. During the 3 months July, August and September 39 per cent. of all specimens, both positive and negative, were received. Experience over a number of years indicated that in Edinburgh more patients attend the clinics during these 3 months than at any other time of the year. No attempt was made to diagnose all herpetic infections in clinic attenders, but the number of positive isolations indicates that at least 1 in 300 were suffering from genital herpetic infection.

Infection of the central nervous system

In addition to the groups of patients described, specimens were submitted for virus isolation studies from 14 patients with a suspected diagnosis of herpes simplex encephalitis. Samples of cerebrospinal fluid from 6 of these patients, a brain biopsy and a post-mortem brain specimen did not yield virus. Serological studies detected a 16-fold rise in antibody titre in 1 of the 7 patients tested, and, although difficult to interpret, herpetic encephalitis was the probable diagnosis in this case; post-mortem histological examination of the brain did not confirm the diagnosis.

A similar increase in antibody titre was found in a 65-year-old female patient with a diagnosis of leptospiral meningitis.

ANTIGENIC TYPING OF HERPES SIMPLEX VIRUS

Standardisation of method

Cell concentration

Suspensions of BHK21 cells containing  $0.1 \times 10^6$ ,  $0.2 \times 10^6$ ,  $0.5 \times 10^6$  and  $2 \times 10^6$  viable cells per ml were prepared in growth medium and 0.02 ml of each suspension added to 1 well of a prepared microtitre plate. Two drops of growth medium were added, the wells were sealed with Sellotape and the plate was incubated at  $35^\circ$ . After 24 hours most cells had attached to the bottom of the wells and had stretched out to assume a typical fibroblast morphology. The pH of the bicarbonate buffered medium became distinctly alkaline in wells inoculated with low cell concentrations and subsequent cell growth was poor. Cell concentrations of  $0.5 \times 10^6$  and greater showed good cell growth. The plates were examined each day for 5 days; by the fourth day wells inoculated with suspensions containing  $0.5 \times 10^6$ ,  $1.0 \times 10^6$  and  $2.0 \times 10^6$  per ml were overgrown. To avoid difficulties in reading due to overgrowth of cells it was decided to use a concentration of  $0.5 \times 10^6$ /ml and to incubate plates for 3 days before reading.

Reproducibility of infectivity titrations

The reproducibility of virus infectivity titrations was tested by performing replicate titrations of stocks of viruses HF and MS grown in BHK cells. Fresh dilutions were prepared on each occasion, as dilution errors seemed to be a probable source of variation in titre. The method proved reproducible. With HF virus, the titres lay within the range  $10^{6.62} - 10^{7.00}$  TCID<sub>50</sub>/0.02 ml with a mean titre and



standard deviation of the mean of  $10^{6.81 \pm 0.17}$ . The titres of the MS virus stock fell within a range of  $10^{4.50} - 10^{5.16}$  TCID<sub>50</sub>/0.02 ml with a mean titre and standard deviation of the mean of  $10^{4.67 \pm 0.25}$ . The individual values are shown in Table 9. The method of Reed and Muench (1938) has been criticised because there is no method of deriving an estimate of error (Dougherty, 1964). The method has been retained in this study, as the typing procedure was basically that of Pauls and Dowdle (1967) who used this method of calculating 50 per cent. endpoints. In addition, the calculation of standard deviation of the mean values was used by Pauls and Dowdle (1967) to establish the reproducibility of the test. To allow comparison with their published results, the same procedure has been followed.

Fig. 6 shows the plates used for titrations and the layout for an infectivity estimation.

As an infectivity titration of each virus stock is performed before antigenic typing can be attempted, it was necessary to show that virus suspended in Eagle's growth medium was stable over a period of some days at  $-65^{\circ}\text{C}$ , and that the infectivity titre did not drop significantly on freezing and thawing. The results of such an experiment showed that over a period of 7 days the titre of a suspension of MS virus was virtually unchanged, the values recorded being  $10^{6.00}$ ,  $10^{6.00}$ ,  $10^{5.83}$  and  $10^{6.00}$  TCID<sub>50</sub>/0.02 ml after each cycle of freezing and thawing. This experiment was performed with MS virus as antigenic type 2 strains have been shown to inactivate more readily than type 1 strains. It can be concluded therefore that storage of virus stocks in the manner described did not lead to an excessive inactivation of virus.

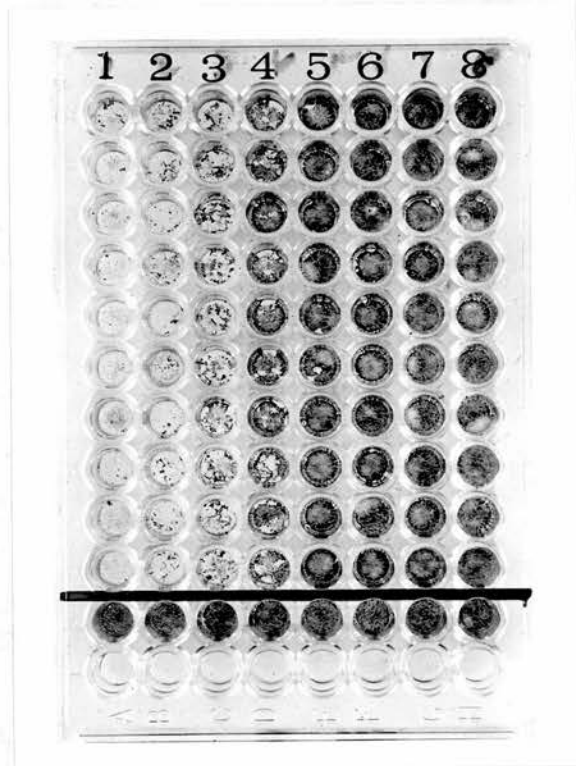
TABLE 9

Mean infectivity titres and standard deviation of the mean  
of 2 virus stocks titrated on 6 occasions

Virus	Observed titres*	Mean titre*	Standard deviation of the mean
HF	6.95 6.95 6.74 6.62 6.62 7.00	6.81	$\pm 0.17$
MS	5.16 4.50 4.58 4.56 4.65 4.55	4.67	$\pm 0.25$

\* Titres expressed as  $\log_{10}$  TCID<sub>50</sub>/0.02 ml.

Virus dilution ( $\log_{10}$ )



Uninfected  
cells

**FIGURE 6.** Virus infectivity titration performed in a microtitre plate.

This plate has been drained and the cell sheets stained with methyl violet. In routine use the cytopathic effect in each well was assessed by microscopical examination of unstained preparations.

X 0.75.

Variation of pN values with virus challenge dose

Antigenic typing of strains of herpes simplex virus by the microquantal technique depends on parallel neutralisation tests of the unknown strain against reference type 1 and type 2 antisera. As described by Pauls and Dowdle (1967), 0.90-ml doubling dilutions of the reference sera are made, and 0.1 ml of a dilution of the virus suspension added. The virus dilution used is determined by a preliminary infectivity titration and is selected to contain 1000 TCID<sub>50</sub>/0.02 ml. In any neutralisation test the observed serum titre depends on the challenge dose of virus and to allow for this variation the calculation of a pN or neutralising potency value for each serum-virus reaction was introduced by St. Groth (1961). To confirm the validity of this calculation two reference rabbit antisera were titrated against homologous virus challenge doses that varied by up to a factor of ten from the recommended value. The results are detailed in Table 10. With one serum (HF-H), the highest serum titre of  $10^{2.74}$  was obtained with the lowest virus inoculum ( $10^{2.0}$  TCID<sub>50</sub>/0.02 ml) and the lowest serum titre of  $10^{2.03}$  with the highest virus concentration ( $10^{4.0}$  TCID<sub>50</sub>/0.02 ml). The recommended  $10^{3.0}$  TCID<sub>50</sub>/0.02 ml) challenge dose of virus resulted in an intermediate value for the serum titre of  $10^{2.31}$ . Thus the serum titre was made to vary by a factor of 5. Calculation of the corresponding pN values gave a reduced variation, the lowest value recorded being 2.71 and the highest 2.94. Similar results were obtained with the other serum (HF-L); the serum titre varied from  $10^{1.13}$  -  $10^{1.67}$  (3-fold) whereas the corresponding pN values were in the range 1.92 - 2.09. As explained below the two sera tested were produced by

TABLE 10

Serum neutralisation titres and pN values of an HF-H and an HF-L serum determined with different concentrations of HF virus in the neutralisation reaction

Serum	Challenge virus titre in neutralisation reaction	Observed serum titre	pN value
HF-H(a)	2.0	2.74	2.89
	2.7	2.52	2.94
	3.0	2.31	2.80
	3.7	2.09	2.72
	4.0	2.03	2.71
HF-L(a)	2.63	1.67	2.09
	3.33	1.41	2.00
	3.63	1.39	2.03
	4.33	1.30	2.05
	4.63	1.13	1.92

Virus titres expressed as  $\log_{10}$  TCID<sub>50</sub>/0.02 ml.

Serum titres expressed as  $\log_{10}$ .

For explanation of serum designations see Table 15.

inoculation of different amounts of virus to rabbits. For these different sera, the calculation of pN values was equally effective in compensating for variation of the virus challenge dose.

The reproducibility of pN values

To establish the reproducibility of the titration technique 3 sera were titrated against their homologous viruses on 4 occasions, and mean pN and standard deviation of the mean values calculated. The results in Table 11 show that pN values were reproducible, the mean and standard deviation of the mean values being  $2.64 \pm 0.07$ ;  $2.68 \pm 0.28$  and  $2.58 \pm 0.20$ . The standard deviations are similar to those published by Pauls and Dowdle (1967).

Effect of varying the challenge dose of virus used in neutralisation tests on the resulting pN values with homologous and heterologous antisera

After demonstrating the validity of the pN calculation it seemed worthwhile to check the effect of varying the virus challenge dose inoculated in the neutralisation reaction on the pN values obtained with homologous and heterologous antisera, as this is the basic procedure in typing unknown isolates. The reference type 2 virus (MS) was reacted against HF-H and MS-L antisera, a pair of sera used subsequently in typing unknown virus isolates. Unknown virus strains are allocated to type 1 or 2 by subtracting the pN2 from the pN1 value ( $pN1 - pN2$ ). The effects of varying the MS virus challenge dose on the resulting pN values with the two reference sera and the calculated pN difference are shown in Table 12. With the lowest virus challenge, the pN1 and pN2 values were almost identical, but with increased virus doses, the heterologous (pN1)

TABLE 11

Reproducibility of pN values obtained in four replicate neutralisation tests made between each of three sera and the homologous viruses

Virus strain	Serum	pN values	Mean pN value	Standard deviation of the mean
1657	1657-H(b)	2.68 2.57 2.60 2.72	2.64	$\pm 0.07$
HF	HF-H (a)	2.62 2.32 2.84 2.95	2.68	$\pm 0.28$
MS	MS-L (a)	2.87 2.55 2.41 2.49	2.58	$\pm 0.20$

For explanation of the antiserum designations see Table 15.

TABLE 12

pN values of an HF-H and an MS-L pair of sera determined with different concentrations of MS virus in the neutralisation tests

MS virus challenge virus titre	Observed pN value with the indicated serum		pN1 - pN2
	HF-H(a) pN1	MS-L(a) pN2	
1.63	2.59	2.58	+ 0.01
2.63	2.35	2.45	- 0.10
3.63	1.82	2.12	- 0.30

Virus titres expressed as  $\log_{10}$  TCID<sub>50</sub>/0.02 ml.



value was less than the homologous (pN2) value. As a result, the pN differences were found to be + 0.01, - 0.10 and - 0.30. As will be seen later these values are all within the type 2 range, so that some variation of virus challenge dose from the recommended  $10^3$  TCID<sub>50</sub>/0.02 ml) can be accepted. The observations also suggest that the pN difference will be most typical with higher challenge doses rather than with lower doses of virus. MS virus was used in this experiment as it had been shown to react with both sera, whereas the type 1 reference strain (HF) was not neutralised by the heterologous serum (see below).

#### Reference antisera

#### Reference viruses

Reference type 1 and type 2 antisera are necessary for typing unknown strains of virus. MS virus (Gudnadottir et al., 1964) was selected as an established type 2 strain (Plummer, 1964; Pauls and Dowdle, 1967). HF virus originally isolated by Flexner and Amoss (1925b) was taken as the reference type 1 virus. The strain employed in this laboratory was obtained from the Institute of Virology, Glasgow. In addition to these two established laboratory viruses, a strain 1657, isolated in Edinburgh from a recurrent vesicular eruption on the buttock of a 5-year-old girl was inoculated into rabbits to produce antisera which were included in the preliminary testing of the method. This local strain of virus has been used to produce complement-fixing and neutralising antigen stocks which were applied in the antibody survey (Smith et al., 1967), described in the Introduction. It seemed worthwhile to establish the type of this

virus and to include another probable type 1 strain in the early experiments.

#### Production of antisera

To reproduce the typing method of Pauls and Dowdle (1967), rabbits were immunised with suspensions of each of the strains described above (one rabbit for each virus). The virus stocks were grown in RK13 cells and immunisation of rabbits was performed according to the schedule of Plummer (1964); Pauls and Dowdle (1967) also followed this procedure. The sera collected 4 weeks after initial inoculation were then tested for neutralising antibody by the method described above. The serum titres and pN values of the 3 sera against all 3 viruses are shown in Table 13. As expected from the previous results, the replicate determinations of pN values were in reasonable agreement. From the cross-reactions shown it appears that the antiserum raised against MS virus is specific; sera against HF and 1657 strains reacted with all 3 viruses, although the highest pN value was obtained with the homologous viruses. Calculation of the pN differences gave the values listed in Table 14. Allowing for the approximate figures quoted for viruses which were not neutralised by the anti-MS serum it is obvious that HF and MS can be separated by this method and that 1657 is the same type as HF. On the basis of this preliminary experiment, MS-like, or type 2, strains should give a pN difference of about 0 or less whereas HF-like, or type 1, strains give a positive pN difference. Dowdle et al. (1967) give similar values, although all their viruses appeared to be neutralised by both type 1 and 2 sera. From their results it was concluded that, in contrast to the findings described above, type 1 antisera were most

TABLE 13

Replicate neutralisation titres and pN values of 3 sera titrated  
against homologous and heterologous virus strains

Virus	Virus challenge dose* in neutralisation test	Observed titres, pN and mean pN values determined against indicated antiserum								
		HF			MS			1657		
		Titre <sup>φ</sup>	pN	Mean pN	Titre	pN	Mean pN	Titre	pN	Mean pN
HF	2.74	2.50	2.93	2.74	**	≤1.00	≤1.43	2.70	3.13	3.04
	2.62	2.15	2.54		≤1.00			≤1.39	2.55	
MS	2.58	1.83	2.21	2.37	2.04	2.42	2.50	2.30	2.68	2.83
	2.56	2.15	2.53		2.20	2.58		2.60	2.98	
1657	3.30	1.75	2.31	2.33	≤1.00	≤1.56	≤1.59	2.20	2.76	2.74
	3.55	1.73	2.34		≤1.00	≤1.61		2.10	2.71	

\* Virus titres expressed as  $\log_{10}$  TCID<sub>50</sub>/0.02 ml

<sup>φ</sup> Serum titres expressed as  $\log_{10}$

\*\* No neutralisation of virus with lowest dilution of serum tested; the pN value calculated for an assumed titre of 10.

TABLE 14

pN values and pN differences of virus strains HF, MS and 1657  
determined in neutralisation tests with antisera to HF and MS viruses

Virus	pN values determined with indicated antiserum		pN difference (pN1 - pN2)
	HF (pN1)	MS (pN2)	
HF	2.74	$\leq 1.41^*$	$\geq 1.33$
MS	2.37	2.50	- 0.13
1657	2.33	$\leq 1.59$	$\geq 0.74$

The pN values quoted in this table are the mean pN values calculated in Table 13.

\* No neutralisation detected with the lowest dilution of serum tested; pN values were calculated for an assumed titre of 10.

effective in typing unknown strains.

There was no obvious explanation for the observed reversal of specificity of the type 2 serum, as virus for immunisation had been grown in a similar manner and rabbits had been inoculated according to the same schedule. It was noted that no mention had been made of the amount of virus inoculated into the rabbits, and as it is known that type 2 strains are generally less thermostable than type 1 strains and are produced to lower titres than type 1 strains, it seemed possible that in this laboratory a low dose of MS virus could have been inoculated into the rabbits. A retrospective titration of the virus suspensions inoculated showed that this had happened. It was decided, therefore, to investigate the effect of varying the dose of virus inoculated on the specificity of the resulting antisera, and to see if the results of Pauls and Dowdle (1967) could be reproduced.

Effect of varying the dose of virus inoculated to rabbits to produce reference antisera

Fresh stocks of virus were produced in RK13 cells, harvested and prepared as described in Materials and Methods, and the infectivity determined by the titration technique described. On the basis of these preliminary results dilutions of each virus stock were prepared in sterile Hanks' salt solution, to give a final titre of less than  $10^{2.0}$  TCID<sub>50</sub>/0.02 ml. These dilutions and the original virus stocks were retitrated and then each was inoculated to two rabbits and the serum collected as described. Table 15 lists the titres of the virus suspensions at the time of first inoculation; the virus stocks were stored at  $-65^{\circ}$  for 3 weeks until the second inoculation.

The sera were designated H or high-dose if they were produced

TABLE 15

Titres of virus suspensions inoculated into rabbits to produce antisera to virus strains HF, MS and 1657. Titres estimated by micro-method and expressed as  $\log_{10}/0.02$  ml.

Virus inoculated	Inoculum Titre	Resulting antiserum designated
HF	3.40; 4.40	HF-H(a) and (b)
HF	1.40; 1.38	HF-L(a) and (b)
MS	5.68; 5.68	MS-H(a) and (b)
MS	1.50; 1.50	MS-L(a) and (b)
1657	4.09; 4.80	1657-H(a) and (b)
1657	1.80; 1.09	1657-L(a) and (b)

H = antiserum from rabbits inoculated with high dose of virus  
(range  $10^{3.40} - 10^{5.68}$ ).

L = antiserum from rabbits inoculated with low dose of virus  
(range  $10^{1.09} - 10^{1.80}$ ).

(a) and (b) are individual rabbits.

A control rabbit was inoculated with uninfected cell extract in serum-free medium.

by inoculation of virus suspensions with a titre of  $> 10^{3.0}$  TCID<sub>50</sub>/0.02 ml and "L" or low-dose if produced in response to virus preparations with a titre  $< 10^{2.0}$  TCID<sub>50</sub>/0.02 ml. In addition, a rabbit was immunised with a suspension of uninfected cells at the same concentration as in the high-dose virus stocks.

Each of the sera was titrated against the homologous and heterologous viruses on at least two separate occasions, and the serum titres and pN values calculated. The control serum produced against uninfected cells did not neutralise any of the viruses at the lowest dilution (10) tested.

#### High-dose (H) sera

Tables 16 and 18 list the serum titres and pN values of the high-dose sera tested against the immunising viruses. There was reasonable agreement between the serum titres and derived pN values for each of the pair of animals inoculated. All the sera neutralised the other virus strains tested, and there was little difference between homologous and heterologous titres. When the pN values were normalised by the usual convention, taking the homologous value as 100, all the other values were within the range 90 - 110 and this would be interpreted to mean that the viruses were closely related if not identical (McBride, 1959). The only exception, 1657-H against MS virus, had a value of 89, and this is near the limits expressed above. On the basis of these results, it seemed unlikely that sera produced in rabbits by inoculation of identical doses of virus would be of value in allocating unknown viruses to either type 1 or type 2.

#### Low-dose (L) sera

The relatively high degree of cross-reactivity noted above was

TABLE 16

Effect of the size of the dose of virus inoculated into rabbits on the neutralising titre and pN value of the resulting antiserum measured against different viruses. 1. Rabbits inoculated with high doses of virus

Serum	Neutralising titre and pN value of serum with indicated virus					
	HF		MS		1657	
	Titre	pN	Titre	pN	Titre	pN
HF-H(a)	2.06	2.68	1.85	2.32	1.79	2.41
HF-H(b)	2.26	2.72	2.11	2.53	2.06	2.61
MS-H(a)	2.00	2.59	2.01	2.46	2.03	2.55
MS-H(b)	2.17	2.63	2.44	2.85	2.20	2.73
1657-H(a)	2.03	2.67	1.63	2.18	2.04	2.64
1657-H(b)	2.10	2.57	2.25	2.66	2.24	2.79

Serum titres expressed as  $\log_{10}$

Each titre listed is the mean of at least 2 observations.



not seen with sera raised in response to low doses of immunising viruses (Tables 17 and 18). No neutralising antibody was detected in serum 1657-L(b) produced by inoculation of two doses of virus of titre  $10^{1.09}$  TCID<sub>50</sub>/0.02 ml, perhaps related to a threshold effect. In contrast, serum MS-L(b) neutralised all 3 test viruses although the homologous pN value was highest; the inoculum in this case had a titre of  $10^{1.50}$  TCID<sub>50</sub>/0.02 ml. The normalised value for HF virus relative to the homologous (MS) pN value is 64, and 66 for 1657 virus, indicating that serum MS-L(b) could be used to classify virus strains. Apart from 1657-L(a) the other low-dose sera only neutralised the homologous virus strain: both the HF-L and 1657-L(a) sera were of low titre. They would be very difficult to work with at a starting dilution of 10; an initial dilution of 5 could have been employed but would have doubled serum consumption.

An exception to the trend of high specificity but low titre is serum MS-L(a) with a neutralising titre in excess of 100 for MS virus, but without detectable neutralisation of HF or 1657 viruses at a dilution of 10. It is apparent that the low-dose MS antisera have high homologous neutralising titres and have greater specificity than the corresponding high-dose antisera. The neutralising titres against MS virus are of the same magnitude as with the high-dose sera. An explanation of this might lie in the reported greater neurovirulence of type 2 strains of virus (Plummer and Hackett, 1966), but no paralysis of the hind legs of the inoculated rabbits was noted.

#### Selection of reference typing sera

It was concluded that inoculation of virus suspensions with titres in the range  $10^{3.0}$  TCID<sub>50</sub>/0.02 ml did not produce sera with

TABLE 17

Effect of the size of the dose of virus inoculated into rabbits on the neutralising titre and pN value of the resulting antiserum measured against different viruses. 2. Rabbits inoculated with low doses of virus

Serum	Neutralising titre and pN value of serum with indicated virus					
	HF		MS		1657	
	Titre <sup>φ</sup>	pN	Titre	pN	Titre	pN
HF-L(a)	1.29	1.89	≤ 1.00*	≤ 1.48	≤ 1.00	≤ 1.61
HF-L(b)	1.37	1.56	≤ 1.00	≤ 1.26	≤ 1.00	≤ 1.66
MS-L(a)	≤ 1.02	≤ 1.31	2.10	2.58	≤ 1.00	≤ 1.61
MS-L(b)	1.29	1.76	2.34	2.76	1.29	1.82
1657-L(a)	1.07	1.47	≤ 1.00	≤ 1.34	≤ 1.06	≤ 1.72
1657-L(b)	≤ 1.00	≤ 1.60	≤ 1.00	≤ 1.48	≤ 1.07	≤ 1.60

φ Serum titres expressed as log<sub>10</sub>

\* No neutralisation detected with lowest dilution of serum tested: pN values were calculated for an assumed titre of 10.

Each titre listed is the mean of at least 2 observations.

TABLE 18

Mean pN values of high- and low-dose antisera determined in titrations  
against homologous and heterologous virus strains

Sera	Mean pN value* and normalised pN of serum with indicated virus					
	HF	NpN <sup>φ</sup>	MS	NpN	1657	NpN
HF-H	2.70	100	2.43	90	2.51	93
HF-L	1.73		≤ 1.37		≤ 1.64	
MS-H	2.61	98	2.66	100	2.64	99
MS-L	≤ 1.54		2.67		≤ 1.72	
1657-H	2.62	97	2.42	89	2.71	100
1657-L	≤ 1.54		≤ 1.41		≤ 1.66	

\* pN values are means of values listed in Tables 16 and 17.

<sup>φ</sup> NpN = normalised pN value. Calculated by taking the ratio of the heterologous pN to the homologous pN and multiplying by 100.

a suitable degree of specificity; but inoculation of very low-titre suspensions was unreliable in producing sera with useful neutralising titres, MS virus being an exception to this rule.

Sera HF-H(a) and MS-L(a) were selected as being most suitable for typing unknown isolates and the experiments reported later are concerned with these two sera.

Before the microneutralisation test was applied on a wide scale, the rabbit antisera were assayed by tube neutralisation and chess-board complement fixation tests to determine if the reference viruses could be distinguished by these methods.

The use of a quantal tube assay in the typing of strains of herpes simplex virus

The sera described in the previous section were each assayed by the neutralisation procedure described in Materials and Methods (Peutherer and Smith, 1966).

The results of the cross-neutralisation tests are listed in Table 19. In general the neutralising titres of the high-dose sera determined by the tube method were higher than the titres obtained by the micro-neutralisation procedure, although the extent of the increase varied, probably due to differences in virus challenge dose, and to the different incubation conditions during the neutralisation reaction. With the low-dose sera, the micro-neutralisation titres were higher than the tube titres perhaps due to the different conditions of the test. The lower avidity of the low-dose sera probably reduced their ability to combine with virus at 4°C, and hence titres were lower by this method.

With the exception of the MS-L sera, no significant differences

TABLE 19

Effect of the size of the dose of virus inoculated into rabbits on the neutralising titres of the resulting antisera measured against different viruses by a quantal assay in tubes

Serum	Neutralising titres and mean titres of antisera assayed against indicated viruses					
	HF	Mean titre	MS	Mean titre	1657	Mean titre
HF-H(a)	2.2*		2.2		1.9	
HF-H(b)	2.2	2.2	1.9	2.1	2.2	2.1
HF-L(a)	1.0		1.0		1.3	
HF-L(b)	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.2
MS-H(a)	≥ 2.5		≥ 2.5		2.5	
MS-H(b)	2.5	≥ 2.5	2.5	≥ 2.5	2.2	2.4
MS-L(a)	≤ 1.0		2.5		≤ 1.0	
MS-L(b)	1.3	≤ 1.2	2.5	2.5	1.0	≤ 1.0
1657-H(a)	≥ 2.5		2.2		2.5	
1657-H(b)	≥ 2.5	≥ 2.5	2.5	2.4	2.5	2.5
1657-L(a)	1.0		1.3		1.0	
1657-L(b)	≤ 1.0	≤ 1.0	≤ 1.0	≤ 1.2	≤ 1.0	≤ 1.0

\* Titres expressed as  $\log_{10}$

Titres estimated by the tube method described. Titre expressed as  $\geq 10^{2.5}$  if complete neutralisation recorded at highest serum dilution tested ( $10^{2.5}$ ).

Serum of rabbit immunised with uninfected RK13 cells did not neutralise any of the viruses.

( > 4 four-fold) in titres were detected by this method.

The use of complement fixation tests in the typing of strains of herpes simplex virus

To investigate this method, a representative serum from each type was titrated in a chessboard test against antigens prepared in BHK21 cells. A preliminary complement titration determined one-HD50 of complement and the optimal sensitising concentration of the horse anti-sheep cell serum, in the presence of the antigen. Antigen dilutions of 4-128 were tested in titrations against two-fold dilutions of inactivated serum with 2HD50 of complement and overnight fixation at 4°C. Serum end-points were taken as the highest dilution showing 50 per cent. or more fixation of complement.

The highest titres obtained for each serum are listed in Table 20; no significant difference ( > four-fold) in titre was found for any serum with the different antigens. The chessboard patterns illustrated in Fig. 7 show little variation, but calculation of the area of each histogram to give an estimate of the total complement fixation reaction resulted in the figures shown in Fig. 7. To calculate these values the estimate of the area of the homologous virus:serum reaction was taken as 100, and the heterologous values calculated according to the formula  $\frac{a_x}{a_h} \times 100$  where

$a_x$  = area of histogram of serum h with antigen x

$a_h$  = area of histogram of serum h with antigen h

By normal convention (McBride, 1959) values in the range 90 - 110 are accepted as indicating antigenic identity. On this basis, Fig. 7 shows that with high-dose sera all three viruses were almost in the

TABLE 20

Complement fixing antibody titres of rabbit antisera titrated  
against homologous viruses

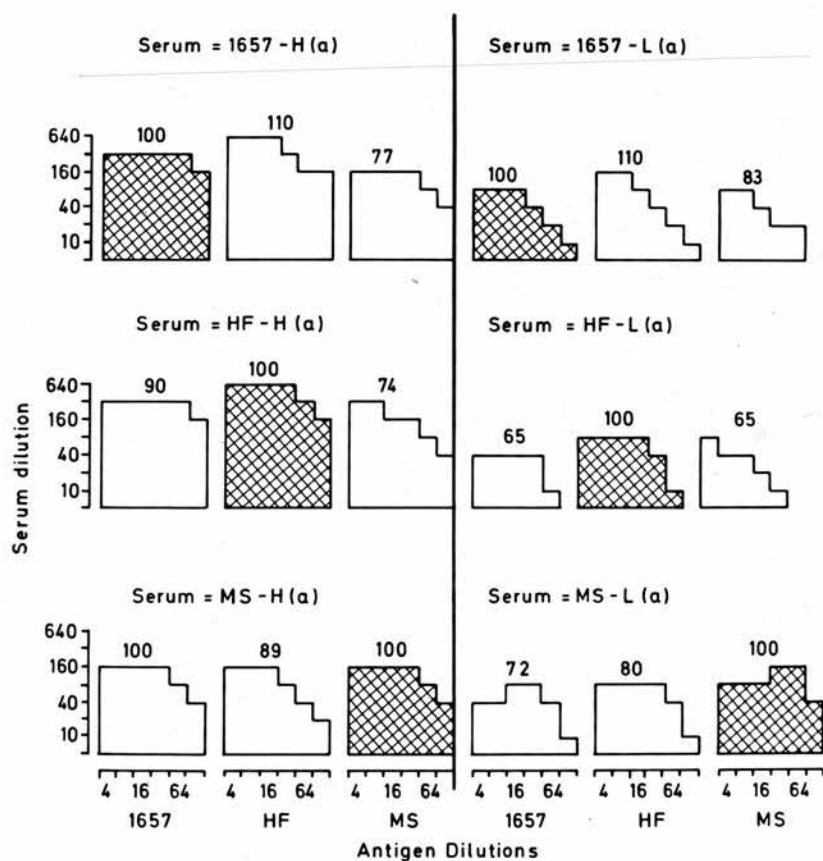
Serum	Complement fixing titres of serum assayed against indicated viruses		
	HF	MS	1657
HF-H(a)	2.8*	2.5	2.5
HF-L(a)	1.9	1.9	1.6
MS-H(a)	2.2	2.2	2.2
MS-L(a)	1.9	2.2	1.9
1657-H(a)	2.8	2.2	2.5
1657-L(a)	2.2	1.9	1.9

\* Serum titres expressed as  $\log_{10}$

An uninfected-cell antigen did not react with any of the sera.

**FIGURE 7.** Chessboard complement fixation reactions performed with high- and low-dose rabbit antisera and the corresponding virus antigens.

The figures above each histogram are a normalised value of the area of the histogram relative to the value for the homologous virus:serum reaction which is represented by the cross-hatched area.





identical range with the MS antiserum, whereas with the HF and 1657 antisera, MS virus was outside the normal limits. These results suggested that the high-dose sera against type 1 strains could be used for typing.

The results with the low-dose sera showed a greater degree of specificity than the high-dose sera; the HF-L(a) and MS-L(a) sera cross-reacted less than the 1657-L(a) serum. The reaction of the 1657-L(a) serum with 1657 and HF antigens indicated that they belong to the same type, although with the HF-L(a) serum, the two viruses were not identical. By complement fixation, serum MS-L(a) showed a greater degree of cross-reaction with heterologous virus than by either of the neutralisation assays. The results suggest that type-specific complement fixing sera could be produced.

Application of neutralisation rate studies to the typing of  
viruses

The sensitivity of the kinetic neutralisation test was investigated using some of the antisera already prepared. In addition, two of the sera were fractionated and IgG and IgM components tested for their ability to classify strains, following the report of Hampar et al. (1970) that late IgM rabbit antibody could be used to differentiate type 1 and type 2 strains of virus.

Tube focal assay

A focal assay of virus in monolayer cultures is used in most kinetic tests; to reduce the number of cells required, a method employing cells growing in standard cell culture tubes was investigated. The method was based on a method for counting rubella plaques in RK13

cell monolayers in tubes reported by McCarthy and Taylor-Robinson (1967); foci of infection were counted, unstained, by low-power microscopy. The foci were viewed by strong oblique illumination while the tube was held above a dark background. Initially RK13 cell monolayers were used, but later studies showed that BHK cells were suitable, and did not differ in sensitivity. A similar method was described by Farnham (1958).

A binocular plate microscope with a total magnification of 8.75x was found to be suitable. Because of the oblique lighting, surface marks and scratches on the tubes could obscure the cell sheet and for this reason, new or almost new tubes were used for these studies.

Virus 1657 was grown in RK13 cells, and the infected cells harvested into the culture medium. This stock was stored at  $-65^{\circ}\text{C}$  and used for the next series of experiments. Ten-fold dilutions of the virus suspension were prepared in skim milk by adding 0.2 ml to 1.8 ml. Virus titres are expressed as focus forming units (f.f.u.) per ml.

#### Investigation of the optimal assay conditions

In the first experiment, established monolayer cultures of RK13 cells in tubes were drained of medium and a 0.1-ml volume of virus dilution added to each of 8 tubes per dilution and the inoculated tubes rolled at  $35^{\circ}\text{C}$  for 4 hr to adsorb virus. At this time 3 procedures were adopted to complete incubation:

1. 2 ml of fresh medium was added and the tubes were incubated in a roller apparatus.
2. 2 ml of fresh medium was added and the tubes were incubated in

racks without rotation.

3. unadsorbed virus was removed by washing twice with 1 ml Dulbecco's solution; 2 ml fresh medium was added and the tubes were incubated in racks without rotation.

Between 28 and 56 hr post-infection, two tubes inoculated with each dilution were removed and counted. The results are given in Table 21. Throughout the experiment tubes treated by procedure 3 were easiest to read, with well defined foci of uniform size. Static incubation of the tubes obviously led to a slower rate of increase of plaque count due to reduced virus spread and secondary plaque formation. The slowest rate of increase was between 35 and 45 hr post-infection.

In a further experiment the effect of adsorbing the virus at 4°C was investigated, since considerable asynchrony of virus replication could arise during an adsorption period of 4 hr at 35°C, resulting in variation of plaque size and a steadily rising focus count with time post-infection. Virus dilutions were prepared as before, 14 tubes were inoculated at each dilution with a 0.1 ml volume of virus suspension, and virus adsorbed for 4 hr at 4°C. Each tube was then washed twice with 1 ml Dulbecco's solution, 2 ml fresh medium added and static incubation continued at 35°C for the time listed in Table 22 when two tubes of each dilution were removed and counted. The results indicated that the number of foci was relatively unchanged over a period from 34 - 42 hr post-infection. After this time, the count began to rise slowly due to secondary plaque formation; a finding in agreement with the results in Table 21.

TABLE 21

Effects of time and conditions of incubation on the number of foci of infection in tubes inoculated with dilutions of a virus stock (1657)

Incubation time (hours)	$\log_{10}$ dilution counted	Number of foci	Calculated titre*	Procedure
28	4	38; 38	$3.80 \times 10^6$	1
35	4	130; 148	$1.39 \times 10^7$	
45	5	40; 36	$3.80 \times 10^7$	
56	6	17; 40	$2.85 \times 10^8$	
28	4	44; 49	$4.65 \times 10^6$	2
35	4	123; 149	$1.36 \times 10^7$	
45	5	21; 34	$2.75 \times 10^7$	
56	5	37; 48	$4.25 \times 10^7$	
28	4	61; 50	$5.55 \times 10^6$	3
35	4	128; 113	$1.21 \times 10^7$	
45	4	192; 190	$1.91 \times 10^7$	
56	5	42; 33	$3.75 \times 10^7$	

RK13 cell monolayers in tubes were inoculated with a 0.1-ml volume of each virus dilution.

\* Titres expressed as focus forming units per ml.

TABLE 22

Effect of duration of incubation on focal counts produced by 1657  
virus inoculated to RK13 cells in tubes

Incubation time (hours)	$\log_{10}$ dilution counted	Number of foci	Calculated titre*
30	4	110; 169	$1.40 \times 10^7$
34	4	217; 266	$2.41 \times 10^7$
38	5	24; 24	$2.40 \times 10^7$
42	5	27; 28	$2.75 \times 10^7$
47	5	31; 34	$3.25 \times 10^7$
51	5	39; 52	$4.55 \times 10^7$
56	5	50; 58	$5.40 \times 10^7$

A 0.1-ml volume of each virus dilution was inoculated to each tube.

\* Titres expressed as focus forming units per ml.

During these experiments it was noted that 0.1 ml inocula did not always cover the entire cell sheet causing uneven distribution of foci and variation of the focus count. To avoid this complication, the inoculum volume was increased to 0.2 ml and the adsorption time was reduced to 2 hr at 4°C. The results are given in Table 23, and are in good agreement with those in Table 22. The titres observed were slightly higher than before, perhaps the more complete coverage of the cell sheet outweighing any decreased efficiency of adsorption due to the larger inoculum volume. The introduction of the 2 hr adsorption period made the method more adaptable, and did not appear to affect the titre. This procedure, with readings between 34 - 44 hr post-infection was used in the experiments reported below.

#### Neutralisation-kinetic studies

The tube focal assay was applied to the study of the rate of neutralisation of viruses 1657, HF and MS by the corresponding antisera. The technique used was based on that of Nakano *et al.* (1963) and employed a short neutralisation time of seven minutes, with initial viral titres in the range of  $10^{4.0} - 10^{5.0}$  (f.f.u.) per ml.

Preliminary tests established the optimal dilution of each serum for use in subsequent cross-neutralisation tests. The sera tested were 1657-H(a), HF-H(a) and MS-L(a) produced as described earlier. Ten-, 20- and 40-fold dilutions were prepared in skim milk and reacted with an equal volume of homologous virus suspension diluted to contain  $10^4 - 10^5$  ffu per ml. The serum dilution selected for further study was the highest dilution capable of reducing the homologous virus titre by  $1.5 \log_{10}$  during the seven minute neutralisation reaction. Both the 1657 and MS antisera were used at

TABLE 23

Effect of increasing the volume inoculated and reducing the adsorption  
time on the resulting focus counts in RK13 monolayers in tubes  
infected with 1657 virus

Incubation time (hours)	$\log_{10}$ dilution counted	Number of foci	Calculated titre*
24	4.7	147: 156	$3.79 \times 10^7$
29	4.7	141: 127	$3.35 \times 10^7$
35	4.7	198: 168	$4.58 \times 10^7$
43	4.7	183: 209	$4.90 \times 10^7$
46	4.7	229: 197	$5.33 \times 10^7$

A 0.2-ml volume of each virus dilution was inoculated to each tube.

\* Titres expressed as focus forming units per ml.

a dilution of 10 and HF serum at a dilution of 20.

The results of a series of cross-neutralisation tests are given in Table 24; in each case, the homologous virus was neutralised at the greatest rate although the distinction was least with the MS antiserum.

Neutralisation rate constant (K) values were calculated for each virus: serum reaction according to the standard formula, and the values are given in Table 25. On the basis of these results, and accepting the normal convention it can be concluded that the 3 strains tested are distinct, although the results show that 1657 and HF viruses are related. Despite this apparent sensitivity in separating strains, it can be argued that the method is too sensitive for epidemiological studies where the definition of antigenic groupings is most useful. Before abandoning this method, it was applied along with the techniques of micro-neutralisation and complement fixation, to the study of the IgM immunoglobulin fractions derived from two of the sera described earlier.

The use of IgM fractions of rabbit antisera in the typing of  
strains of herpes simplex virus

Serum fractionation

The sera fractionated were HF-H(b), previously described in Table 16, and MS-L(a). A preliminary kinetic neutralisation test established that the percentages of virus surviving after seven min. neutralisation at 37°C were in close agreement with the previous results. A 10-fold dilution of the anti-MS serum reduced the homologous virus titre to 19 per cent. and HF virus titre to 67 per cent. of the original; the HF-H(b) serum proved comparable to the HF-H(a)



TABLE 24

Results of cross-neutralisation kinetic tests with antisera to 1657, HF and MS strains

against the corresponding viruses

Virus	Initial virus titre	Control virus titre at 7 min.	Surviving virus titres and percentage survival at 7 min. with indicated serum		
			HF	MS	1657
HF	$1.2 \times 10^5$	$1.0 \times 10^5$	$8.3 \times 10^3$ (8.3)*	$5.6 \times 10^4$ (56)*	$2.1 \times 10^4$ (21)*
MS	$2.1 \times 10^4$	$2.0 \times 10^4$	$1.5 \times 10^4$ (75)	$4.7 \times 10^3$ (24)	$1.3 \times 10^4$ (65)
1657	$5.6 \times 10^4$	$6.3 \times 10^4$	$1.4 \times 10^4$ (22)	$4.7 \times 10^4$ (75)	$1.8 \times 10^3$ (2.9)

\* Percent. virus surviving calculated according to formula:

$$\frac{\text{Titre at time 7 min.}}{\text{Virus control titre at 7 min.}} \times 100$$

TABLE 25

Neutralisation rate constants and normalised values of antisera to 1657, HF and MS strains determined against the corresponding viruses

Antiserum	Neutralisation rate constant (K) and normalised (NK) values against indicated virus					
	HF		MS		1657	
	K	NK	K	NK	K	NK
HF-H(a)	7.11	100	0.83	12	4.28	60
MS-L(a)	0.83	40	2.06	100	0.42	20
1657-H(a)	2.23	44	0.62	12	5.08	100

Neutralisation rate constant (K) values calculated according to formula given in Materials and Methods.

Normalised K values (NK) calculated by taking homologous K value as 100 and expressing heterologous values as a proportion of this.

serum in that a 20-fold dilution reduced the homologous virus titre to 4 per cent. and the MS virus titre to 77 per cent. of the original.

These two sera were layered on top of sucrose density gradients and fractionated as described in Materials and Methods. Previous experiments had established the locations of the IgG and IgM antibody peaks in the gradient, and in the preparations used here, fractions 4 and 5 from the three gradients of a single ultracentrifuge run were pooled and designated the IgG fraction; fractions 7 and 8 were pooled and called the IgM fraction. At this stage the two preparations were tested against the corresponding viruses by the micro-neutralisation method but only the IgG fraction of serum HF-H(b) was found to possess low neutralising ability against HF virus. For this reason, the pooled serum fractions were concentrated from the starting volume of 3 ml to 1 ml.

#### Assay of IgM fractions by neutralisation-kinetic tests

Neutralisation rate studies were performed; in this instance, the serum fractions were not diluted in setting up the neutralisation reactions. The ability of the IgM serum fraction to reduce the test virus titre was found to be greatly reduced compared with the whole serum. The HF-H(b) serum yielded an IgM component which was able to reduce the homologous virus to 50 per cent. and MS virus to 73 per cent. of the virus control titres. The MS-L(a) serum IgM component reduced HF and MS viruses to 54 and 59 per cent. of the control titres respectively. On a  $\log_{10}$  scale, a 50 per cent. reduction in virus titre is barely outwith the experimental titration error.

#### Assay of serum fractions by complement fixation

The concentrated serum fractions were tested against the BHK

complement fixing viral antigens by the standard technique using 2HD50 complement. The dose of complement necessary for each test was determined by titration of complement in the presence of a 10-fold dilution of each serum fraction: IgM fractions required higher doses of complement than IgG fractions. A single antigen dilution of 4 was used throughout as earlier studies with serum fractions (Smith and Peutherer, unpublished results) had shown that high antigen concentrations were necessary to demonstrate complement fixation with serum IgM fractions. The results of these tests are given in Table 26. An obvious problem was that the overall recovery of antibody appeared to be low. As with the whole sera no significant differences in titre were found.

From these studies it was concluded that the kinetic neutralisation test might be too sensitive when applied to a large group of virus strains, and that no advantage was gained by using serum IgM fractions instead of whole sera.

It was decided therefore to adopt the Pauls and Dowdle (1967) method modified as described earlier, using HF-H and MS-L reference antisera. Before proceeding with the attempted typing of unknown virus strains, the duration and temperature of incubation of the neutralisation reaction were investigated.

Effect of varying the time and temperature of incubation of the neutralisation reaction on the resulting pN values

For these experiments the neutralisation reactions were set up in large volumes in universal glass containers. Nine-ml doubling dilutions were prepared from 10 to 1280 and a 1-ml volume of virus

TABLE 26

Complement fixing antibody titres of two rabbit sera and their IgG  
and IgM fractions determined with the corresponding antigens

Serum	Serum Fraction	Complement fixing titre with indicated antigen		
		HF	MS	Control
HF-H(b)	Whole	2.8*	2.5	≤ 1.0
	IgG	2.2	1.9	1.0
	IgM	1.9	1.6	1.3
MS-L(a)	Whole	1.9	2.2	≤ 1.0
	IgG	1.6	1.6	1.0
	IgM	1.0	1.0	≤ 1.0

\* Titres expressed as  $\log_{10}$

dilution was added to each: a series prepared in this way was then incubated at 4°, 22° and 37°C and sampled after 30 min., 3, 5 and 9 hr incubation by direct inoculation of prepared plates. A single dilution series was used rather than replicate dilutions to obtain a more accurate estimate of the effects of the temperature and duration of incubation. The dilutions were prepared in growth medium and over the duration of the experiments, the pH of the medium rose: the extent of the rise was uniform in both serum and virus dilutions.

A pair of high-dose sera HF-H(a) and MS-H(a), and a pair of low-dose sera HF-L(a) and MS-L(a) were tested in this way. The results of these experiments are represented graphically in Fig. 8 and Fig. 9 respectively.

It was observed that even after 9 - 10 hr at 37°C the virus control titres had not decreased to a significant extent. In one experiment, the HF virus control titre was  $10^{5.44}$  TCID<sub>50</sub>/0.02 ml after 30 min. at 37°C and this declined to  $10^{4.71}$  TCID<sub>50</sub>/0.02 ml after nine and a half hr. Similarly the MS virus control titre did not drop significantly: the decrease recorded in one experiment being from  $10^{6.00}$  TCID<sub>50</sub>/0.02 ml to  $10^{5.83}$  TCID<sub>50</sub>/0.02 ml despite the reported greater thermal lability of type 2 strains. Similar observations were made with the virus control titrations at 22°C and 4°C. A significant decrease would probably have been recorded if a focal assay of virus had been employed as it was noticed that the degree of CPE detected in wells at or near the end-point did diminish over the period of the experiment; in a quantal assay a just detectable and a complete CPE are both scored as positive in calculating the virus titre.

In Fig. 8 and Fig. 9, pN values for a particular virus are graphed together so that the pN difference and hence the relative discriminating power of the sera could be assessed. All homologous pN values were greater than heterologous with the single exception of the high-dose sera incubated at 37°C; the values with both viruses were almost equal but are reversed after 3 hr and 9 hr incubation. With the high-dose sera the pN values were highest after incubation at 37°C; the values obtained at 22°C were slightly higher than those at 4°C. Increasing the time allowed for neutralisation resulted in an increased pN value, although with the exception of the 37°C experiment, there was no tendency for the pN difference to diminish.

The results obtained with the low-dose antisera are illustrated in Fig. 9. The low-dose sera were found to be relatively type specific and under certain conditions they did not neutralise the heterologous virus; sera showing no neutralisation were assumed to have a titre of 10 and the pN values calculated accordingly. As with the high-dose sera the pN values were highest after incubation at 37°C and neutralisation of the heterologous virus was detectable sooner at this temperature than at 22°C or 4°C. Incubation at 4°C produced least cross-reaction even after several hours of neutralisation.

It was concluded that incubation of the neutralisation reaction at 37°C produced the highest pN values, but the greatest degree of cross-reaction; only small differences were found between the values obtained at 22°C and 4°C over neutralisation periods up to 3 hr. The basic conditions of the test as described by Pauls and

Dowdle (1967) were not altered.

Comparison of the pN values obtained for each virus against one serum showed that the high-dose MS serum reacted equally well with both HF and MS viruses, whereas the high-dose HF serum showed a greater difference between HF and MS viruses. The same general trend was seen with the low-dose sera; neutralisation of the heterologous virus was detected sooner with the MS serum compared with the results obtained with the HF serum. These observations are in general agreement with the reports of Schneeweis (1962a) and Pauls and Dowdle (1967).

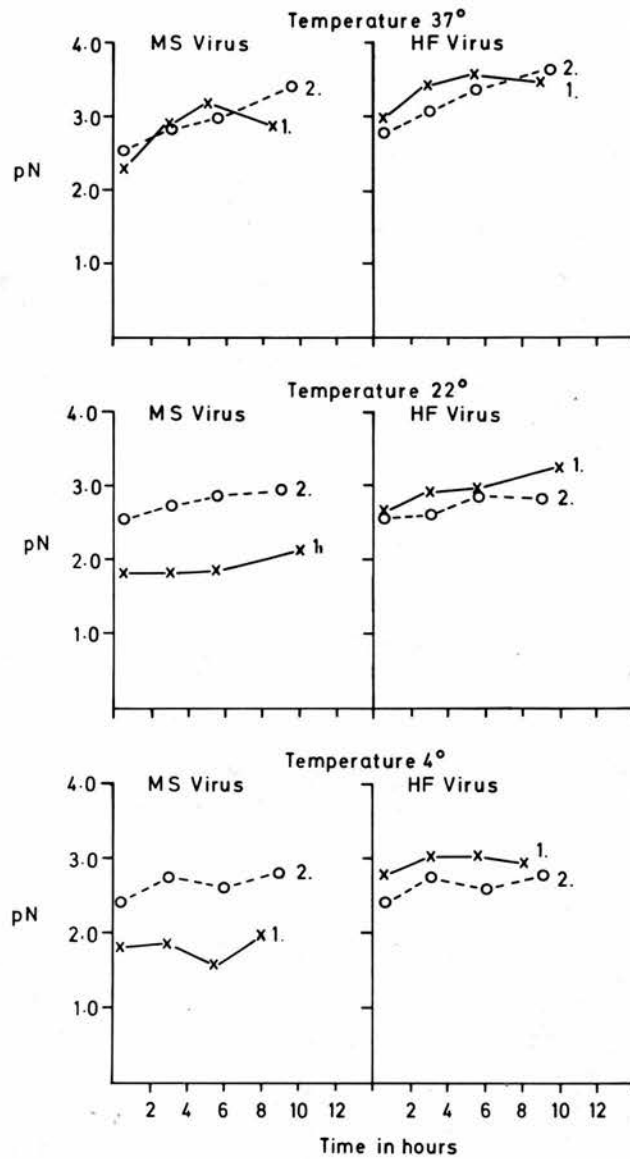
A pair of the above reference sera, HF-H(a) and MS-L(a), were chosen for the initial typing of unknown virus isolates.

#### Antigenic typing of laboratory strains of herpes simplex virus

The results of 5 independent titrations of the reference strains HF and MS against the HF-H(a) and MS-L(a) antisera are given in Table 27. These results confirm that the method is reproducible, and that 1657 is HF-like or type 1. The classification of strain Watson as type 1 and strain Dawson as type 2 agrees with previous results (Plummer et al., 1968).

The failure of the MS antiserum to neutralise many type 1 strains is a major difference between the Edinburgh study and that of Dowdle et al. (1967): for this reason, the relevant pN values are represented as "less than or equal to". The sera used by Dowdle et al. (1967) neutralised both homologous and heterologous types of virus, and as a result, their pN difference values for type 1 strains lay within the range + 0.5 to + 1.0, with a mean of + 0.66 ± 0.09.





**FIGURE 8.** The effect of varying the duration and temperature of incubation of the neutralisation reaction on the calculated pN values of two high-dose rabbit antisera determined with the corresponding viruses.

1 = serum HF-H(a); 2 = serum MS-H(a).

The time indicated is the duration of incubation of the virus: serum mixtures before inoculation to plates.

**FIGURE 9.** The effect of varying the duration and temperature of incubation of the neutralisation reaction on the calculated pN values of two low-dose rabbit antisera determined with the corresponding viruses.

1 = serum HF-L(a); 2 = serum MS-L(a)

The time indicated is the duration of incubation of the virus: serum mixtures before inoculation to plates.

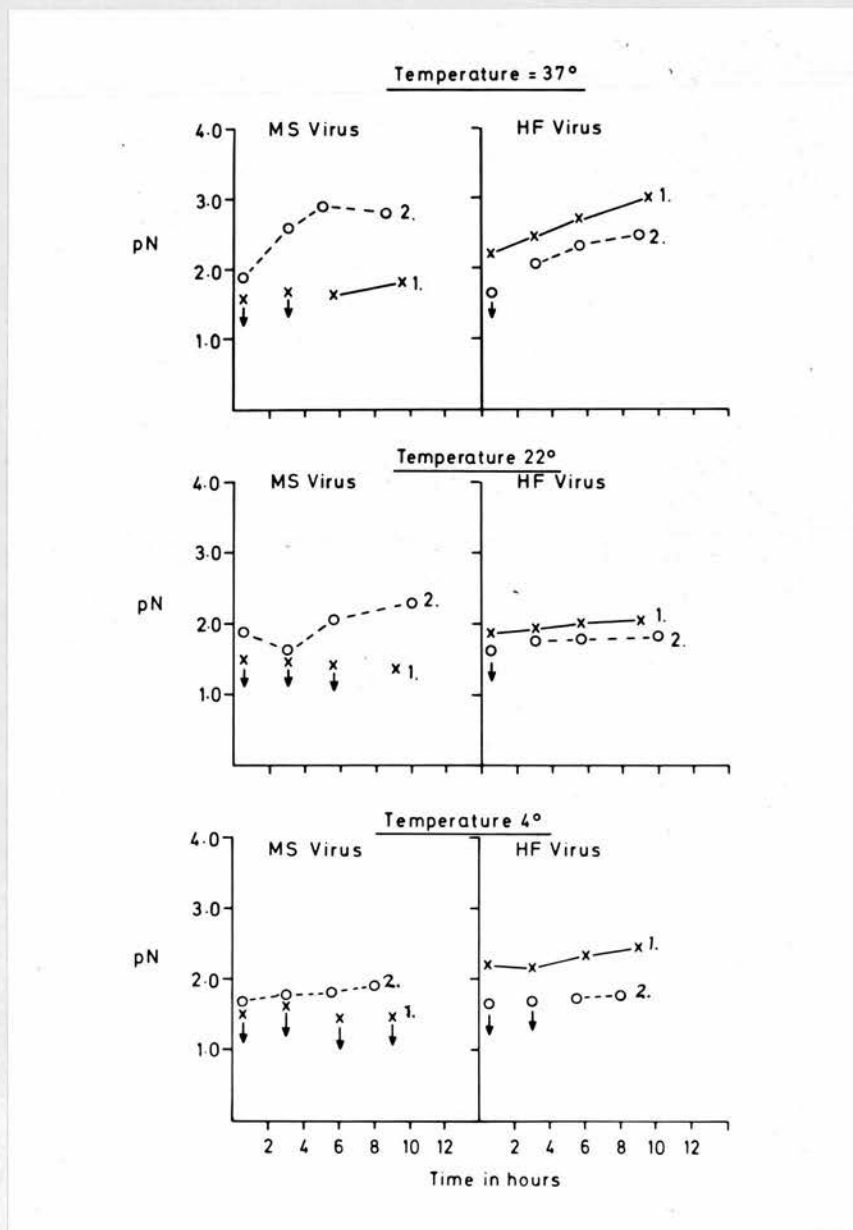


TABLE 27

Mean, and standard deviation of the mean, pN values of reference strains HF, MS and 1657 determined against HF-H(a) and MS-L(a) antisera, compared with the pN values of laboratory strains Watson, Dawson and MP with the same antisera

Virus strain	Mean and standard deviation of the mean, pN values determined against indicated antisera				Mean pN difference S.D.		Type of virus indicated
	HF-H(a) mean pN1	S.D.	MS-L(a) mean pN2	S.D.			
HF*	2.68	± 0.28	≤ 1.57	± 0.15	≥ 1.12	± 0.30	1. Reference strain
MS*	2.07	± 0.32	2.29	± 0.24	- 0.22	± 0.11	2. Reference strain
1657*	2.34	± 0.08	≤ 1.62	± 0.04	≥ 0.72	± 0.07	1
Watson φ	2.40	-	≤ 1.38	-	≥ 1.02	-	1
Dawson φ	1.95	-	2.10	-	- 0.15	-	2
MP φ	1.95	-	≤ 1.49	-	≥ 0.46	-	1

\* Mean pN values determined from 5 separate titrations

φ Single determinations of pN values.

S.D. = standard deviation of the mean.

The values designated ≤ or ≥ are derived from titrations in which no neutralisation of virus was detected at the lowest dilution tested (10).

Exact calculation of the mean values and standard deviations of the mean of the type 1 pN differences is not possible with the results in this thesis for the reasons mentioned above; however, the values in Table 27 for type 1 strains are  $\geq 0.72$ . The mean value (Table 27) for MS virus is  $- 0.22 \pm 0.11$  and is in good agreement with the value  $- 0.18 \pm 0.12$  reported by Dowdle et al. (1967) for type 2 strains. The pN difference values for Dawson and Watson are also in agreement with the published results. Strain MP was found to have a pN difference of  $+ 0.46$  which is less than all the other type 1 strains: Fig. 10 confirms that this is located at the low end of the type 1 range.

During the study the supply of MS-L(a) antiserum was exhausted and a new serum was produced. This antiserum (MS(2)) was raised by inoculating virus diluted to contain  $10^{2.5}$  TCID<sub>50</sub>/0.02 ml. This concentration of virus is less than that inoculated to produce the high-dose sera, but slightly greater than the concentration used to produce low-dose sera ( $< 10^{2.0}$  TCID<sub>50</sub>/0.02 ml). This serum neutralised both MS and HF viruses, and did not show such a high degree of type specificity as the earlier serum, but adequate distinction was possible between strains when this serum was used in conjunction with serum HF-H(b). Replicate titrations of these two sera with the reference viruses HF and MS gave the results indicated in Table 28, with mean pN difference values for HF and MS virus of  $+ 0.55$  and  $- 0.26$  respectively. In addition, 7 virus strains were retyped with the second pair of reference sera (Table 36); there was complete agreement in the indicated type.

In conclusion, the antigenic typing method described appears

TABLE 28

Neutralising titres, pN values and pN differences of second pair  
of typing sera determined on two occasions with the reference  
strains HF and MS

Virus	Titres and pN values determined with indicated serum				pN difference pN1 - pN2
	HF-H(b)		MS (2)		
	Titre	pN1	Titre	pN2	
HF	1.97	2.42	1.51	1.96	+ 0.46
	1.96	2.45	1.33	1.82	+ 0.63
MS	1.30	1.68	1.60	1.98	- 0.30
	1.43	1.89	1.65	2.11	- 0.22

Serum titres expressed as  $\log_{10}$

Titration performed on separate occasions.

to be reliable and gives results in agreement with published reports, although comparison of  $p^N$  difference values is difficult due to the different degrees of cross-neutralisation of the typing sera.



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ANTIGENIC TYPING OF STRAINS OF HERPES SIMPLEX VIRUS

The reports of Dowdle et al. (1967) and Nahmias and Dowdle (1968) indicated that there was a fairly strict association of type 2 strains with infections of the genital tract and associated areas of skin. However, the population examined by Dr. A. J. Nahmias and his co-workers in Atlanta, Georgia, was predominantly negroid and previous serological studies have shown that this type of population is characterised by widespread primary infection in young children (Buddingh et al., 1953). The sero-epidemiological survey performed on sera collected in the Edinburgh area (Smith et al., 1967) indicated that the proportion of young adults without antibody was high enough to allow primary infection to occur in this age group: this could be with type 1 or type 2 virus.

Strains of herpes simplex virus isolated from a variety of clinical conditions were submitted to antigenic typing to examine the association of virus type and site of infection.

Age distribution and sites of infection of patients

Table 29 gives the age distribution of the 208 patients from whom the viruses were isolated. Comparison of this table with the overall age distribution of patients given in Fig. 2 and Table 2 shows that the selected patients included almost all those aged 15 - 34 years with infections of the mouth, skin or eye. In addition isolates from younger and older patients were studied, including a number from patients with eye infections. This last group was included because antigenic typing of eye isolates had not been widely reported, and also because the virus could have been acquired at a younger age

TABLE 29

Typing of herpes simplex virus strains: age and site of infection  
of source patients

Age group (years)	Number of virus strains typed from each of the indicated sites				Total
	Mouth	Skin	Eye	Genital tract	
0 - 4	11	4	0	0	15
5 - 9	9	6	2	0	17
10 - 14	4	5	0	0	9
15 - 19	16	6	4	8	34
20 - 24	15	12	4	25	56
25 - 29	2	2	2	15	21
30 - 34	1	1	4	9	15
35 - 39	0	1	1	3	5
40 - 44	0	0	2	2	4
45 - 49	0	3	3	2	8
50 - 54	1	1	10	0	12
55 - 59	1	0	2	0	3
60 - 64	0	2	0	0	2
65 - 69	0	0	2	2	4
70 +	0	0	3	0	3
Total	60	43	39	66	208



possibly by sexual contact.

The majority of the strains isolated from genital tract infections were typed; the age distribution of these patients has already been described.

#### Sex distribution of patients

The overall ratio of male:female patients is 1:1.1 (Table 30), although the results for individual sites show considerable variation from this figure. The high proportion of female patients in the oral infection group is due to the inclusion of 16 strains from girls aged under 15 years. Variation in the ratio in lip, face, skin and finger groups probably arises from the small number in each category, although the ratio of 1:2.3 in the finger group is accounted for by the inclusion of 6 nurses. The ratios of the eye and genital tract patients reflect the expected sex distribution in these categories.

#### Reference antisera

Table 31 shows that 112 strains were examined with the first pair of sera, and 96 with the second pair of sera. Oral strains were typed with both sets, eye strains with the first set and the majority of genital tract isolates by the second set. Full lists of the patients included in the typing study are included in Appendix C where the age, sex, serum titres, pN values and typing sera are detailed.

#### Reproducibility and consistency of the method in practice

The preliminary studies with the reference antisera and a few laboratory strains established that the typing method was reproducible,

TABLE 30

Typing of herpes simplex virus strains: age and sex distribution  
of source patients

Site	Male patients(M) number of strains typed from patients aged:			Female patients(F) number of strains typed from patients aged:			Total ratio (M:F)	Total number of patients
	<15 years	≥ 15 years	Total	<15 years	≥ 15 years	Total		
Mouth	7	15	22	16	22	38	1:1.7	60
Lip	0	6	6	2	2	4	1.5:1	10
Face	2	6	8	2	3	5	1.6:1	13
Skin	3	0	3	2	5	7	1:2.3	10
Finger	3	0	3	1	6	7	1:2.3	10
Skin (total)	8	12	20	7	16	23	1:1.2	43
Eye	1	21	22	1	16	17	1.3:1	39
Genital tract	0	36	36	0	30	30	1.2:1	66
Total	16	84	100	24	84	108	1:1.1	208

Skin (total) includes viruses isolated from lip, face, skin and finger sites.

TABLE 31

Number of strains and sites of isolation of viruses typed by first  
and second pairs of reference antisera

Site of isolation	Number of strains typed with:		Total
	First sera	Second sera	
Mouth	37	23	60
Skin	18	25	43
Eye	39	0	39
Genital tract	18	48	66
Total	112	96	208

First sera = HF-H(a) and MS-L(a)

Second sera = HF-H(b) and MS(2)

at least over a short period of time. This defined experiment did not reflect the conditions in practice when the typing method was applied to a wide range of viruses. For this reason, a number of strains were retested to confirm that the reference antisera had not deteriorated during storage at  $-30^{\circ}\text{C}$ . The results of studies with 8 virus isolates are given in Table 32, which shows that there was complete agreement of allocated type. In 4 instances there was good agreement of pN1 - pN2 or pN difference values; in another case, although the change in the pN difference was just greater than the others mentioned it did result in the pN difference changing from a positive value to a negative value. As discussed later, both values are within the type 2 range with the first pair of reference antisera. In the other 3 instances, comparison was difficult as the pN difference is represented as "greater or equal to", due to the failure of the type 2 reference serum to neutralise these viruses. The calculation convention adopted has been described, but this gives rise to apparently divergent numerical values (Table 32); when plotted with the other results (Fig. 10) they are both in the type 1 region.

The consistency of the typing method is indicated by the results detailed in Table 33. Simultaneous virus isolations were made from separate specimens collected from the same site of infection or closely related areas of 5 patients. Two pairs of eye specimens are included, the virus being isolated from both conjunctiva and cornea, as well as 3 pairs of isolates from lesions of the vulva and the cervix uteri. Finally, two viruses isolated from recurrent penile infections were examined: there was an interval of 10 weeks between isolations. In no instance was there any change in the type

TABLE 32

Replicate neutralising titres, pN values and pN differences of 8 viruses determined with the first set of reference antisera

Patient	Virus number	Site of infection	HFT	MST	pN1	pN2	pN1-pN2	Type indicated
E32	3366 "	cornea "	2.03	1.05	2.59	1.61	+ 0.98	1
			2.29	1.44	2.69	1.84	+ 0.85	1
S13	2827 "	finger "	2.04	1.13	2.76	1.85	+ 0.91	1
			1.87	1.05	2.27	1.45	+ 0.82	1
G5	4035 "	labia "	1.12	1.02	1.72	1.62	+ 0.10	2
			2.20	2.30	2.22	2.32	- 0.10	2
S8	5518 "	lumbar region	1.40	1.38	1.78	1.76	+ 0.02	2
			1.72	1.63	2.08	1.99	+ 0.09	2
E20	2879 "	cornea "	1.95	≤ 1.00	2.35	≤ 1.40	≥ 0.95	1
			1.67	≤ 1.00	2.28	≤ 1.61	≥ 0.67	1
S22	2018 "	lip "	1.53	≤ 1.00	2.28	≤ 1.75	≥ 0.53	1
			2.00	≤ 1.00	2.33	≤ 1.33	≥ 1.00	1
G7	5285 "	vulva "	1.43	≤ 1.00	2.04	≤ 1.61	≥ 0.43	1
			1.80	≤ 1.00	2.24	≤ 1.44	≥ 0.80	1
G62	4159 "	penis "	2.29	1.15	2.67	1.53	+ 1.14	1
			2.70	1.54	2.85	1.69	+ 1.16	1

HFT = titre of HF (type 1) reference serum with virus. MST = titre of MS (type 2) reference serum with virus. All serum titres expressed as log<sub>10</sub>

TABLE 33

Neutralising titres, pN values, and pN differences of 6 pairs of virus isolates with the first set of reference antisera

Patient	Virus number	Site of infection	HFT	MST	pN1	pN2	pN1-pN2	Type indicated
E36	φ 0044 0045	conjunctiva cornea	2.11	1.03	2.43	1.35	+ 1.08	1
			1.33	≤ 1.00	2.00	≤ 1.66	≥ 0.33	1
E13	3909 3910	cornea conjunctiva	1.73	≤ 1.00	2.13	≤ 1.40	≥ 0.73	1
			1.39	≤ 1.00	1.92	≤ 1.53	≥ 0.39	1
G4	6653 6654	cervix labia	2.43	2.29	2.33	2.19	+ 0.14	2
			1.99	1.99	1.97	1.97	0.00	2
G3	6587 6588	vulva vulva	1.75	1.53	2.11	1.89	+ 0.22	2
			2.09	1.91	2.07	1.89	+ 0.18	2
G7	5285* 5286	vulva cervix	1.62	≤ 1.00	2.14	≤ 1.53	≥ 0.61	1
			1.36	≤ 1.00	2.12	≤ 1.76	≥ 0.36	1
G11	φφ 5179 5456	penis penis	2.05	1.84	2.41	2.20	+ 0.21	2
			1.16	1.12	1.58	1.54	+ 0.04	2

\* Mean of values listed in table 32.

HFT = titre of HF (type 1) reference serum with virus.

MST = titre of MS (type 2) reference serum with virus.

All serum titres expressed as  $\log_{10}$

φ The pairs of viruses with consecutive numbers were isolated from separate specimens collected on the same day.

φφ Specimen 5179 was collected on 23.4.68 and specimen 5456 on 2.7.68.

of virus determined by the microneutralisation method: virus strains 6587 and 6588 were both at the upper end of the range of pN difference values for type 2 strains.

Similar observations were made with the second set of reference sera and the results are detailed in Tables 34 and 35. As discussed later the pN difference values for type 1 and 2 strains are different from those with the first typing sera; both sera neutralised all viruses tested, and as a result the pN difference values are more closely grouped. This is reflected in the close agreement of the individual results (Table 34) for 5 viruses isolated from the face, labia and cervix uteri. No type 2 strains are included in this table, but results with both simultaneous and spaced isolations of type 2 strains are shown in Table 35: the pN differences of these strains are in good agreement. In addition to the 4 pairs of viruses isolated on the same day from the same or related lesions of the thigh, labia and cervix, viruses isolated from a patient at intervals of 7 and 6 weeks were examined.

Six viruses were typed by both pairs of sera to confirm that the two sets gave similar typing results. Table 36 shows that no change in antigenic type was noted. During the time when the second pair of sera were in use, viruses were isolated from two patients whose original isolate had been typed by the first pair of sera. One of these patients, a 26-year-old female with an earlier virus isolation from the cervix uteri returned 15 months later with an infection of the mons pubis area; both strains were type 2. The other patient, a 40-year-old male, returned more than 3 years later with a recurrence of penile lesions; virus was isolated, and typed. In this

TABLE 34

Replicate neutralising titres, pN values and pN differences for 5 viruses determined against the second set of reference antisera

Patient	Virus number	Site of infection	HFT	MST	pN1	pN2	pN1-pN2	Type
G32	7637 "	cervix "	2.08	1.75	2.44	2.11	+ 0.33	1
			2.31	1.90	2.72	2.31	+ 0.41	1
G21	7927 "	labia "	2.37	1.98	2.64	2.25	+ 0.39	1
			2.43	2.08	2.68	2.33	+ 0.35	1
S39	9319 "	chin "	2.28	1.84	2.77	2.33	+ 0.44	1
			1.63	1.00	2.34	1.71	+ 0.63	1
S41	7552 "	face "	2.05	1.43	2.65	2.03	+ 0.62	1
			2.05	1.63	2.61	2.19	+ 0.42	1
G29	9291 "	cervix "	2.36	1.97	2.80	2.41	+ 0.39	1
			2.52	2.07	2.87	2.42	+ 0.45	1

HFT = titre of HF (type 1) reference serum with virus

MST = titre of MS (type 2) reference serum with virus

Serum titres expressed as  $\log_{10}$



TABLE 35

Neutralising titres, pN values, and pN differences of 4 pairs of simultaneous virus isolations and consecutive virus isolations from one patient, determined against the second set of reference antisera

Patient	Virus number	Site of infection	Isolation date	HFT	MST	pN1	pN2	pN1-pN2	Type indicated
G29	9290	vagina	31.12.70	2.32	1.97	2.78	2.43	+ 0.35	1
	9291*	cervix	31.12.70	2.44	2.02	2.84	2.42	+ 0.42	1
G26	8681	labia	11. 3.70	2.20	1.83	2.80	2.43	+ 0.37	1
	8682	labia	11. 3.70	2.34	2.03	2.79	2.48	+ 0.31	1
G23	9007	cervix	18. 9.70	2.35	2.55	2.59	2.79	- 0.20	2
	9008	Bartholin's gland	18. 9.70	2.10	2.33	2.24	2.47	- 0.23	2
G35	9431	labia	16. 2.71	2.88	2.20	3.18	2.50	+ 0.68	1
	9434	thigh	16. 2.71	2.41	1.55	2.46	1.60	+ 0.86	1
G24	8730	labia	11. 3.70	1.47	1.71	2.09	2.33	- 0.24	2
	8740	labia	28. 4.70	1.72	2.14	1.94	2.36	- 0.42	2
	8771	labia	12. 6.70	2.09	2.23	2.67	2.81	- 0.14	2

\* Mean of values listed in Table 34

HFT = titre of HF (type 1) reference serum with virus

MST = titre of MS (type 2) reference serum with virus

Serum titres expressed as  $\log_{10}$

TABLE 36

Neutralising titres, pN values, and pN differences for a series of viruses determined against both pairs of reference antisera: separate virus isolates or repeat determinations for one virus strain

Patient	Virus number	Site of infection	Date	HFT	MST	pN1	pN2	pN1-pN2	Type	Reference sera
G13	1718	penis	23.11.66	1.73	1.65	2.22	2.14	+ 0.08	2	1st
		"	"	3.00	3.08	1.23	1.31	- 0.08	2	2nd
G9	2107	labia	29.12.66	1.05	1.30	1.64	1.89	- 0.25	2	1st
		"	"	1.73	1.96	2.27	2.50	- 0.23	2	2nd
G11	5179	penis	23. 4.68	2.05	1.84	2.41	2.20	+ 0.21	2	1st
		"	"	2.45	2.80	1.53	1.88	- 0.35	2	2nd
O12	0497	mouth	9. 4.66	1.77 ≤	1.00	2.25 ≤	1.48	≥ 0.77	1	1st
		"	"	2.50	1.85	3.20	2.55	+ 0.65	1	2nd
G10	3668	penis	3.10.67	1.20	1.75	1.61	2.16	- 0.55	2	1st
		"	"	1.60	2.41	2.17	2.98	- 0.81	2	2nd
O33	7332	mouth	7. 4.69	2.55	1.47	3.07	1.99	+ 1.08	1	1st
		"	"	1.95	1.52	2.47	2.04	+ 0.43	1	2nd
G8	5423	cervix	26. 6.68	1.43	1.50	1.67	1.74	- 0.07	2	1st
	8003	mons pubis	18. 9.69	1.64	2.01	2.04	2.41	- 0.37	2	2nd
G62	4159*	penis	12.12.67	2.50	1.35	2.76	1.61	+ 1.15	1	1st
	9864	"	6. 9.71	2.11	2.09	2.71	2.69	+ 0.02	2	2nd

\* Mean of values listed in Table 32

HFT = titre of HF (type 1) reference serum with virus

MST = titre of MS (type 2) reference serum with virus

All serum titres expressed as log<sub>10</sub>

instance, the only one recorded in this study, the virus type was found to have "changed" from type 1 to type 2. The classification of strain 4159 as type 1 had been confirmed (Table 32) as it was one of the first genital type 1 strains identified. The change in type could be accounted for if the second episode represented a reinfection rather than a recurrence.

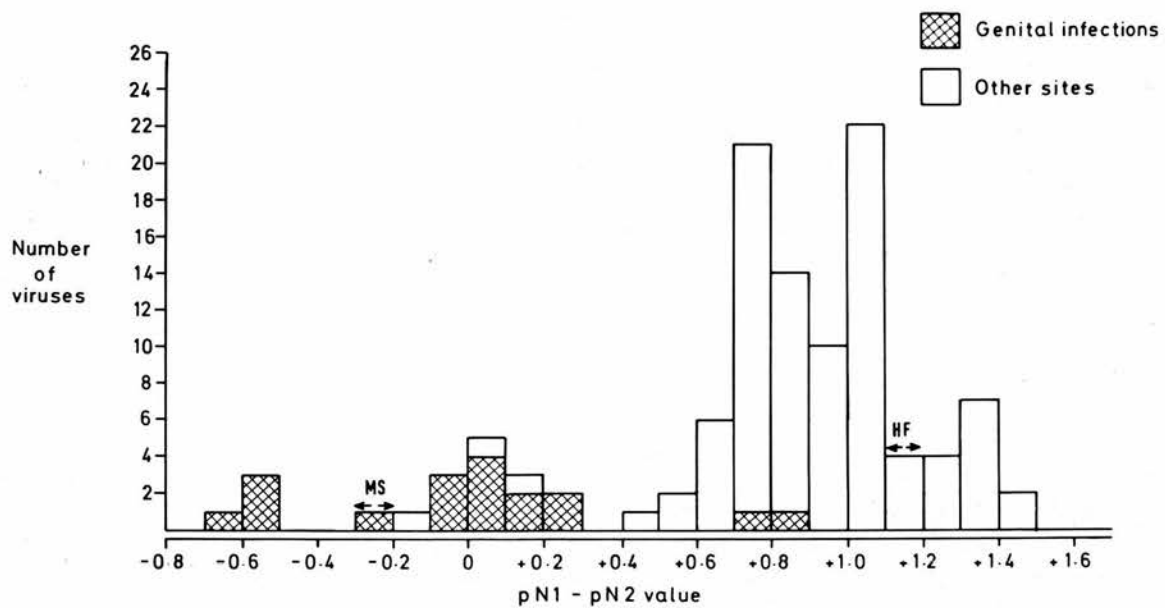
Results of antigenic typing of unknown strains of  
herpes simplex virus

The results of the antigenic typing of the strains listed in Table 29 are illustrated graphically in Fig. 10 and Fig. 11. The clearest appreciation of the grouping of the isolates into two types is obtained from the histograms in Fig. 10 for the first set of reference antisera, and in Fig. 11 for the second set of antisera.

Results with the first pair of reference antisera

Strains of herpes simplex virus fell into two main groups one of which included the reference type 1 virus (HF) and the other the type 2 reference strain (MS). The HF-like group were designated type 1 and the MS-like strains type 2. The type 1 strains had pN difference values of + 0.48 and greater, up to a maximum recorded of + 1.49, with 67 of the 93 values (72 per cent.) between + 0.60 and + 1.10. The mean value of all 93 strains was  $\geq 0.94$  with a standard deviation of the mean of  $\pm 0.22$  (Table 37).

The convention adopted for strains not neutralised by the type 2 antiserum has been described; results of this type were obtained with 69 (74 per cent.) HF-like strains. The two peaks of pN



**FIGURE 10.** Distribution of pN1 - pN2 values and site of isolation of 112 strains of herpes simplex virus determined with the first pair of reference antisera.

TABLE 37

Mean and standard deviation of the mean values of the pN differences of virus strains typed with the first pair of reference antisera

Type	Number of viruses tested	Mean pN difference	Standard deviation of the mean
1 *			
(HF-like)			
a	69	$\geq 0.90$	$\pm 0.21$
b	24	1.06	$\pm 0.19$
Total	93	$\geq 0.94$	$\pm 0.22$
2			
(MS-like)	19	- 0.09	$\pm 0.27$

\* Type 1 strains are divided into groups on the basis of the observed neutralisation titre with the type 2 (MS) antiserum.

a = virus not neutralised by type 2 antiserum

b = virus neutralised by type 2 antiserum

difference values shown in Fig. 10 between 0.7 - 0.8 and 1.0 - 1.1 are not related to this convention. Comparison of various factors such as age, sex and clinical site of infection also failed to reveal any correlation with either of the two distribution peaks in the type 1 range. The significant factor appears to be the challenge dose of virus inoculated to the neutralisation reaction. Analysis of the groups with estimated values of  $< 1.0$  and  $> 1.0$  showed that virus challenge doses in excess of  $10^{3.5}$  TCID<sub>50</sub>/0.02 ml had been inoculated in 43 per cent. of cases with values  $< 1.0$  as opposed to 4 per cent. of the  $> 1.0$  group. The excess virus depresses the type 1 (HF) antiserum titres and since there cannot be a corresponding reduction in the "fixed" type 2 (MS) antiserum titre, the resulting pN difference value is reduced and the point is shifted towards the mid-point on the histogram. This is not a defect of the typing method, but rather a reflection of the use of too high virus doses, in conjunction with a serum with little cross-neutralising ability. An alternative method of dealing with the results would have been to call the pN2 values 0 for the viruses not neutralised by type 2 (MS) antiserum. However, this would produce a pronounced bimodal distribution, with the zero notional value strains having high pN difference values in the range + 2.05 to + 3.19.

The MS-like group of strains are designated type 2 viruses. The highest pN difference found in this group was + 0.22, and the lowest - 0.61. The mean value was - 0.09 with a standard deviation of the mean of  $\pm 0.27$ . This is a small group, and although the beginning of a normal distribution curve is apparent, further strains would need to be typed to determine the exact range of values.

Effect of the age of source patient on the pN difference  
of type 1 viruses

Table 38 gives the results of an analysis of the pN difference values of all type 1 strains, dividing the viruses into groups according to the ages of the source patients and the reaction with the type 2 antiserum. The means and standard deviations of the mean for the patients aged over 15 years were  $\geq 0.91 \pm 0.23$  and  $0.99 \pm 0.14$  respectively. For patients younger than 15 years values of  $\geq 0.88 \pm 0.20$  and  $1.26 \pm 0.19$  were calculated. Further statistical analysis was not undertaken because (i) notional values had to be allocated for most of the strains and (ii) the small size of the groups. If the results of the two sections are pooled, the mean pN value for the patients under 15 years becomes  $\geq 0.96$  and  $\geq 0.95$  for those over 15 years.

It was concluded that the age of the patient was not related to the pN difference value of type 1 viruses and that, within the limitations of the test, strains isolated from patients over 15 years were identical to those from younger patients.

Association of antigenic type of virus with site of infection

The mean and standard deviation of the mean values for the pN differences of viruses isolated from the 4 main clinical sites are listed in Table 39. The type 1 strains are subdivided into two groups according to their reaction with the MS antiserum. The differences between the mean values of type 1 strains from different sites, could be attributed to the small number of strains in some categories. The

TABLE 38

Analysis of the pN difference values with the first reference antisera of type 1 viruses according to the age of the source patient

Group	Age of source patients	Number of viruses	Mean pN difference	Standard deviation of the mean
a	< 15	24	$\geq 0.88$	$\pm 0.20$
	$\geq 15$	45	$\geq 0.91$	$\pm 0.23$
b	< 15	6	1.26	$\pm 0.19$
	$\geq 15$	18	0.99	$\pm 0.14$

a = virus not neutralised by type 2 antiserum

b = viruses neutralised by type 2 antiserum



TABLE 39

Analysis of the pN differences with the first reference antisera of type 1 virus strains according to the site of infection of the source patient

Type	Category	Site of infection of source patient	Number of strains	Mean pN difference	Standard deviation of the mean
1	a *	mouth	25	$\geq 0.88$	$\pm 0.25$
	b †	"	12	1.07	$\pm 0.23$
	a	skin	12	$\geq 0.91$	$\pm 0.18$
	b	"	3	1.14	$\pm 0.24$
	a	eye	30	$\geq 0.91$	$\pm 0.22$
	b	"	9	1.01	$\pm 0.09$
	a	genital tract	2	$\geq 0.77$	$\pm 0.05$
	2	-	skin	3	+ 0.02
-		genital tract	16	- 0.11	$\pm 0.29$

\*a = Virus not neutralised by type 2 antiserum

†b = Virus neutralised by type 2 antiserum

overall means for the 3 main sites - mouth, skin and eye - are in good agreement and are  $\geq 0.93$ ,  $\geq 0.94$  and  $\geq 0.96$  respectively. The site from which a type 1 virus was isolated did not appear to have any effect on the  $pN$  difference value. The typing results can be summarised as follows:

- i) viruses isolated from the mouth. All 37 strains were found to be type 1.
- ii) viruses isolated from the skin. Fifteen of the 18 strains examined were allocated to type 1; the remaining 3 were type 2. Two strains isolated from lip lesions, 4 from face infections, and 4 strains recovered from finger infections including 3 from nurses, all belonged to type 1. The remaining 5 type-1 strains were isolated from a variety of skin sites in children aged less than 12 years, including one strain from a recurrent lesion of the buttock. This patient was 5 years of age, and this may explain the recovery of a type 1 strain from a site usually associated with type 2 viruses. The 3 type-2 strains in this category were isolated from infections of the lower lumbar region, buttock and perianal skin; these patients were adults, aged 60, 21 and 18 years respectively.
- iii) viruses isolated from the eye. All 39 strains were found to be type 1.
- iv) viruses isolated from the genital tract. Viruses isolated from 9 male and 9 female patients were typed. Sixteen viruses were allocated to type 2 including those from the 9 male patients with penile lesions, and 5 female patients with vulvar infections. Two of the 3 strains isolated from the cervix uteri were found to be anti-genic type 2; the third, from a 24-year-old patient, was a type 1

strain. One other type 1 virus was isolated from a labial infection of a 23-year-old patient.

All viruses isolated from the eye, mouth and skin of lip, face and other sites except the lumbar and buttock regions were antigenic type 1. Type 2 strains were associated with infections of the genital tract and associated areas of skin in both male and female patients. The isolation of two type-1 strains from these sites, including the cervix, was of interest, and to confirm these results another 96 strains were typed with the second pair of reference antisera: these included a further 48 genital isolates, 25 from skin sites and 23 from intraoral infections (Table 31).

Results with the second pair of reference antisera

Fig. 11 shows the distribution of the  $pN$  difference values of 96 strains of herpes simplex virus according to genital and non-genital sites of isolation. The most obvious difference from Fig. 10 is that the values form two clearly defined groups, one related to HF virus (type 1) and the other to MS virus (type 2). This results from the reduced specificity of the type 2 reference serum, although the calculation of  $pN$  values was simplified due to its ability to neutralise both types of virus. One other result is that the upper limit of the type 2 group (+ 0.02) and the lower limit of the type 1 group (+ 0.12) are closer together than with the first sera. This observation emphasises that there are no absolute values of  $pN$  differences for type 1 and 2 strains, but rather that the limits have to be defined for each pair of reference antisera.

The mean and standard deviation of the mean of the  $pN$  difference

**FIGURE 11.** Distribution of pN1 - pN2 values and site of isolation of 96 strains of herpes simplex virus determined with the second pair of reference antisera.

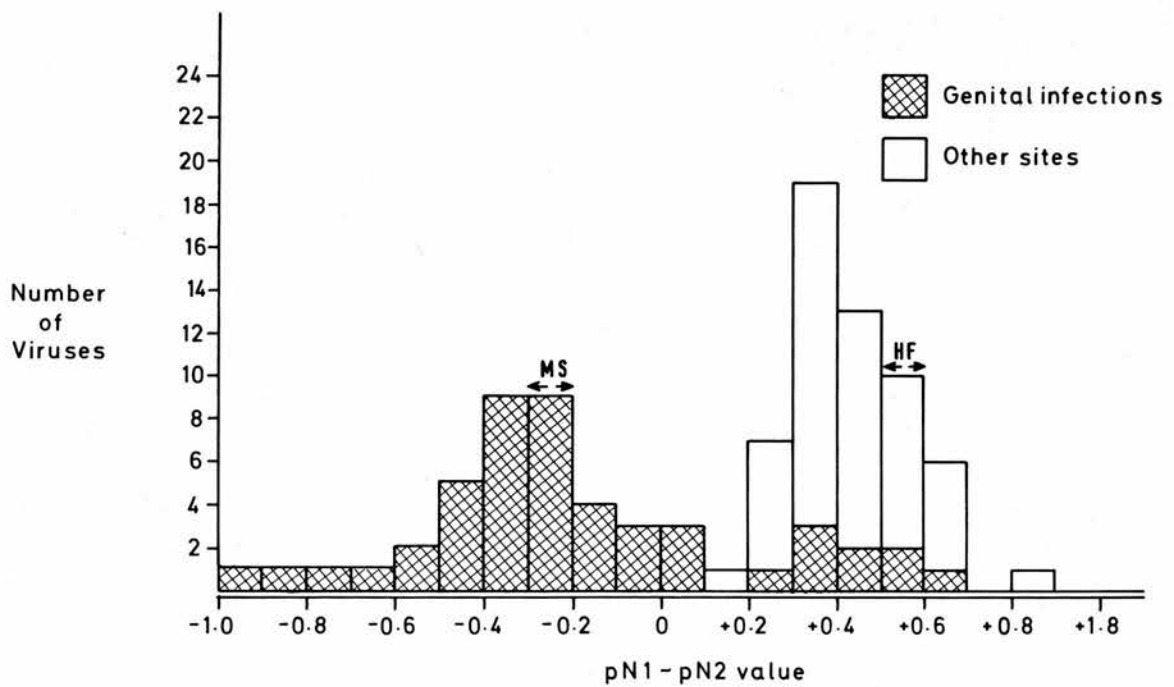


TABLE 40

Mean, and standard deviation of the mean values of the pN difference values with the second pair of reference antisera of virus strains isolated from different sites of infection

Type	Site of infection of source patient	Number of strains	Mean pN difference	Standard deviation of the mean
1	mouth	23	+ 0.47	$\pm$ 0.15
	skin	25	+ 0.37	$\pm$ 0.11
	genital tract	9	+ 0.42	$\pm$ 0.14
	Total	57	+ 0.42	$\pm$ 0.14
2	genital tract	39	- 0.32	$\pm$ 0.21

values for the two groups are given in Table 40. For type 1 strains these ranged from + 0.12 to + 0.87 with a mean and standard deviation of the mean of  $+ 0.42 \pm 0.14$ . Eighty-six per cent. of the values lay between + 0.20 and + 0.59. Type 2 values varied from - 0.92 to + 0.02 with a mean and standard deviation of the mean of  $- 0.32 \pm 0.21$ . This group covers a wide range, and as a result, only 69 per cent. of the values are between - 0.49 and - 0.10.

Effect of the age of the source patient on the pN  
difference value of type 1 viruses

Examination of the pN differences for the 10 patients under 15 years of age yielded mean and standard deviation of the mean values of  $+ 0.40 \pm 0.18$ ; for the 47 patients over the age of 15 years, the results were  $+ 0.45 \pm 0.13$ . These values are in good agreement and it can be concluded that the age of the patient is not related to the pN difference value of the virus isolated.

Association of antigenic type of virus with site of infection

Analysis of the results according to the clinical site of infection is shown in Table 40, and it is obvious that type 1 strains do not differ with site. Further statistical analysis was not justified due to the lack of sensitivity of the method and the good agreement of the results for the different sites. The results can be summarised as follows:

- i) viruses isolated from the mouth. All 23 oral strains were of type 1.
- ii) viruses isolated from the skin. No type 2 strains were found

among the 25 isolates from skin sites: these included 8 strains from patients aged 14 - 45 years with lip infections; 9 from face infections of patients aged 1 - 60 years and 6 from herpetic whitlows including 3 strains from nurses. The two skin strains, both from young women, one infected on the dorsum of the hand and the other on the trunk, were also type 1.

iii) viruses isolated from the genital tract. Forty-eight strains from genital infections of 21 female and 27 male patients were typed, including a random isolation from the urine of a 67-year-old man. Thirty-nine of these viruses were type 2, 14 from female and 25 from male patients. The males all suffered from penile infections; of the female patients 12 isolates were from labial infections. The other two type-2 strains were isolated from the cervix uteri; there was a simultaneous isolation from the vulva in one patient. The remaining 9 strains in the genital category were typical type 1 strains, with a distribution of pN difference values identical to the results for type 1 strains from all sites. Two of these strains were isolated from penile infections and labial infections accounted for another 5 isolates. The other two strains were isolated from the cervix uteri; in one case this was the only specimen collected, but in the other, type 1 virus was isolated from a labial lesion. Material was not collected from the cervix of the 5 patients with labial type 1 infections; in one case, a 26-year-old, there was a simultaneous isolation of type 1 virus from the skin of the inner aspect of the thigh.

Summary of antigenic typing results

Table 41 summarises the overall findings of the association of antigenic type of virus and site of virus recovery. All strains from the mouth, eye, lip, face and skin above the waist were of type 1. In this study, all the finger isolates, including 6 from herpetic whitlows of nurses were type 1. Three out of 4 strains isolated from the lower lumbar, perianal and buttock region were type 2; the fourth strain, from a recurrent buttock infection in a 5-year-old girl, was type 1. A surprising finding was that a significant number of genital strains were type 1; 11 of the 66 genital strains (16.7 per cent.) fell into this category. The frequency of type 1 strains was two of 36 strains (5.6 per cent.) from males and 9 of 30 (30 per cent.) from female patients. The results of the typing studies of strains from the female genital tract are detailed in Table 42.

Too few isolations were attempted to allow any conclusions to be reached about the role of the cervix in maintaining infection in the female genital tract. It is of interest that 30 per cent. of viruses isolated from the female genital tract were type 1, and that two of the 6 strains recovered from the cervix were type 1.

One possible source of these type 1 viruses is the oral cavity either by direct transfer from the mouth of the patient or by orogenital contact with an infected partner. There was a history of contact with a partner apparently suffering from cold sores in only 1 instance: as described below, this patient apparently contracted a primary genital infection.

Once infection of the genital tract is established, recurrent episodes could occur either by reactivation of a latent virus at the



TABLE 41

Association between site of infection and antigenic  
type of herpes simplex virus

Isolation site	Number of isolates in each antigenic type		Total
	type 1	type 2	
mouth	60	0	60
lip	10	0	10
face	13	0	13
finger	10	0	10
skin*	6	0	6
buttock <sup>-</sup>	1	3	4
eye	39	0	39
genital - male	2	34	36
genital - female	9	21	30
TOTAL	150	58	208

\* This group includes virus isolates from skin of trunk, neck and limbs (except finger).

- This group includes strains from lower lumbar, perianal and buttock sites.

TABLE 42

Summary of isolation and antigenic typing of strains of herpes simplex virus from the female genital tract

Site of attempted isolation		Antigenic type of virus isolated		Total
cervix	labia	type 1	type 2	
+	+	1	3	4
+	ND	1	1	2
ND	+	6	15	21
ND	+(thigh also)	0	1	1
-	+	1	1	2
Total		9	21	30

+ = Virus isolation successful

- = Virus not isolated

ND = Virus isolation not attempted

site of the eruption or by endogenous reinfection from the cervix, or by reinfection by contact with an infected partner. Repeat isolations were made from two female and two male patients at intervals up to 3 years. In all but one instance the virus type isolated at the time of the recurrence was the same as at the initial isolation: over a period of 3 months the same type of virus was isolated on 3 occasions from one female patient. Strains 4159 and 9864 isolated from the same patient have been described. For the purposes of the typing studies, this patient has been classified as suffering from a type 2 infection.

#### Serological evidence of nature of infection

The finding that a number of the young adult infections of the mouth were primary in nature has been described. From the evidence presented in this typing section these would all be primary type 1 infections. Infections of the skin yielded 3 type-2 strains from adult patients, and serological studies showed that two of these, the 19-year-old girl and the 60-year-old patient, had antibody in the acute phase serum indicating that the episodes studied were recurrent in nature. No serum samples were submitted from the third patient.

Thirteen serological conversions were found on examining the 42 pairs of serum samples from patients with genital infections. Among the patients from whom type 2 virus was isolated, 3 of the 20 male patients, and 5 of the 14 female patients showed evidence of primary infection. Of the patients who did not show an antibody response two did not develop antibody and the others had antibody in the early serum sample. Paired serum samples were tested from 8

patients, one male and 7 female, from whom type 1 virus was isolated. One male and 4 of the female patients were undergoing a primary infection at the time of virus isolation. One patient did not develop an antibody response, but antibody was present in the acute serum sample from the remaining two patients. The results of the examination of paired serum samples from patients with cervical involvement are included above: one patient was suffering from a primary infection. Single serum specimens were available from the other 4 patients with proven cervical involvement and all contained antibody.

It can be concluded that primary type 2 genital infection is more frequent in females. In the small group of patients from whom type 1 virus was isolated, 4 of the 7 examined were suffering a primary infection.

BIOLOGICAL CLASSIFICATION OF STRAINS OF HERPES SIMPLEX VIRUS

To confirm the finding that a high proportion of strains isolated from the genital tract were antigenic type 1 these and other strains were characterised by biological marker tests. The next section deals with the results of the typing of strains by pock production on the chorioallantoic membrane, cytopathic effect in cell culture, electron microscopical examination of virus infected cells and thermal stability.

The diameter of pock produced on the chorioallantoic membrane

The size of pock produced by strains of herpes simplex virus has been used as a typing character by a number of workers (see Introduction).

To confirm that the strains included in this study were biologically as well as antigenically types 1 and 2, 99 of the virus isolates referred to in the previous section, including 65 from genital infections, were tested for their ability to grow on the chorioallantoic membrane of 10-12-day-old developing chick embryos. As described in Materials and Methods 0.2-ml volumes of dilutions of virus suspensions grown in BHK21 cells were inoculated and incubation continued for 7 days. The diameters of at least 6 separate pocks were measured with a hand vernier lens after the excised membranes had been fixed in formal saline. This method of measurement is not entirely accurate and some variation in the height at which the lens was held above the membrane must have occurred, although the need to have both the pock and the engraved scale in focus would standardise

the measuring system to a great extent.

All the strains examined, apart from the reference strains referred to below, were within a few (less than 10) cell culture passages from isolation.

#### Pock diameters of reference strains

The two reference strains, HF and MS, and strains Dawson, Watson, MP and the local 1657 were examined for the size of pock they produced. Table 43 lists the mean diameters; HF strain gave rise to pocks with a mean diameter of 0.63 mm, whereas the pocks produced by MS virus had a mean diameter of 1.38 mm. The other type 2 strain, Dawson also gave rise to large pocks (1.75 mm). Strains Watson, MP and 1657, all antigenic type 1, produced pocks with diameters less than 1.0 mm, Watson being the largest at 0.80 mm. This initial experiment indicated that the size of pock measured at 7 days was a useful means of characterising isolates.

#### Pock diameters of 99 strains of herpes simplex virus

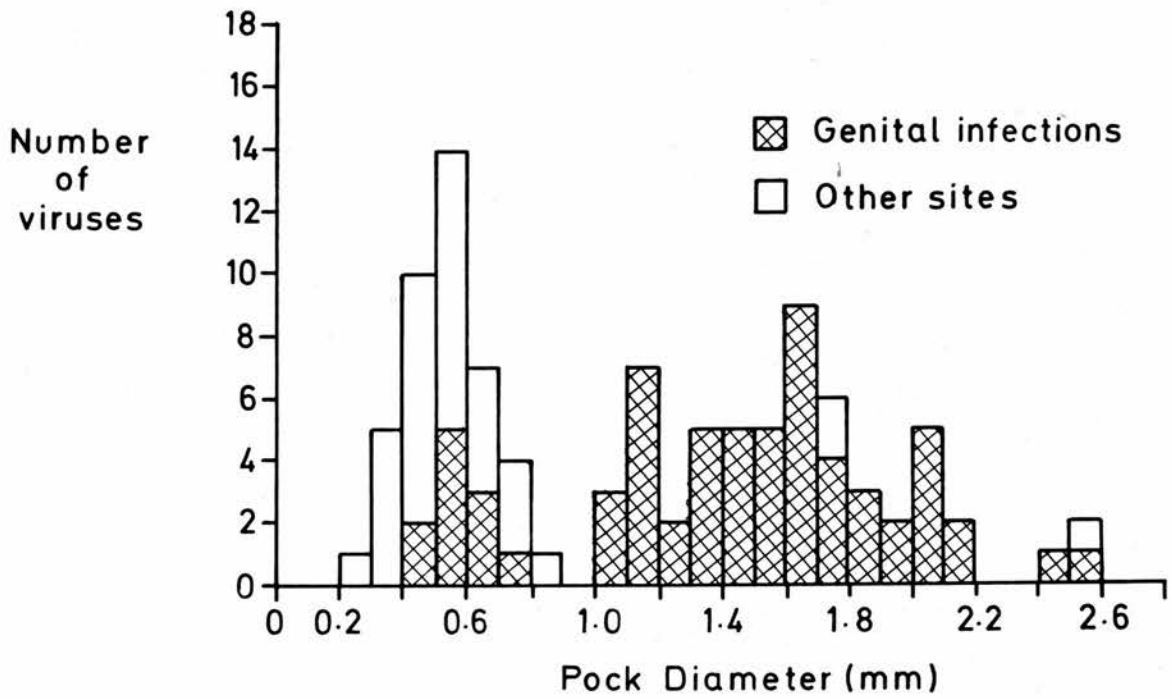
Included in this study are the following virus isolates; 65 strains isolated from infections of the male and female genital tract; 16 from oral infections; 14 from the skin and 4 from the eye. Fifty-two of the viruses examined were from female patients, and the ages of all patients ranged from 1 - 60 years. The overall distribution of the pock diameters is shown in Fig. 12; two groups of pock sizes are apparent. The small pocks are within a narrower range than the larger pocks, as shown also in Table 44 where the mean diameters, and standard deviations of the mean, of the two groups are listed. The small pocks had a mean diameter of 0.52 mm with a standard deviation of the mean of  $\pm 0.13$  mm. The corresponding

TABLE 43

Pock diameters of reference and established strains of herpes simplex virus after 7 days incubation

Strain	Antigenic type	Pock diameter (mm)
HF	1	0.63
MS	2	1.38
Dawson	2	1.75
Watson	1	0.80
MP	1	0.73
1657	1	0.25

All these strains had undergone a large number of passages in cell culture at the time of the experiment. Of the group, 1657 had probably undergone fewest, although it had been passaged many times (>10).



**FIGURE 12.** Poek production on the choricallantoic membrane of the developing chick embryo: distribution of the diameters of lesions produced by 99 strains of herpes simplex virus iselated from different clinical sites of infection.



TABLE 44

Mean diameters and standard deviations of the mean of small and large pocks produced by 99 strains of herpes simplex virus

Type of Pock	Small	Large
Number of strains examined	42	57
Mean diameter (mm)	0.52	1.60
Standard deviation (mm)	$\pm 0.13$	$\pm 0.37$
Maximum diameter (mm)	0.80	2.58
Minimum diameter (mm)	0.25	1.00

measurements for the large pocks were  $1.60 \pm 0.37$  mm. The distinction between the two groups can be made on the basis that the small pocks were never more than 0.80 mm in diameter, whereas the large pocks were never less than 1.00 mm on average. Some representative pocks are illustrated in Fig. 13.

The uniform distribution of the small-pock diameters, and the low standard deviation of the mean, indicated that a significant number of strains had not been adapted by laboratory passage to produce large pocks. If this had occurred, a second distribution peak would have been expected in the upper part of the range.

The greater variation in the diameter of large pocks could be explained in several ways apart from natural variation of pock size. Secondary pock production will not be a source of confusion over short incubation periods but could become a significant feature when incubation times are extended to 7 days. Also, over this period, premature death of the embryo could occur either from injury or contamination introduced at inoculation. Careful examination of the embryo and membranes when harvesting could exclude the most obvious examples of such problems, but they are probably the major cause of the variation noted.

Analysis of the results of pock diameters of type 1 strains according to the age of the source patients and the site of isolation of virus was attempted. Interpretation of the results listed in Table 45 is hampered by the small numbers in some of the groups and the small number of pocks measured; no conclusion could be reached of an association between the age of the patients or the site of infection and the pock diameter of the virus isolated.

TABLE 45

Mean and standard deviation of the mean of pock diameters of type 1 strains of herpes simplex virus according to the age and site of infection of the source patients

Site of infection	Age Group (years)	Number of strains tested	Mean pock diameter (mm)	Standard deviation of the mean
mouth	< 15	6	0.50	$\pm 0.11$
	$\geq 15$	10	0.52	$\pm 0.16$
skin	< 15	3	0.36	$\pm 0.09$
	$\geq 15$	8	0.61	$\pm 0.12$
eye	$\geq 15$	4	0.41	$\pm 0.09$
genital tract	$\geq 15$	11	0.57	$\pm 0.09$
Total	< 15	9	0.45	$\pm 0.12$
Total	$\geq 15$	33	0.54	$\pm 0.13$

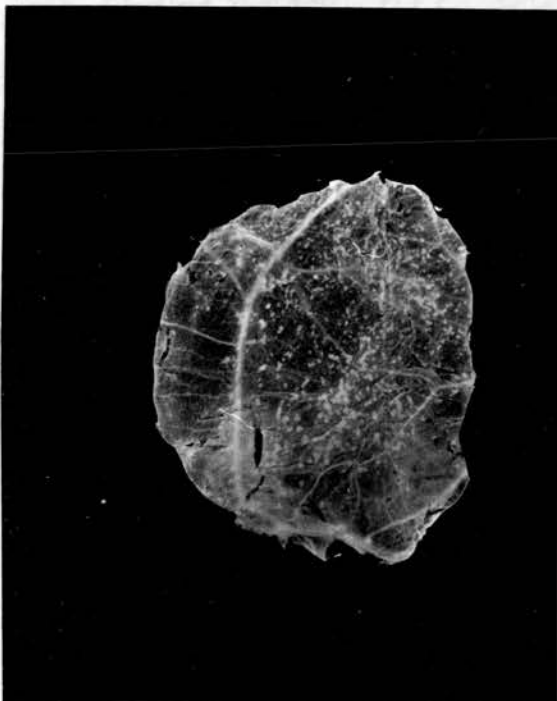
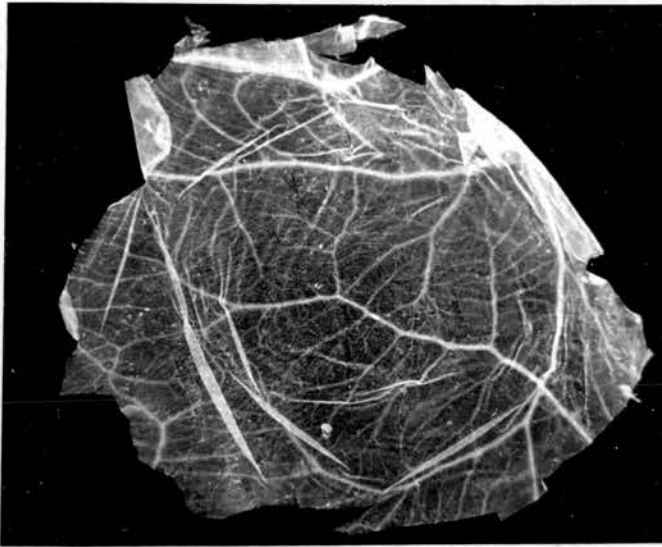
**FIGURE 13.** Pock production on the chorioallantoic membrane (CAM) of the developing chick embryo: appearance of membranes infected with different strains of herpes simplex virus.

Fixed in formal-saline

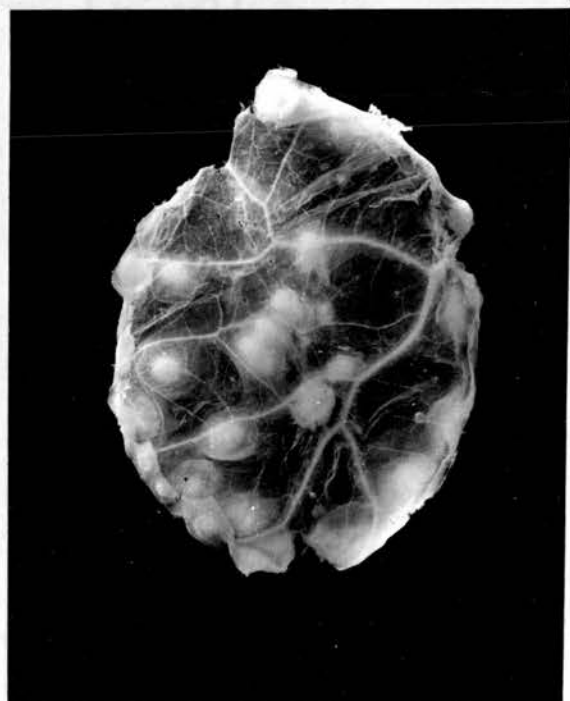
X 2

- A = uninfected CAM
- B = CAM infected with strain Watson (type 1)
- C = CAM infected with strain Dawson (type 2)

A



B



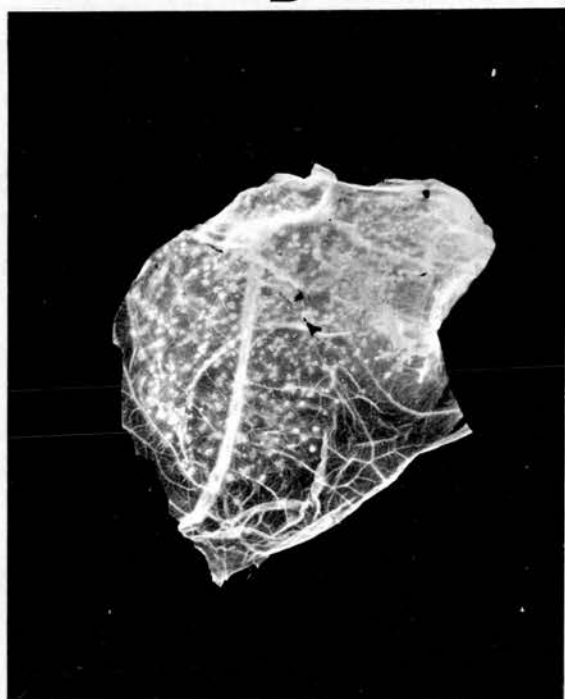
C

FIGURE 13 (continued)

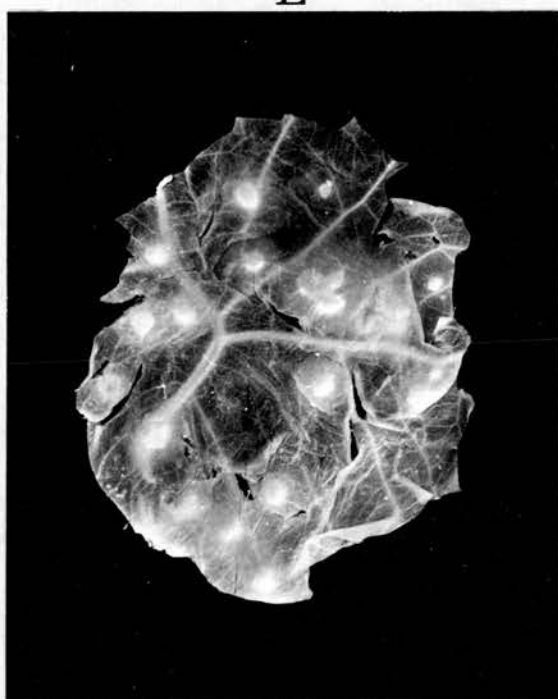
- D = CAM infected with strain 6248 (type 1)
- E = CAM infected with strain 9816 (type 2)
- F = CAM infected with strain 4159 (type 1)

These 3 strains were isolated from infections of the genital tract.

D



E

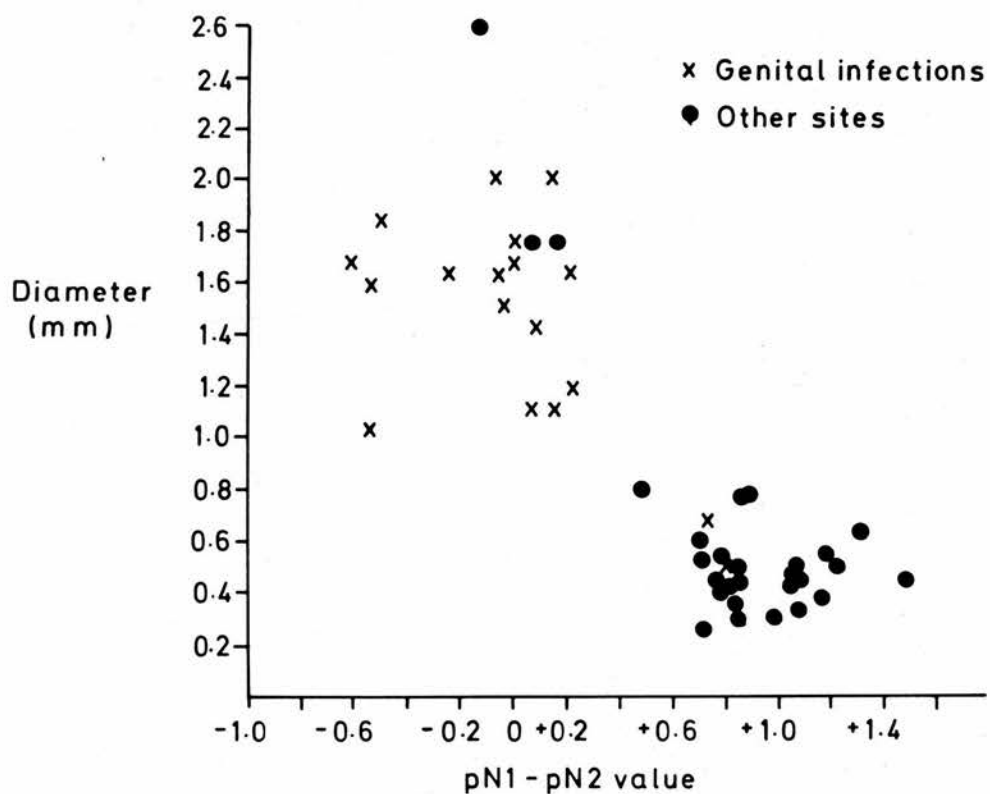


F

It was concluded that the diameter of the pock resulting from inoculation of the chorioallantoic membrane with herpes simplex virus strains could be used to divide strains into two groups. To confirm that the small pock strains coincide with the antigenic type 1 group and the large pock group with antigenic type 2 strains, the  $pN$  difference values obtained with the two sets of reference antisera were graphed against the mean pock diameters (Fig. 14 and 15). These show convincingly that virus isolates in the type 1 range according to  $pN$  difference give rise to small pocks on the chorioallantoic membrane, whereas type 2 strains by  $pN$  difference are large pock-producing viruses. Further evidence that the range of  $pN$  difference values found for type 1 strains with the first set of reference antisera was due to experimental technique rather than intrinsic variation of the viruses was provided by the results of the pock diameter study which indicated that all the type 1 group were small pock-producing strains.

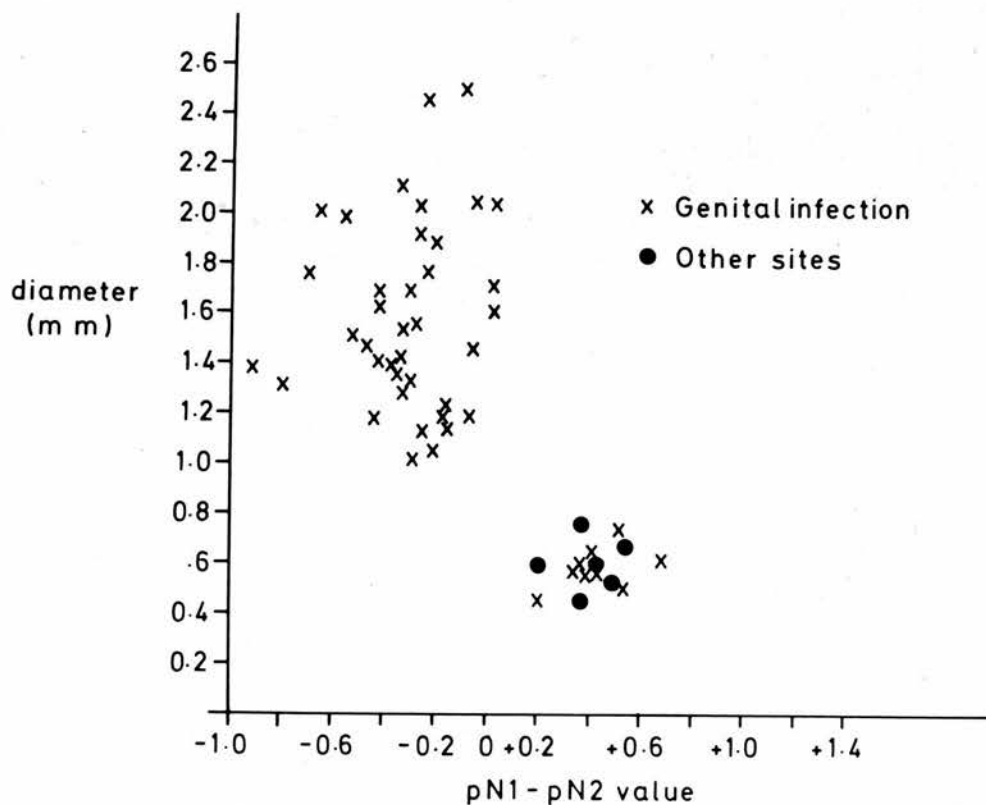
As indicated in Fig. 12, there was a clear association of the large pock strains with genital tract infections. The 11 small pock strains are the same 11 type-1 strains discussed in the previous section. The 3 non-genital, large pock strains are the type 2 strains isolated from skin infections of the lower lumbar, buttock and perianal regions. All the oral, eye, lip, face, finger and other skin strains produced small pocks.

An anomalous situation mentioned in the typing section with strains 4159 and 9864 was confirmed. Although the second isolate, 9864, was type 2 and large-pock-producing, strain 4159 was found to be antigenic type 1, but large pocks were found on the chorioallantoic membrane.



**FIGURE 14.** Correlation between the diameter of pock produced on the chorioallantoic membrane and the pN1 - pN2 value determined with the first pair of reference antisera for 45 strains of herpes simplex virus isolated from different clinical sites.

**FIGURE 15.** Correlation between the diameter of pock produced on the chorioallantoic membrane and the pN1 - pN2 value determined with the second pair of reference antisera for 55 strains of herpes simplex virus isolated from different clinical sites.





The cytopathic effect produced in cell cultures

From the early reports of the growth of herpes simplex virus in cell cultures it was apparent that strains differed in their effects on cultured cells. The characteristic late appearance of the CPE in epithelial or fibroblast cell cultures consists of clumps of round refractile cells with occasional ballooned cells: later they detach from the glass and clusters of round cells can be seen floating in the medium. Some stable laboratory strains can produce cell fusion.

The use of the CPE-type as a means of classification depends on two main considerations; firstly the type of cell culture, and secondly the passage history of the virus strain. The previous passage history is particularly important as the laboratory strains MP and HF epitomise (Table 46). No attempt was made to study the effects of passage on CPE-type, but some observations were made with strain 1657 which confirmed that syncytial formation was a constant, although variable, feature of the CPE after a period of years in culture. The syncytia were never as large as those produced by MP and HF, the term "flat" being an appropriate description. To avoid confusion, the type of CPE produced by a wild strain was determined with as low passage virus as possible, although inevitably many were at the fifth to tenth pass.

RK13 cell cultures were inoculated during the early stages of the isolation studies. This epithelial cell line is as sensitive as any other cell type (Dr Isabel W. Smith, personal communication), and can show syncytium formation. At this stage of the study virus

isolation was attempted from infections of the eye and mouth; only type 1 strains were isolated and no difference in the appearance of the CPE were recognised. Later BHK21 cells were substituted, and differences in CPE became apparent when infections of the genital tract were studied. All isolates gave rise to round refractile cells, but most genital strains produced fewer round cells in foci of infection and these usually contained one or two small elongated or fusiform syncytia. These changes were most apparent during the early stages of the development of a focal CPE in cultures inoculated with a diluted virus suspension: as the virus spread through the cell sheet and affected cells began to fall off the glass, the small syncytia were still apparent, although eventually they retracted and detached also.

The confirmation that the type of CPE is indeed related to the antigenic type of the virus is summarised in Table 46. All 21 freshly isolated type 1 strains produced a similar round cell CPE in fibroblast cultures whereas all 57 type-2 strains gave rise to loose foci of round cells and fusiform syncytia. The CPE of the 6 laboratory strains examined is also described in Table 46. Strains MS and Dawson were still recognisable as type 2, but greater differences were seen with the type 1 strains. The trend to syncytium formation was noted with 1657 and Watson, although this was not as prominent as with strains MP and HF. Some representative CPE appearances are illustrated in Fig. 16.

Figueroa and Rawls (1969) reported that type 2 strains grew in chick embryo fibroblast cultures, but that type 1 viruses did not grow. To investigate this a number of type 1 and 2 strains were

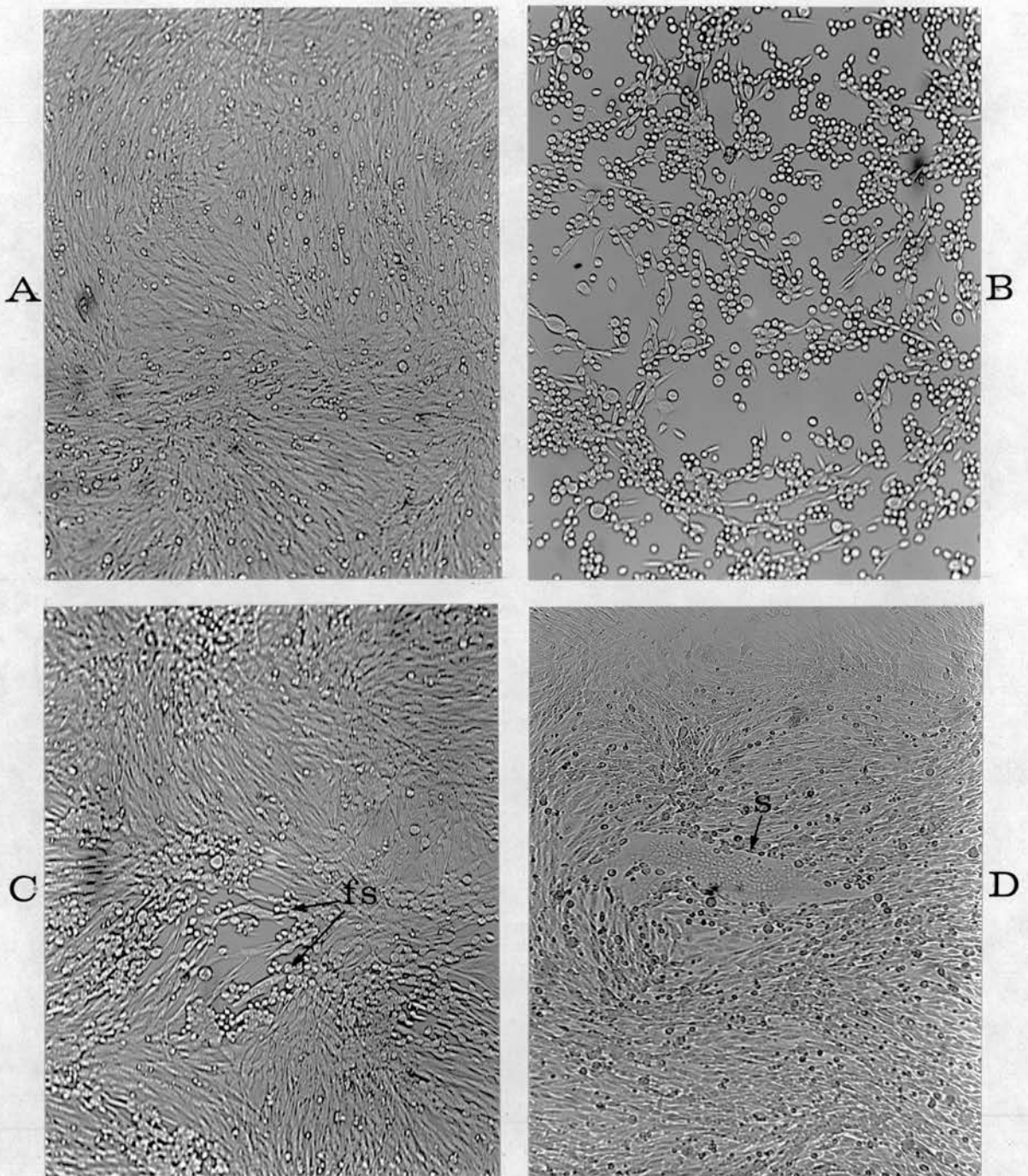
TABLE 46

Classification of 6 laboratory and 78 freshly isolated strains of herpes simplex virus according to the cytopathic effect in cell cultures

Antigenic type	Origin or site of isolation	Number of isolates tested	Cytopathic effect
1	HF *	1	syncytia
1	MP *	1	syncytia
1	Watson *	1	some flat syncytia, round cells predominate
1	1657 *	1	some flat syncytia, round cells predominate
2	MS *	1	round cells, some fusiform syncytia
2	Dawson *	1	loose foci, fusiform syncytia
1	mouth, skin, eye	10	round cells
1	genital tract	11	round cells
2	buttock	3	loose foci, fusiform syncytia
2	genital tract	54	loose foci, fusiform syncytia

\* Laboratory strains

Monolayer cultures of BHK21 cells in tubes were examined unstained by low-power microscopy.



**FIGURE 16.** Cytopathic effect (CPE) in BHK21 cells of strains of herpes simplex virus. Unstained.

A = un inoculated BHK21 cells x 70

B = CPE of strain 4545 (type 1) x 70

C = CPE of strain 2107 (type 2) x 70

D = CPE of strain HF (type 1) x 70

f.s. = fusiform syncytium    s = syncytium

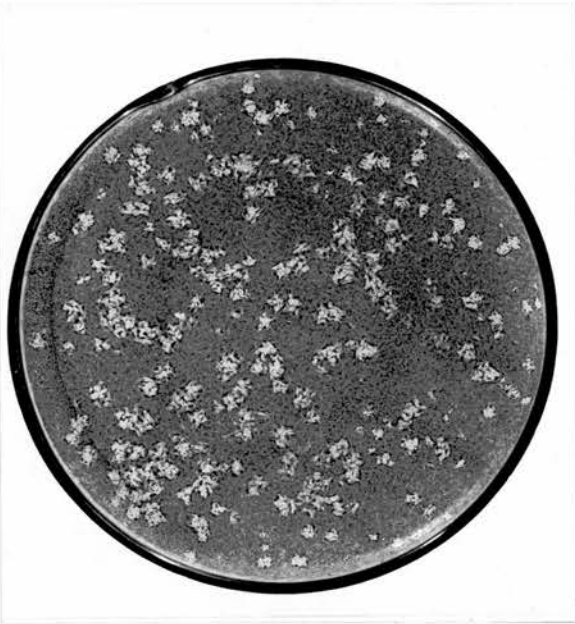
passaged in BHK21 and chick fibroblast cells. Some correlation between CPE and antigenic type was noted, but there was no clear distinction; large inocula of type 1 strains produced rounding of chick fibroblast cells. Some form of plaque assay to detect the production of infectious virus would seem to be necessary to make this a useful method.

It can be concluded that the genital type 1 strains were identical to type 1 strains isolated from other sites such as the mouth, eye and skin. The 3 type-2 strains recovered from skin infections of the lumbar, buttock and perianal regions behaved as typical genital tract type 2 strains.

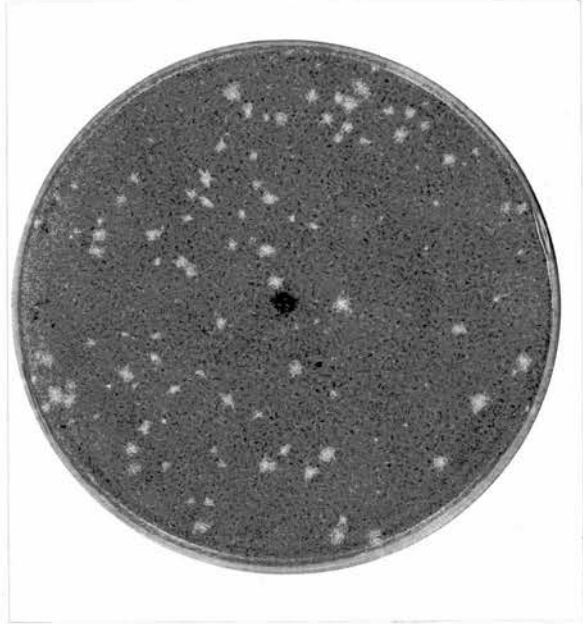
#### Plaque morphology

The size and appearance of the plaques produced in monolayer cultures under a methocel overlay were investigated. Plaques were produced in BHK21 cell cultures, as described in Materials and Methods; plates were drained and stained after 3 days incubation. By this method, differences were apparent between HF and MS viruses (Fig. 17). HF virus produced larger plaques with a hard sharp edge consisting of a rim of retracted syncytium. The MS virus plaques were not so sharp-edged, although they were of the same size. Examination of 4 fresh isolates by this method gave the results illustrated in Fig. 17. Plaque size varied but the main difference can be seen in the edge of the plaques produced by type 2 strains 1718 and 6248 compared with type 1 strains 4210 and 4545. The former pair did not produce as sharp-edged plaques, apparently due to the lack of cell proliferation and the presence of small syncytia in the

A



B



C

**FIGURE 17.** Plaque morphology of laboratory strains HF and MS and four freshly isolated strains of herpes simplex virus in BHK21 cells.

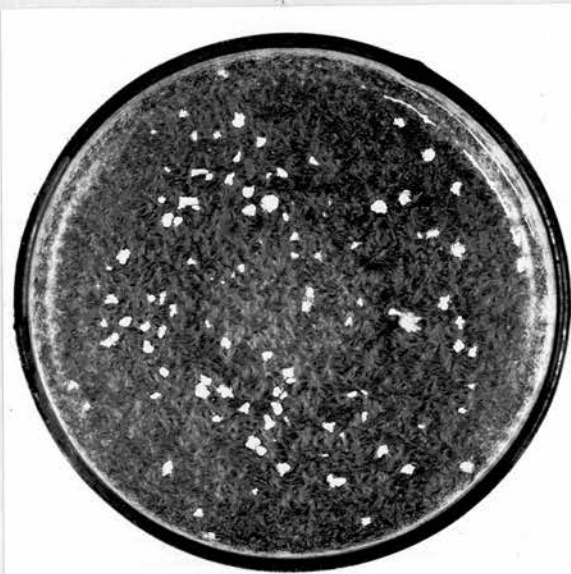
Formal-methyl violet      x 1

- A - uninfected BHK21 cell monolayer
- B - plaques of laboratory strain HF (type 1)
- C - plaques of laboratory strain MS (type 2)

**FIGURE 17 (continued)**

- D = plaques of strain 4545 (type 1)
- E = plaques of strain 4210 (type 1)
- F = plaques of strain 6248 (type 2)
- G = plaques of strain 1718 (type 2)

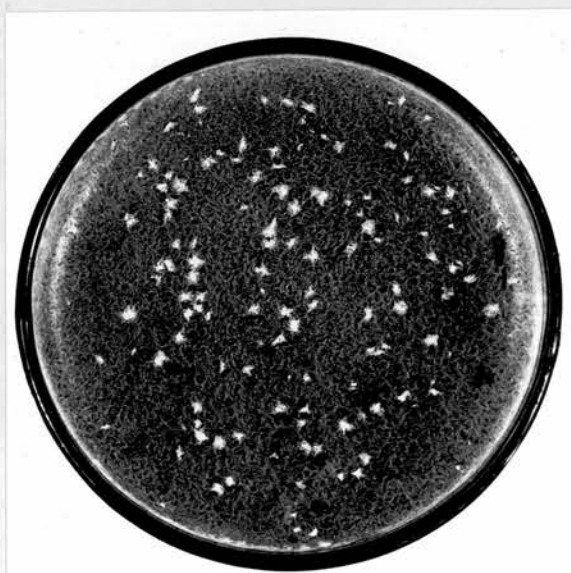
D



E



F



G



margins. Although this method could be used to classify strains, the differences in plaque morphology are not great enough to make this method practicable.

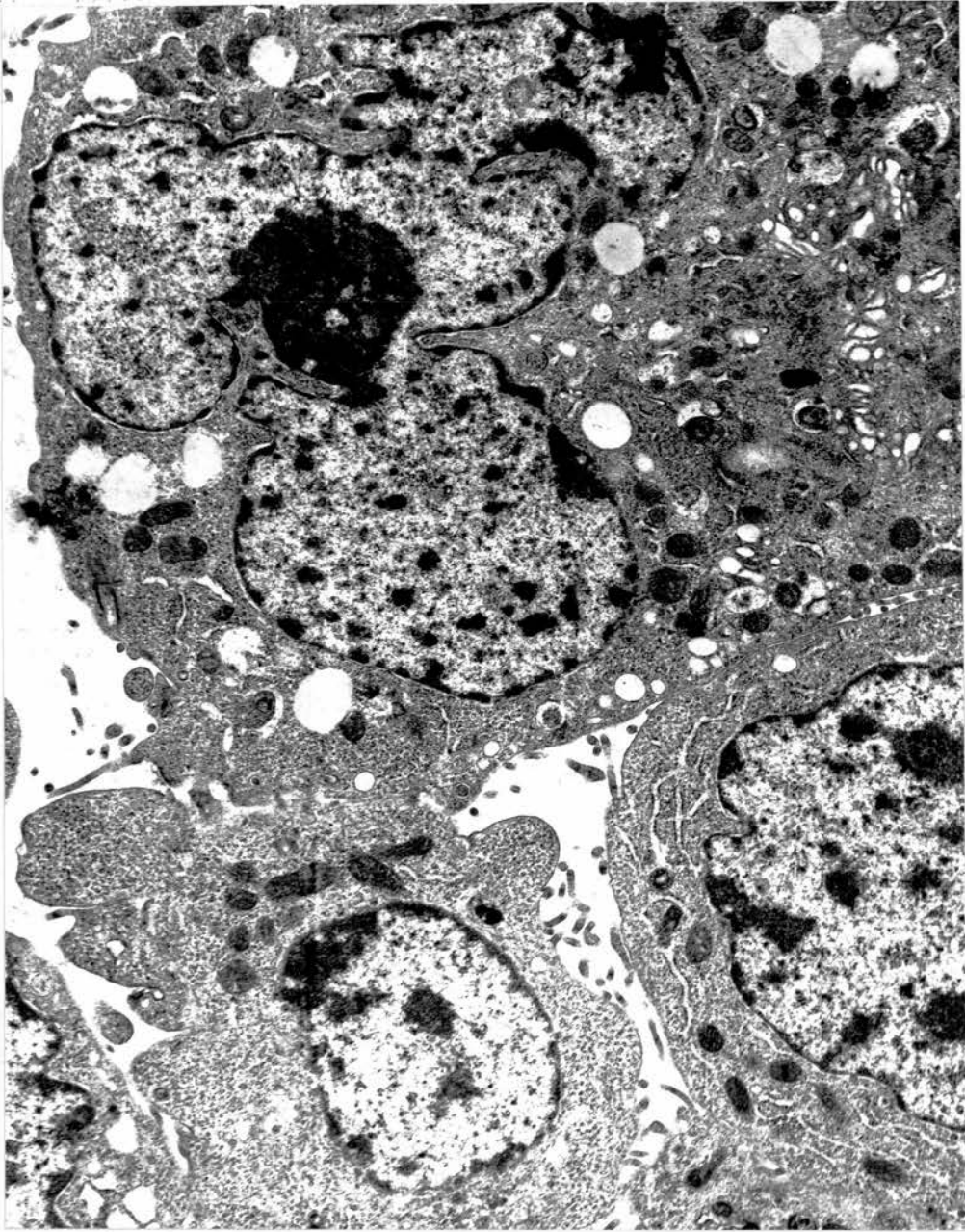
The electron-microscopical examination of infected cells

Monolayer cultures of BHK21 cells were inoculated with sufficient virus to infect the majority of cells and incubated until the CPE was complete; the cells were rolled off the glass with glass beads, fixed and processed as described. Examination of sections allowed each virus to be scored as positive or negative for intranuclear or cytoplasmic microtubules. Two forms of intranuclear structure were visible; a series of parallel filaments or tubules spaced approximately 15 nm apart, or linked "chain-mail" aggregates of diameter 15 nm. The dimensions of the four-sided mesh suggest that it represents the filaments or tubular structures cut in cross-section. Figures 18 - 23 illustrate these findings. The length of the filaments varied considerably; sometimes they passed through the nuclear membrane or were present in the cytoplasm.

Attention was concentrated on virus isolates from the genital tract to establish that the filaments were universally present in type 2 infected cells, and that the 11 genital type 1 isolates did not produce these structures.

All 52 type-2 strains examined showed intranuclear filaments, whereas the 11 type-1 strains did not produce filaments or similar structures. One type-2 strain could not be examined as it was lost due to fungal contamination and one type-2 strain did not show filaments, despite examination at various times ranging from 6 - 48 hr





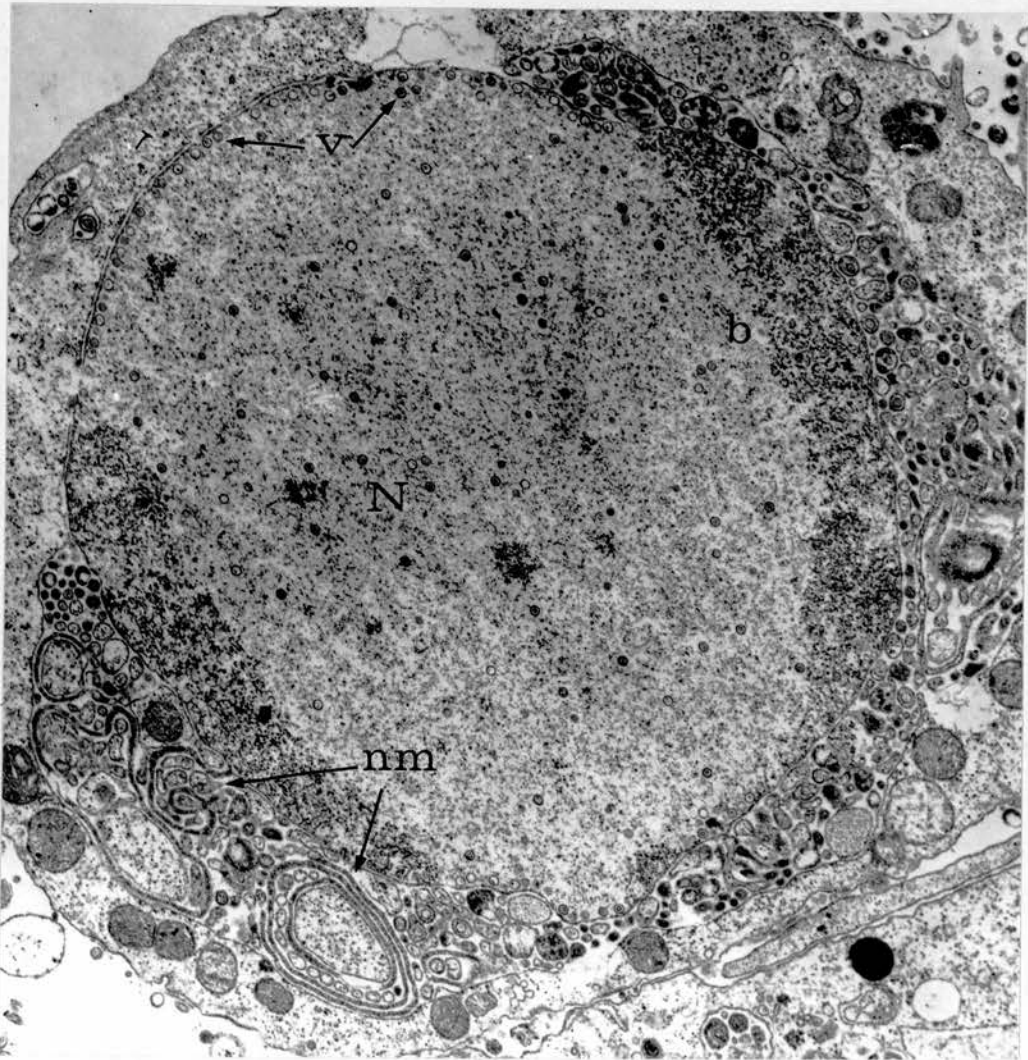
**FIGURE 18.** Electronmicrograph of uninfected BHK21 cells.  
Lead citrate and uranyl acetate X 6.000

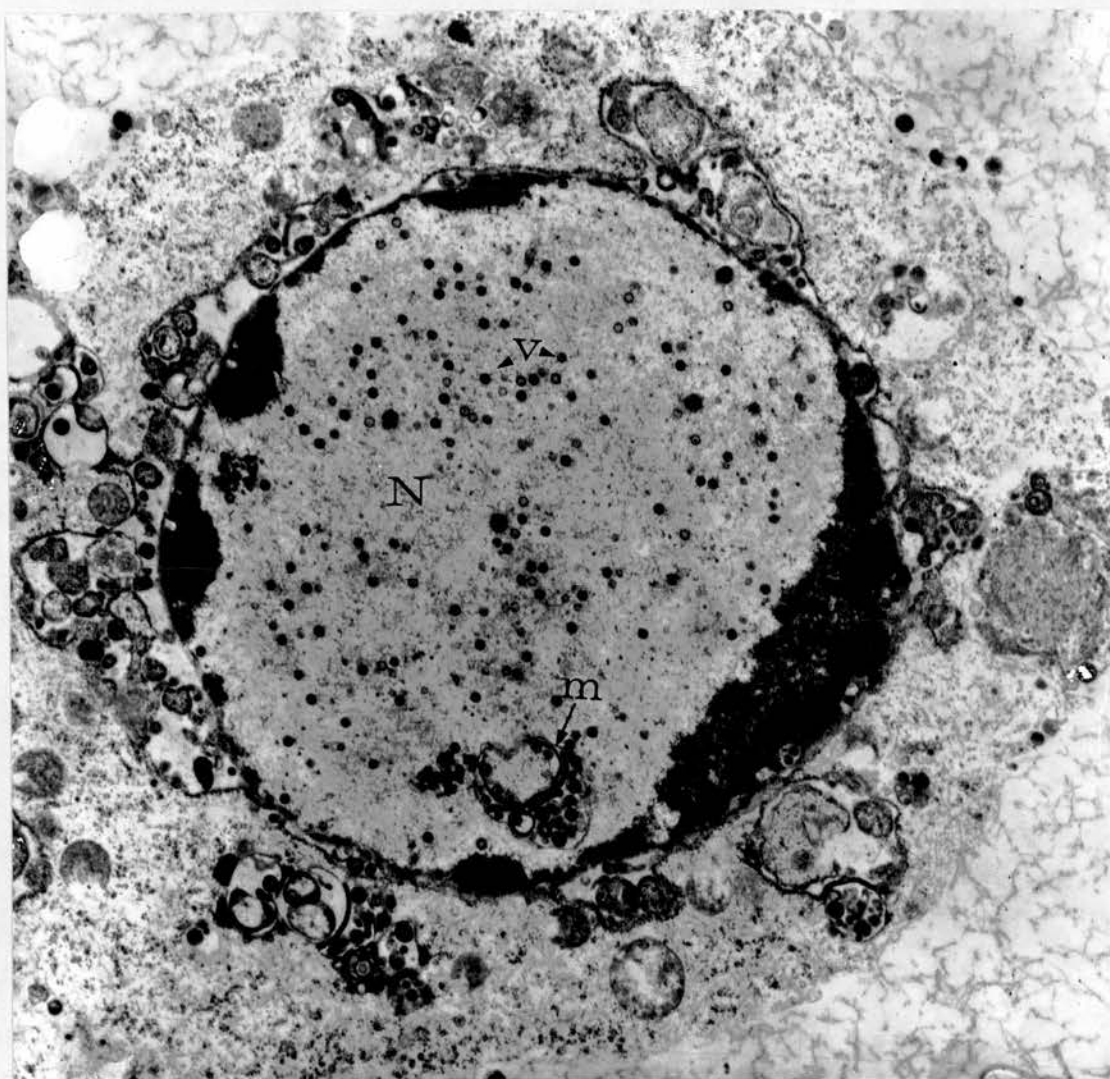
**FIGURE 19.** Electronmicrographs of BHK21 cell infected with herpes simplex virus strain 9165 (type 1).

Lead citrate and uranyl acetate

X 12,000

Nucleus (N) contains unenveloped virus particles (v)  
Marginated host chromatin (b) and extensive reduplication of  
the nuclear membrane (nm) are apparent.





**FIGURE 20.** Electronmicrograph of BHK21 cell infected with herpes simplex virus strain 9712 (type 1).

Lead citrate and uranyl acetate                      x 12,000

The nucleus (N) contains unenveloped virus particles (V).  
Reduplication of the nuclear membrane (m) is apparent.

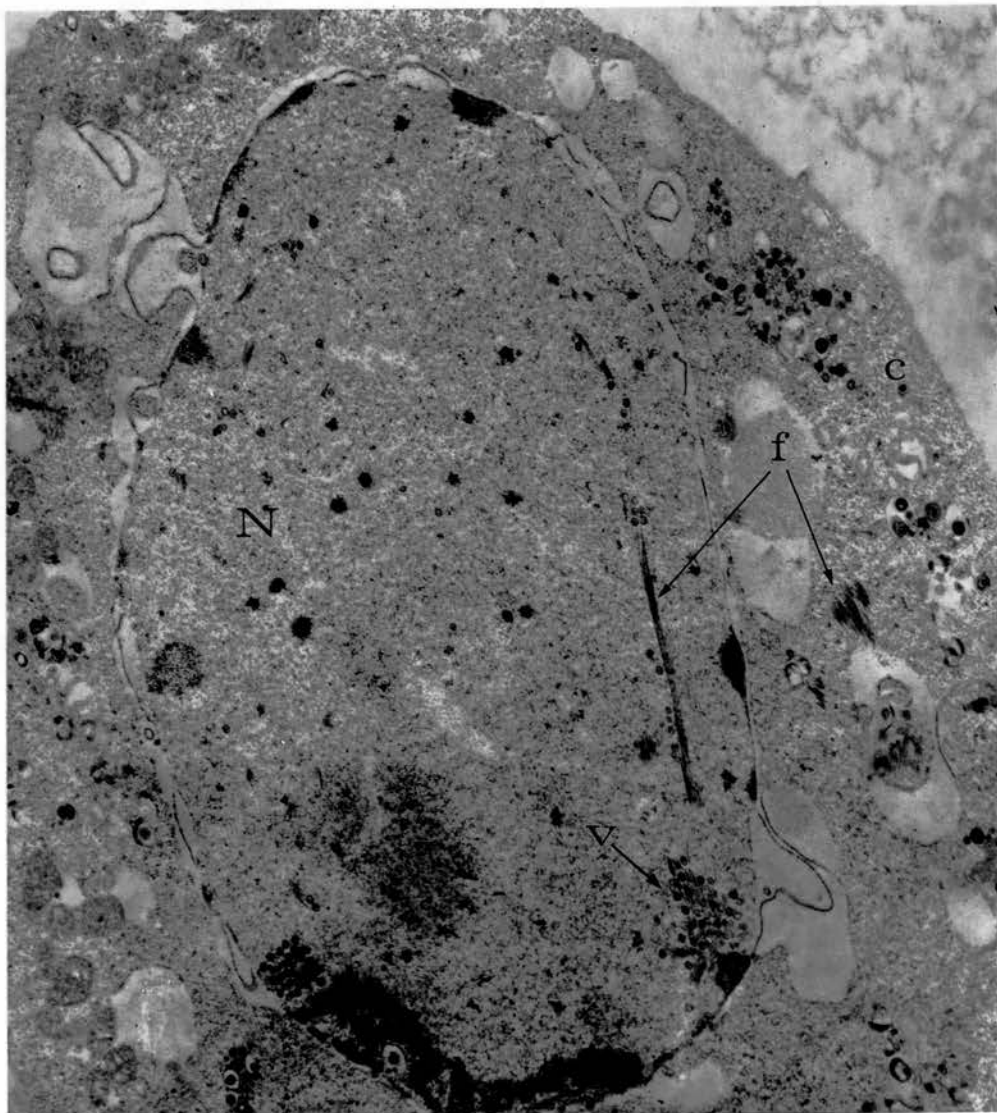
**FIGURE 21.** Electronmicrograph of BHK21 cell infected with herpes simplex virus strain 8928 (type 2).

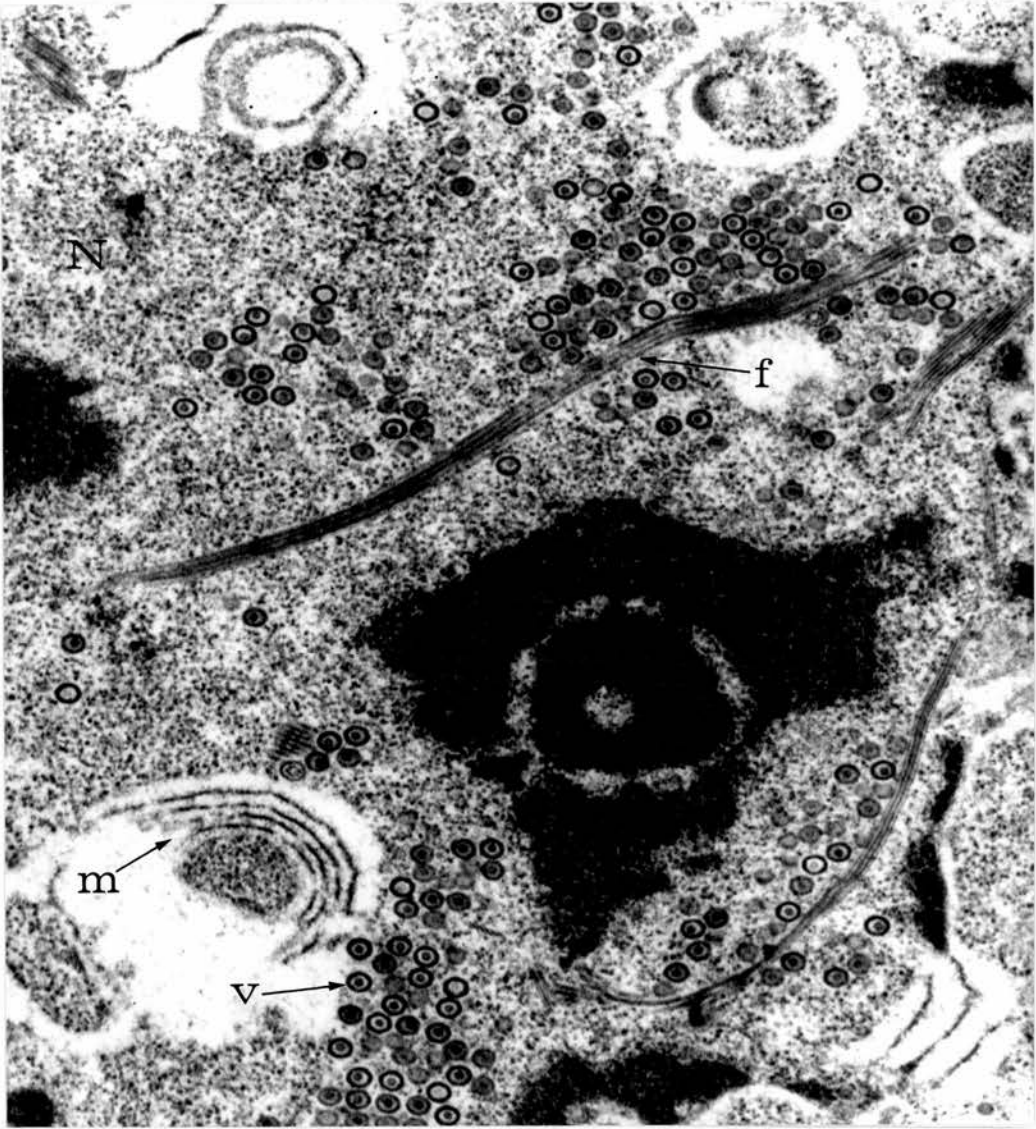
Lead citrate and uranyl acetate

X 10,000

The nucleus (N) contains unenveloped virus particles (V).

Filaments (f) in parallel array present in nucleus and cytoplasm (c).





**FIGURE 22.** Electronmicrograph of BHK21 cell infected with herpes simplex virus strain 9796 (type 2).

Lead citrate and uranyl acetate X 40,000.

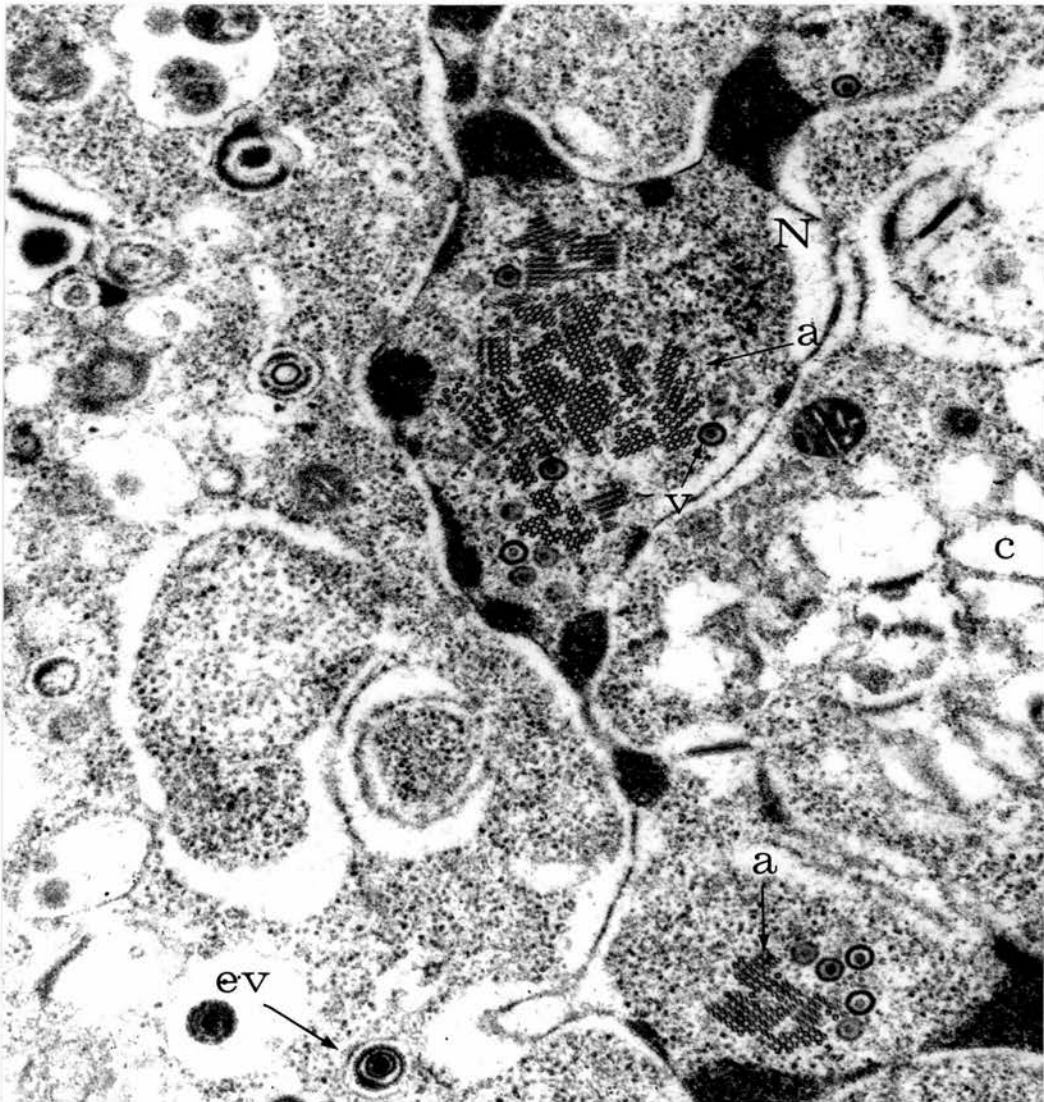
Unenveloped virus particles (V) are present in nucleus (N). The nuclear membrane (m) is reduplicated, but this is distinct from the filaments (f).

**FIGURE 23.** Electronmicrograph of BHK21 cells infected with herpes simplex virus strain 9796 (type 2).

Lead citrate and uranyl acetate

X 40,000

Nucleus (N) contains unenveloped virus particles (V), whereas enveloped virions (ev) are present in the cytoplasm (c). The lattice or chain-mail aggregates (a) are located in the nucleus.



post inoculation. As discussed later this strain (3668) was type 2 according to pock size and CPE, but differed in stability at 4°C.

Thermal stability of type 1 and type 2 strains of  
herpes simplex virus at 4°C and 37°C

Plummer et al. (1968) and Figueroa and Rawls (1969) have shown that type 2 strains are inactivated more readily than type 1 strains and this property was investigated with a small group of type 1 and type 2 strains. Virus stocks propagated in BHK21 cells were ultrasonicated and titrated by the focal method in tube cultures. If the titres were within the range  $10^{5.0}$  -  $10^{6.0}$  ffu/ml the suspensions were used for the thermal stability studies; a ten-fold dilution was made in Eagle's growth medium containing 10 per cent. calf serum, in screw-capped universal glass containers. Samples were incubated in a water bath at 37°C, or in a refrigerator: a rise in pH occurred in all experiments, most rapidly at 37°C.

Preliminary experiments showed that less than 10 per cent. of type 1 and 2 strains survived after incubation for 24 hr at 37°C. At 4°C, 23-67 per cent. of type 1 virus survived and up to 25 per cent. of type 2 strains. An incubation time of 6 hr was selected and virus suspensions prepared and incubated as described. Table 47 lists the results obtained with type 1 and 2 strains at 37°C. No clear distinction was apparent, the mean survival being 56 per cent. for type 1 strains and 45 per cent. for type 2 strains. Considerable variation in survival was noted, and there was overlap between the two groups. The effect of incubation at 4°C was investigated, and the results are given in Table 48. In this instance, a clearer

TABLE 47

Inactivation of type 1 and type 2 strains of herpes simplex  
virus at 37°C

Antigenic type	Virus strain	Initial virus titre	Virus titre after 6 hr at 37°C	Percentage of virus surviving
1	HF	$3.10 \times 10^4$	$1.80 \times 10^4$	58
	6222	$3.73 \times 10^4$	$2.60 \times 10^4$	70
	4545	$1.25 \times 10^6$	$6.28 \times 10^5$	50
	4210	$1.05 \times 10^6$	$5.95 \times 10^5$	57
	6428	$5.35 \times 10^4$	$2.00 \times 10^4$	37
	6359	$6.28 \times 10^4$	$3.98 \times 10^4$	63
2	MS	$3.42 \times 10^4$	$2.33 \times 10^4$	68
	6248	$2.83 \times 10^5$	$7.40 \times 10^4$	26
	1718	$3.23 \times 10^4$	$1.03 \times 10^4$	32
	6587	$6.93 \times 10^4$	$2.58 \times 10^4$	37
	6653	$1.73 \times 10^4$	$9.50 \times 10^3$	55
	5760	$2.65 \times 10^4$	$1.20 \times 10^4$	45
	3668	$1.65 \times 10^6$	$9.08 \times 10^5$	55



TABLE 48

Inactivation of type 1 and type 2 strains of herpes simplex  
virus at 4°C

Antigenic type	Virus strain	Initial virus titre	Virus titre after 6 hr at 4°C	Percentage of virus surviving
1	HF	$8.40 \times 10^4$	$6.80 \times 10^4$	81
	6222	$3.73 \times 10^4$	$3.88 \times 10^4$	104
	4545	$1.88 \times 10^6$	$1.68 \times 10^6$	89
	4210	$1.45 \times 10^6$	$1.18 \times 10^6$	81
	6428	$3.18 \times 10^4$	$2.98 \times 10^4$	94
	6359	$5.60 \times 10^4$	$5.10 \times 10^4$	91
	2865	$5.93 \times 10^5$	$5.03 \times 10^5$	85
2	MS	$2.30 \times 10^5$	$1.23 \times 10^5$	54
	6248	$2.83 \times 10^5$	$1.22 \times 10^5$	43
	1718	$6.00 \times 10^4$	$3.33 \times 10^4$	55
	6587	$1.00 \times 10^5$	$4.90 \times 10^4$	49
	6653	$3.05 \times 10^4$	$1.48 \times 10^4$	49
	5760	$4.18 \times 10^4$	$2.15 \times 10^4$	51
	3668	$1.36 \times 10^6$	$9.75 \times 10^5$	72

distinction was found between type 1 and 2 strains. For the 7 type-1 strains tested, the mean survival after 6 hr at 4°C was 89.3 per cent., the lowest value recorded being 81 per cent. The mean survival of the 7 type-2 strains was 53.5 per cent. the values lying in the range 43 - 72 per cent. If this last figure is omitted as being greater by 17 per cent. than all other type 2 values, then the mean 6 hr. survival at 4°C becomes 50.1 per cent. The exclusion of this aberrant strain 3668 seemed justified as this isolate had already been found to be atypical in that it did not produce intranuclear filaments.

No attempt was made to establish the various environmental conditions which could have influenced the sensitivity of the method. The proportion of virus inactivated after 6 hr at 37°C was not great and titres were reduced by little more than 1  $\log_{10}$ . At 4°C, the loss of titre with type 2 strains was about 50 per cent. and this is close to the experimental variation of the titration system.

As the other methods had proved reliable this method of distinguishing virus strains was not examined further. The results obtained were in agreement with previous findings and some extra information concerning the atypical strain 3668 was obtained.

Virus isolates showing abnormal features

Strains 4159 and 9864

A strain of virus, 4159, was isolated from penile lesions of a 40-year-old patient. Antigenic typing clearly indicated that this was a type 1 strain, but large pocks were produced on the chorio-allantoic membrane (Fig. 13). Both these observations were confirmed. It was considered that the strain designated 4159 might be a mixed culture, and therefore 10 discrete pocks were cut out from good membranes, rinsed in saline, placed in 1.5 ml of 1 per cent. skim milk (with penicillin and streptomycin) and frozen and thawed three times. These pock-derived viruses were then inoculated to cells in BHK21 tubes, and when the CPE was complete, the contents of each was frozen and thawed and passed to further BHK21 cells to produce pock-derived virus stocks. Suspensions were typed by the usual method, re-inoculated to the chorioallantoic membrane, and at low input multiplicity to further tube cultures of BHK21 cells to examine the type of CPE produced. The results of these studies are recorded in Table 49; 7 of the 10 pock-derived strains were antigenic type 1 and 3 were allocated to type 2. The other characterisation methods produced the results indicated in that the CPE-type was in agreement with the antigenic type, whereas the pock diameters were consistently large except in two instances, when the average size observed was less than 1.00 mm (0.91 and 0.93 mm). These values are within the limits for a type 1 strain although they are at the upper end of the type 1 pock diameter distribution. Some difficulty was encountered in deciding the type of CPE produced, as these strains had been passed

TABLE 49

pN differences, antigenic type and pock size of strains 4159 and 9864 and 10 pock-derived stocks of strain 4159

Virus strain	HFT	MST	pN1	pN2	pN Diff.	Antigenic type	Pock diameter (mm)	CPE in BHK cells
4159 a*	1.95	≤ 1.00	2.46	≤ 1.51	≥ 0.95	1	1.00	round and ballooned
" b	1.71	1.67	2.11	2.07	+ 0.04	2	1.53	small spindle syncytia
" c	1.75	≤ 1.00	2.43	≤ 1.68	≥ 0.75	1	1.43	round and ballooned
" d	1.90	≤ 1.00	2.60	≤ 1.70	≥ 0.90	1	0.91	flat syncytia
" e	1.99	≤ 1.00	2.50	≤ 1.51	≥ 0.99	1	0.93	flat syncytia
" f	1.61	1.58	2.09	2.06	+ 0.03	2	1.45	small spindle syncytia
" g	2.04	1.98	2.40	2.34	+ 0.06	2	2.00	spindle syncytia
" h	2.15	1.20	2.57	1.62	+ 0.92	1	1.56	large flat syncytia
" i	1.99	≤ 1.00	2.54	≤ 1.55	≥ 0.99	1	1.33	flat syncytia
" j	1.71	≤ 1.00	2.40	≤ 1.69	≥ 0.71	1	1.26	round and ballooned
4159* (isolated 12.12.67)	2.50	1.35	2.76	1.61	+ 1.15	1	1.50	round cells
9864 (isolated 6.9.71)	2.11	2.09	2.71	2.69	+ 0.02	2	2.04	fusiform syncytia

\* Mean of values listed in Table 32. 4159a-j typed with 1st reference antisera.

an additional 3-4 times including one pass on the chorioallantoic membrane and as with laboratory strains of virus the CPE was mixed and showed both round cells and small "flat" syncytia. The typical spindle-shaped or fusiform syncytia seen with type 2 strains could be distinguished from this appearance, mainly by the breadth of the syncytial formation.

The same patient reappeared almost four years later, and a further isolation of virus was made from the same site. This virus was found to be a typical type 2 strain.

#### Strain 3668

Strain 3668 was isolated from penile lesions of a 19-year-old patient; serologically and by CPE it was a type 2 strain. The diameter of pock produced was 1.01 mm, a value that is just within the type 2 range (Fig. 12). In addition, the absence of intranuclear filaments has been described: this does not agree with the antigenic type. Failure to detect the filaments could explain this, although these structures were readily detected with other type 2 strains, but could not be found at any time post-infection with strain 3668.

Because of these abnormalities, this virus was examined for its thermal stability, and as illustrated in Table 48, 3668 was found to be intermediate between type 1 and type 2 strains.

Summary of results of characterisation of herpes simplex virus isolates

The preceding sections have demonstrated that strains of herpes simplex virus can be divided into two types by serological studies and a range of biological characteristics. Table 50 summarises the results for strains that have been allocated to type 1 or type 2 by at least two of the 4 main methods described. Good agreement has been demonstrated between all the methods and the results of the antigenic typing, with the two exceptions described.

The characterisation of strains of herpes simplex virus was necessary to confirm that the serological classification of strains was reliable. This has been established, and the finding that 16.7 per cent. of genital viruses are type 1 is confirmed.

TABLE 50

Summary of findings of characterisation  
of 104 strains of herpes simplex virus

Number of strains examined	Antigenic type	Size of pock	CPE type	Filaments in nucleus
11	1	small	round cells	absent
5	1	small	round cells	ND
26	1	small	ND	ND
5	1	ND	round cells	ND
52	2	large	fusiform syncytia	present
1	2	large	fusiform syncytia	absent
4	2	large	fusiform syncytia	ND

ND = not examined for this property



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DISCUSSION

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The results of our serological survey of patients in the Edinburgh region (Smith et al., 1967) suggested that serological conversion or primary infection often occurred in the 15 - 25 year age group; this finding led to a study of the isolation of herpes simplex virus from different clinical sites. The results of any study such as this must always be influenced by the population studied, and interpretation made in the same way. The age distribution of all patients from whom the virus was isolated shows that the virus does infect young adults, and in the following sections the results of the present study are discussed in relation to previous reports of infection at each of the major sites.

#### ISOLATION OF HERPES SIMPLEX VIRUS FROM DIFFERENT SITES

##### Oral infections

The patients with oral infections came from two main sources, a general practice and a periodontal out-patient department, and the two peaks in the age distribution are related to these sources. The general practice study with Dr. J. D. E. Knox accounted for most of the 67 isolations from children less than 9 years of age. The majority of the isolations were from children with clinical stomatitis, and the estimated incidence was 5.0 per 1000 per annum; most children were aged 2 - 3 years (Knox, 1967). These observations are in agreement with earlier studies; Dodd et al. (1938) and Scott et al. (1941) found that 70 per cent. of their hospital stomatitis patients were less than 6 years old. The overall isolation rate of 77 per cent.

for Edinburgh children almost certainly resulted from the prompt collection of specimens from children with a well-defined clinical condition.

Only 16 of the children were proved to be suffering from a primary herpetic stomatitis, a reflection of the few serum samples collected due to the unwillingness of the doctor to perform venepuncture on patients of this age.

Herpes simplex virus was isolated from the mouths of 40 patients aged over 15 years. Of interest is the finding that 25 of these were proved to be primary infections. It could be claimed that the adult cases were reinfections, the antibody produced in response to the childhood primary stomatitis having declined to undetectable levels. In these circumstances the serological response to reinfection would share the features of a secondary or immune response and should occur very rapidly. In the patients tested, the time post-infection at which antibody was detected was similar to that in the children with stomatitis: the pattern of the response also showed the same features (Smith and Peutherer, unpublished results).

The isolation of herpes simplex virus from the oral cavity has to be interpreted with caution in view of the findings of Buddingh et al. (1953) who isolated the virus from symptomless patients. As mentioned in the Introduction their isolation rate varied from 20 per cent. of children to 2.5 per cent. of the accompanying adults; no serological investigations were undertaken to establish if these were related to subclinical primary infections. Other studies have not confirmed these findings and isolation rates of less than 1 per cent. from unselected cases have been reported

(Herrmann, 1967; Haynes, Azimi and Cramblett, 1968).

Undoubtedly some of the isolations recorded in the present study could be of this type; the isolations from the patient with pericarditis and from the mouth of another following a dental extraction were associated with antibody in the acute phase serum sample, and no change in titre was found. An association between stomatitis and dental anaesthesia has been described (Reade, 1961); Griffin (1965) and Southam et al. (1968) have recorded similar cases, including a 20-year-old patient with a gum boil, but they did not perform antibody studies. The clinical diagnosis of primary herpetic stomatitis was made in the great majority of the Edinburgh Dental Hospital patients, and this was confirmed by the significant number of serological conversions detected.

This series of isolations in conjunction with the serological evidence is an indication that primary herpetic stomatitis in adolescents and young adults is not a rare disease. This age group is also susceptible to other infections such as Vincent's stomatitis and the problem of diagnosis, coupled with the delay in the patient reaching an out-patient department, probably explains the poor isolation rate of 41 per cent. compared with the higher rate in children seen in general practice.

Herpetic stomatitis in adults has been described on a number of occasions: Youmans (1932) and Long (1933) described the disease and isolated the virus. Scott et al. (1941) and Rogers et al. (1949) reported 4 serologically confirmed cases and Kilbourne and Horsfall (1951b) described two patients; the ages ranged from 21 - 42 years. Thirty-six patients were seen by Farmer (1956) during a 3-year

period; virological confirmation was available in 21 cases. These patients were aged 16 - 46 years, and serological studies showed that 12 were primary infections. A history of contact with cold sores and mouth ulcers was given by 16, in agreement with the observation that two of the Edinburgh patients said that their boy friends had cold sores some days before the onset of stomatitis.

Other reports have been unsupported by virus isolation or serological studies, and the diagnosis has been based on the clinical picture and severity of the illness (Cooke, 1958; Southam et al., 1968). Reade (1961) in reviewing the subject of oral herpes simplex states that 10 - 15 per cent. of the population are either adolescents or adults when they first experience this infection. There is no evidence in the paper to support this claim, and no reference is made to another source. No other study could be found to support such a high attack rate in these older ages. Sheridan and Herrmann(1971) reviewed the literature to that date and found reports of 82 cases in which there was some laboratory confirmation of intraoral infection in adults between the ages of 17 and 67 years. They also presented their own results of the isolation of the virus from 82 adults with oral lesions during an 8½-year study. The mean age of their patients was 31.5 years, and 30 were males. Surprisingly, 20 per cent. were immunologically deficient as a result of therapy for some other disease; a similar case was seen in the Edinburgh study. The severe lesions occurring in patients who are immunologically suppressed for renal transplantation (Montgomerie et al., 1969) have usually involved the face, as well as the mouth, oesophagus and other sites: herpes labialis was present in half of these patients, and serological

studies of the 4 patients described in detail showed that they all had antibody to the virus from the onset of symptoms, and no changes in titre were observed. These observations suggest that at least some of the patients described by Sheridan and Herrmann (1971) were not primary infections.

Davies and Longson (1970) in describing herpetic infection of the fingers of nail-biters, recorded that they had made the clinical diagnosis of acute gingivostomatitis in 25 adult patients in a period of 2 years. During a 4-year period Juel-Jensen and MacCallum (1972) made the diagnosis in 18 patients from a population of students, nurses and technicians. Antibody studies indicated that 17 of these were probably primary infections and the degree of systemic upset and lymphadenopathy in adults made this a severe disease, compared with the presentation in childhood.

The finding of 25 young adult patients with confirmed primary herpetic stomatitis, and another 12 with the clinical features of the disease indicates that in the Edinburgh region this disease is not uncommon: the significant proportion of the young adult population without antibody aiding the spread of the virus.

#### Skin infections

Infections of the lip, mucocutaneous junction and face are the most frequently recognised forms of herpetic infection, and their true prevalence in the population is not reflected in the numbers of patients included in this study. The reasons for this are (i) infection at these sites is usually recurrent in type, little systemic upset is present and the disease is seldom so severe as to merit

attention; (ii) these forms of recurrence frequently complicate some other infection or febrile illness and (iii) the diagnosis is usually obvious on clinical grounds alone. Virus can be isolated from labial and facial lesions in young children but the number in this study is due to the interest of Dr. Knox in following the progress of children with previously diagnosed herpetic stomatitis. Larger studies based on clinic attendances suggest that most patients with skin involvement are between 21 and 40 years. In the American study of Gold, Stewart and McKee (1965) there were very few children under 10 years of age and 80 per cent. of infections were said to be recurrent in type. Similar age distributions of patients attending clinics have been recorded by Eilard and Hellgren (1965) and Orusco and Brun (1969). The latter study in Geneva detected 388 cases out of 22,835 clinic attendances over a 10-year period. Included in the total are patients with genital herpes, and more than a quarter of the patients were aged 20 - 30 years. Both Gold et al. (1965) and Orusco and Brun (1969) found that herpes labialis and facialis were more frequent in women, although this could reflect a greater concern for appearance as well as the known association with menstruation. The male:female ratios recorded in Edinburgh are based on small numbers of patients and this may explain the lack of female patients. The frequent association of labial and facial herpes with upper respiratory tract infection would suggest that the greatest number of cases should be seen in the autumn and winter months: such an association was seen with the lip infections in the present study,

The finding that labial and facial herpes simplex is often precipitated by another illness was confirmed by the diagnosis of herpes

during the treatment of syphilis, lead toxicity and benign encephalitis. As expected these were serologically recurrent in type, as were all the other patients tested with the exception of one patient with herpes labialis following a renal transplant and 3 patients with primary facial infections: two of these were adult patients. Primary infection of these sites could have been reflected in the oral cavity, but no clinical evidence to support this was reported.

Apart from its small size the most obvious feature of the skin infection group is the low isolation rate. This must be due to the selection of the difficult case for investigation, where confusion could exist between a variety of vesicular skin lesions. The affected areas ranged from the back to the buttock and perianal regions and in 3 cases, including a 21-year-old girl, a primary infection was diagnosed.

Despite the small number of patients, some of the features of herpes simplex of the skin were illustrated. The primary form with scattered lesions on the trunk and limbs and the localised recurrent form are both represented. The possible confusion with zoster (Juel-Jensen and MacCallum, 1972) is emphasised by the patient with recurrent buttock lesions. The association with trauma commented on by these authors is also reflected in the 18-year-old girl who apparently transferred virus from a lip lesion to a cut on the hand. Trauma of another sort was involved in the activation of perianal infection in an 18-year-old following a septic abortion.

#### Herpetic whitlows

Two age distributions have been described for herpetic

infection of the fingers, and these may be related to different sources of the virus. During primary infection, virus is present in the saliva and transfer to the finger may be achieved by finger sucking and nail biting; this has been described in young children (Buddingh et al., 1953; Knox, 1967) and in adults (Davies and Longson, 1970). One of the children described in the present study had clinical evidence of stomatitis. Among older patients, herpetic whitlows have been described among medical and dental personnel (Stern et al., 1959; Hambrick et al., 1962). In Edinburgh, the nurses did not appear to have worked in a particular area of the hospital before the lesions appeared. Six were primary infections, although one girl with a history of cold sores and a recurrent type of antibody pattern contracted infection despite the presence of antibody. Another nurse gave a history that suggested an alternative source of virus: her boy-friend suffered from cold sores and he had a recurrence about 3 weeks before the whitlow developed.

#### Eye infections

The number of patients diagnosed as suffering from herpetic keratitis was large enough to allow some analysis of clinical severity, and the success of isolation studies with the two types of specimen collected.

The lowest isolation rate was found with clinical grade 1 lesions where only a small ulcer was present: not all of these would be herpetic in origin. Greater success was found with more obvious dendritic ulcers, although in many cases symptoms had been present for 2 - 3 weeks before the virus isolation was made. Gundersen (1936)



found a similar distribution of the duration at isolation, and even succeeded in recovering the virus up to 10-11 weeks after the onset of symptoms.

Not surprisingly, isolation was made more reliably from corneal scrapings from the edge of the lesion, rather than from a simple conjunctival swab.

The isolation rate of 72 per cent. is in agreement with other studies: Coleman, Tsu and Jawetz (1968) isolated virus from 21 of 27 patients (78 per cent.) and found that the administration of idoxuridine in the preceding 48 hr greatly reduced the success rate. No such therapy had been administered to any of the Edinburgh patients before they attended the clinics. Gundersen (1936) had a 63 per cent. success rate. Coleman et al. (1968) commented that the virus was most readily isolated from typical early dendritics in the very young. This age group does not seem to be adequately represented in the Edinburgh study as larger series have found a significant number of patients under 10 years of age. Gold et al. (1965) in a study of 2,695 patients diagnosed infection in 227 (8.4 per cent.) children under 10 years of age; Norn (1970) found 15 per cent. of his patients were less than 10 years old. No explanation is available for the small number (3 per cent.) under 10 years in Edinburgh, as the Ophthalmology clinics are not restricted to adults, and both in-patients and clinic attenders are included in the study. The diagnosis of primary ocular involvement is often made in children and young adults (Jones, 1959), but the laboratory definition of these infections as true primaries does not always appear to have been undertaken. To the ophthalmologist, primary herpetic keratitis can mean the first involvement of the eye

often associated with conjunctivitis, rather than an infection associated with serological conversion. This may account for the different frequencies reported; 4.5 - 7 per cent. of all cases (Gundersen, 1936; Norn, 1970), compared with 52.5 per cent. (Gold et al., 1965). In Edinburgh no true primary infections were seen: all patients appeared to be undergoing a recurrent form of infection, including some experiencing their first corneal involvement.

The overall age distribution of patients in Edinburgh is in general agreement with other studies, confirming that this is a disease of older patients. Gundersen (1936), Norn (1970) and Gold et al. (1965) all found that adults in the 40 - 50 year age-range were the most frequently affected; in Edinburgh the mean age of patients was greater than 50 years. All the series discussed found that male patients were in the majority; this has been related to minor trauma to the eye as a predisposing factor. The Edinburgh series showed the same trend, although a history of trauma was rarely elicited.

The finding that just less than half of the patients were seen in the early part of the year is in broad agreement with reports from other parts of the world; these have shown that the disease is commonest in the Autumn in England (Jones, 1959), and New England (Gundersen, 1936) and Spring in other parts of the United States of America (Norn, 1970). Most patients give a history of cold sores (Norn, 1970) although in Edinburgh only 3 patients had evidence of concurrent herpes facialis, but 7 had suffered from an upper respiratory tract infection shortly before eye involvement had developed.

The results in this thesis confirm that herpetic corneal

infection is recurrent in type, and is often transferred to the eye from another site of infection. No information from the study can throw any light on the pathogenesis of recurring eye infections. The greater frequency in older patients is reminiscent of zoster: perhaps the special conditions in the eye, and the efficiency of cell-mediated immune responses influence the incidence.

### Genital tract infections

The finding that young adults and teenagers were the major source of virus isolations is in agreement with the known age distribution of patients attending venereal disease clinics. The recognition of infection in almost equal numbers of male and female patients has not always been reported. Hutfield (1967) working in a large clinic in London made the diagnosis frequently in males but only one-tenth of all patients were female. This could be due to difficulties in recognising subclinical cervical infection and the report of Nahmias et al. (1969c) has confirmed that equal numbers of patients may be seen. The low isolation rate (38.8 per cent.) in Edinburgh compared with other sites of infection could be related to difficulties in accurate clinical diagnosis and the duration of the lesions when the patients attended the clinic.

Other studies have reported higher success rates: Nahmias et al. (1969c) made the diagnosis in 74 per cent (17/30) of female and 56 per cent. (31/43) of male patients. The report by Hutfield (1967) of 140 patients records an isolation rate of 55.5 per cent., the success rate depending on the duration of the illness. These reports do not include any information that could explain the greater

isolation rates.

In the Edinburgh study no attempt was made to detect all possible cases of herpetic infection and the true occurrence of the disease must be considerably greater than 1 in 300 of all new attendances.

The sites of infection in male and female patients were typical of this condition. In female patients most lesions were superficial and were located on the labia, inner aspect of the thigh, perianal region and the mons pubis. Infection of the cervix was demonstrated simultaneously in 4 cases; the cervix could be the sole site of infection, although examination of 6 patients with acute cervicitis or cervical erosions did not suggest that herpetic infection was a common cause of this condition. The clinical findings are similar to those recorded by Lazar (1955), Dooley et al. (1957) and Diddle et al. (1963): the more extensive investigations of Dr. A. J. Nahmias and his colleagues have confirmed these findings and established that the cervix is the site most frequently involved (Josey et al., 1968). Yen, Reagan and Rosenthal (1965) also came to a similar conclusion, and showed that cervical infection could be clinically inapparent.

Asymptomatic infection of the male has been claimed to be an important reservoir of infection by Jeansson and Molin (1970; 1971) who isolated the virus from the urethra of healthy patients. Recently, Centifanto et al. (1972) in a short article described their isolation studies with 190 specimens of urethral secretions, prostatic fluid, prostatic biopsies, and vas deferens sections. Their overall isolation rate was 15 per cent., and the highest rates

of 29 and 23 per cent. were from vas and prostatic fluid samples respectively; urethral swabs also yielded virus, 11 of 144 (7.6 per cent.) were positive. These are very high recovery rates, but they have not been confirmed. Traub et al. (1973) failed to isolate the virus from vasectomy specimens and in Edinburgh no virus was recovered from over 200 samples of seminal fluid and vasectomy specimens (Smith and Peutherer, unpublished observations). Alexander (1973) gives similar results in that he could recover the virus from less than two per cent. of serially examined male clinic patients. In a study of military personnel in Taiwan only two of 512 urethral specimens were positive; failure to isolate virus from 125 vasectomy specimens was also recorded. In the present study the isolation from the urine of a man with suspected cytomegalovirus infection is the only case comparable to those of Centifanto et al. (1972).

The ability of genital infection to recur (Sharlitt, 1940; Lazar, 1955) is illustrated by the Edinburgh results; recurrences were confirmed in both male and female patients either at the same or related sites. Virus isolation was repeated from penile lesions in one patient more than 3 years after the original episode, although the two isolates were found to differ in certain respects.

Serological studies indicated that one-quarter of genital infections are primary in nature; this finding is in agreement with the comments of Nahmias et al. (1969c) that one-third of all genital infections are of this form. Rawls et al. (1971) in a study of two social groups apparently confirmed the diagnosis of primary genital infection in 27 per cent. (14/52) of patients from a higher socio-economic group, but in only 14.5 per cent. (10/69) of patients

attending a social hygiene clinic. These distributions are in agreement with the known importance of social factors in determining the proportion of the population with antibody to herpes simplex virus (Smith et al., 1967). The clinic patients tested by Rawls et al. (1971) appeared to be comparable to the group studied by Nahmias et al. (1969c), although the latter study detected more primary infections.

A recent study by Kaufman et al. (1973) of private practice patients found that the high proportion of 61 per cent. (41 of 67) of female genital infections were primary in type.

The use of different methods to detect neutralising antibody does not explain this difference as Rawls et al. (1970a) compared various neutralisation techniques and concluded that there was reasonable agreement between the kinetic and microquantal methods.

It can be concluded that the recorded differences must be due to variations in the social background of the two groups. One-third of the higher socio-economic group of Rawls et al. (1971) had no detectable antibody to either virus type; the study of Kaufman et al. (1973) was carried out in the same city, and the populations studied must be comparable. Only 9 per cent. of the clinic patients were without antibody to herpes virus (Rawls et al., 1971). The population studied by Dr. A. J. Nahmias and colleagues appears to be intermediate between the two extremes described, as 20.5 per cent. did not have antibody to either type of virus (Nahmias et al., 1970b). In Edinburgh the original survey (Smith et al., 1967) and a more recent study of patients attending the venereal disease clinics found that just over one-quarter of this population had no antibody to the virus.

CHARACTERISATION OF STRAINS OF HERPES SIMPLEX VIRUS

The viruses isolated in this study were submitted to antigenic typing and biological characterisation to test the hypothesis that antigenic type 2 strains are associated with genital infections, and type 1 strains with the mouth, eye and other sites. This seemed a relevant population to test as the antibody survey referred to had confirmed that more than 50 per cent. of students, nurses and the general population in the 10 - 14 year age group were fully susceptible to infection with either type of virus.

Antigenic typing methods

The experiments reported confirm that the microquantal neutralisation procedure, modified as described, is a reproducible and accurate method of titrating virus infectivity and neutralising antibody. The reproducibility of the titres for both assays is in good agreement with the results reported in the original description of the method by Pauls and Dowdle (1967). The major differences from their technique are (i) the use of BHK21 cells instead of primary rabbit kidney cells, (ii) incubation for 3 days instead of 5 days and (iii) the use of calibrated glass dropping pipettes.

The three modifications make the method more applicable, in that they provide greater speed and avoid the need to produce primary rabbit kidney cell cultures. Economy is also improved as the use of a continuous cell line is cheaper than maintaining rabbits and preparing primary cultures: simple glass droppers cost a great deal less than the purchase price of sufficient commercial dropping pipettes to allow

the use of one pipette per serum dilution for at least 4 sets of 8 serum dilutions.

Variation of the temperature and time of neutralisation showed that for short periods of 30 min. to 1 hr incubation could be either at 22°C or 4°C; cross-reactions became more apparent at 37°C.

The investigation of tube-neutralisation and complement fixation methods did not suggest that they were sufficiently sensitive for the typing of a large number of strains. The chessboard complement fixation technique could perhaps have been adapted with the selection of more specific antisera: the trend of the results agrees with those of Schneeweis (1962a) using a similar technique.

The neutralisation-kinetic test was confirmed as a sensitive method of distinguishing between viruses (Ashe and Scherp, 1963), and although its usefulness was enhanced by the development of a tube focal assay of virus infectivity, it is a difficult test to apply on a large scale, and has been claimed to have considerable variability (Rawls et al., 1970a). The method has been used to classify sera as type 1 or 2 reacting in sero-epidemiological studies of cervical carcinoma (Rawls et al., 1968b; Skinner, Thouless and Jordan, 1971).

The failure to demonstrate increased specificity with rabbit serum IgM fractions could be due to the method of preparation of the fractions. Differences in the methods of immunising the rabbits to produce the antisera would seem to be a more probable explanation of the lack of agreement with the results of Hampar et al. (1970).

The method of preparation of antisera has been shown to be important in determining the extent of cross-reaction with heterologous virus; variation of the dose of virus inoculated to rabbits



affects the specificity of the resulting antisera.

#### Preparation of antisera

Many studies have shown that type-specific reactions can be obtained with sera after absorption with heterologous virus-infected cells. However, unabsorbed sera to the two types show different degrees of cross-reaction that could either represent antigenic differences between strains or variation in the specificity of the antisera. Many different inoculation or infection schedules have been used and comparisons are rendered difficult by a general lack of information about the titre of virus inoculated. This is not an accurate guide to the antibody response as it is well-established that both virus types can infect the rabbit which is the most popular experimental animal.

Most workers have used lengthy inoculation schedules: Ashe and Scherp (1963) inoculated rabbits with 12 or 18 doses of  $10^{6.8}$  plaque forming units over a period of 4-5 weeks and then used a sensitive kinetic neutralisation test to divide strains into 4 groups. Schneeweis (1962a) inoculated  $10^{4.6}$  -  $10^{5.6}$  plaque forming units of virus in adjuvant on 4 occasions and boosted the animals one month later. Type 2 antisera produced in this way reacted almost equally with homologous and heterologous strains, whereas type 1 antisera gave a reliable differentiation between the two types. Neutralisation tests showed this effect most clearly, but similar results were obtained with chessboard complement fixation tests. Rawls et al. (1968a) inoculated 10 or 11 doses of virus of unknown titre to rabbits; they concluded that antisera to non-genital strains showed greater specificity. Pauls and Dowdle (1967) used the immunisation schedule first described

by Plummer (1964): three-ml injections of rabbit kidney propagated virus are given both intra-muscularly and intra-peritoneally at an interval of 3 weeks. No information about the dose of virus inoculated is given in either publication. Different neutralisation techniques were used, and Pauls and Dowdle (1967) obtained results in close agreement with Schneeweis (1962a) but Plummer (1964) was able to classify strains with antiserum to either virus type.

Other workers have studied the neutralisation of virus by rabbit antisera prepared by infecting the scarified skin and boosting antibody titres during convalescence (Ejercito et al., 1968). Kinetic neutralisation tests with each type of serum were reported to identify the homologous virus reliably, although the extent of the cross-reactions was not recorded.

Nahmias and Dowdle (1968) concluded from their own results and those of other workers that strains could be distinguished efficiently with type 1 antisera and that type 2 antisera showed almost equal reaction with both virus types. To date no firm evidence has been produced from biochemical studies to support the idea of a "one-way cross" between type 1 and 2 strains. An alternative explanation would be that in some of the studies reported, low titres of type 2 virus were inoculated into the animals, due to the relatively poor yields of type 2 virus from most cell culture systems. For this reason, it would be expected that type 1 suspensions for inoculation will contain greater quantities of type-specific components and will apparently be more discriminating than "corresponding" type 2 antisera.

Another result of the various inoculation schedules is apparent from the studies of Hampar et al. (1970) who showed that 19S (IgM)

and 7S (IgG) antibodies collected early and late in the immunisation of rabbits had different discriminating abilities. Sera were raised by intravenous inoculation of  $10^{6.0} - 10^{7.0}$  plaque forming units of virus per week over a 5-week period. Early sera were collected at 7 days and late sera after 7 weeks; comparison of these showed that late 19S antibody fractions were the most specific. This antibody reacts with virus antigens incorporated into cell membranes and with sub-envelope sites in virions (Hamper et al., 1971).

In the present work, inoculation of doses with a titre less than  $10^{2.0}$  TCID<sub>50</sub>/0.02 ml has been shown to produce relatively type-specific antisera. The specificity of this low-dose serum is counteracted by a low titre compared with the higher titres and marked cross-reactivity of high-dose sera. Perhaps by chance, the low-dose MS antisera were the most useful, combining a high homologous titre with no detectable heterologous neutralising ability.

It can be concluded from these studies that the degree of cross-reactivity of sera can be altered by varying the amount of virus inoculated. Similar findings to those of Pauls and Dowdle (1967) were obtained with high-dose sera; antisera to type 2 virus showed a greater degree of heterologous neutralisation. High-dose antisera can be used to separate closely related strains: the results of Ashe and Scherp (1963) establish this, but a sensitive kinetic neutralisation test is necessary. This observation may depend on the presence of minor differences in structural proteins that may constitute only a very small proportion of the total virus-coded protein, and as a result Ashe and Scherp could subdivide a group of type 1 strains. The kinetic neutralisation tests reported in this thesis showed that strains HF

and 1657 were distinct although both are type 1 according to the micro-quantal method. Skinner, Thouless and Gibbs (1972) reported by Wildy (1972) used a kinetic neutralisation test and sera prepared in mice by inoculation of virus suspensions containing  $10^8$  and  $10^6$  plaque forming units over a period of 5 months. On the basis of reciprocal kinetic neutralisation tests, oral and genital strains were shown to form two clusters, although the two groups were found to overlap.

#### Antigenic typing of virus isolates

The results with a small series of reference viruses confirmed that the typing sera allocated strains Watson and Dawson to the same types as described (Plummer et al., 1968). The MS virus of Gudnadottir et al. (1964) was shown to be different from HF virus, and these two viruses are acceptable reference type 1 and 2 strains. The use of HF as a reference type 1 strain could have led to confusion as Dowdle et al. (1967) reported that some HF strains appeared to be of type 2, although the majority were type 1. Contamination of this particular strain, obtained from the Institute of Virology, Glasgow, does not seem to have occurred. The MP virus obtained from Dr. Roizman some years before was found to lie just within the type 1 range; other studies have either placed this in type 1 (Dowdle et al., 1967) or classified it as intermediate between types 1 and 2 (Ejercito et al., 1968).

The results with both sets of reference antisera confirm that virus isolates can be divided into two groups: one related to HF virus (type 1) and the other to MS virus (type 2). The method adopted by

Dowdle et al. (1967) of determining the pN difference (pN1 - pN2) was used: there are no absolute values for each group, and the upper limits of type 2 and the lower limits for type 1 strains have to be determined for each pair of reference sera by examination of a number of viruses.

In practice, there is no advantage in calculating pN1 and pN2 values for one virus against each of the reference antisera. The pN formula was devised to allow for the dose of virus inoculated in a neutralisation test: when the virus is tested against the two antisera by a standard technique, the virus dose and the volume constants are identical and the pN difference is equivalent to the difference between the serum 50 per cent. end-point titres. The pN values were calculated in the studies reported in this thesis to facilitate comparison with the published results of Pauls and Dowdle (1967) and Dowdle et al. (1967).

Calculation of pN values is of greater importance in serological studies where a single serum is reacted with both virus types as differing doses may be inoculated to the neutralisation reactions.

The microneutralisation method is probably not suitable for routine application: virus stocks have to be prepared, titrated and reacted with the reference antisera. In a doubtful case, however, this would be a useful reference technique.

#### Biological characterisation of herpes simplex virus strains

##### Pock production on the chick embryo chorioallantoic membrane

The results of the present study are in close agreement with those of Parker and Banatvala (1967) who showed that marked differences

in pock size could be found after incubation for 7 days. Nahmias et al. (1968b) confirmed these observations and established that type 1 strains produced small pocks whereas type 2 strains gave rise to large pocks.

In the present work this difference was found to be true for the laboratory strains tested, although the pocks of the laboratory type 1 strains were larger than those of the newly isolated strains. This is of interest as older studies had shown that frequent passage in culture or in ovo caused the pock size to increase (Anderson, 1940; Coriell et al., 1949). Thus, typical pocks should be produced despite the fact that the wild-type viruses had been passaged a number of times (less than 10) before pock production was examined.

A greater variation in diameter of the large pocks was found compared to the small pocks. This could be due to a number of factors including secondary pock formation and premature death of the embryo: both would cause a reduction in the average pock size. The finding of a group of strains with recorded pock diameters just greater than 1.0 mm could be due to these effects. Nahmias et al. (1968b) reported that a few strains (9 of 105) of both antigenic types did not grow on the choricallantoic membrane. No failure of growth was recorded among 99 strains examined in this study.

Comparison of pock diameter with established antigenic type confirmed the observations of Nahmias et al. (1968b) that type 1 strains produce small pocks and type 2 strains large pocks. The one exception is discussed below.

This method of characterisation is simple to perform, and requires only a supply of fertile hen eggs and a suitable incubator.

One problem is that several dilutions of the virus suspension have to be inoculated to give some membranes with well-spaced pocks. At least 4 or preferably 6 eggs are required for each virus. The incubation period of 7 days used in this study is longer than that of other reports (Nahmias et al., 1968b) in which pock sizes were determined after 3 days incubation. Although the shorter incubation period makes the method more practicable, it does mean that relatively small differences in pock diameter have to be measured. The longer incubation period, despite several drawbacks, does produce good distinction between types; antigenic type 1 strains produce pocks with a diameter of less than 0.80 mm and the pocks of type 2 strains are greater than 1.00 mm in diameter. The longer incubation period can be an advantage in that by this time contamination and premature death of the embryo are readily detected and therefore erroneously small pocks should not be estimated.

#### Cytopathic effect (CPE) in cell cultures

The examination of unstained, infected cell cultures was found to be a simple and rapid method of typing strains. The results of the antigenic and CPE typing were in agreement.

Examination of reference strains showed that considerable variation in CPE could be seen, ranging from cell rounding and proliferation to syncytium formation. Variation in CPE has been recorded on many occasions in the literature and stable virus clones of differing CPE have been produced. The most obvious effect of multiple passage of virus is the appearance of syncytia (Scott and McLeod, 1959). This effect may appear within a few passages or may even be present from first isolation (Barski and Robineaux, 1959;

Kleger and Prier, 1968). Some isolates initially cause a mixed CPE (Scott and McLeod, 1959; Hutfield and Longson, 1968), but serial passage may alter the CPE by the fourth to the ninth passage (Kohlage and Schieferstein, 1965). In contrast, Plummer et al. (1970) detected change in two genital and two non-genital strains after 30 passages in primary rabbit kidney cultures; after serial passage in mice or chronic infection of rabbits, two syncytial strains were derived from non-genital strains (type 1). Both type 1 and 2 viruses were examined by Smith et al. (1971) before and after 20 serial passages in rabbit corneal cells, but no syncytial forms were found, although variation in plaque size was noted.

All the syncytial strains described above were derived from virus stocks either known to be of type 1 or isolated from sites usually associated with type 1 infection. Schneweis (1962b) studied the effect of passage on the chorioallantoic membrane on syncytial formation and found that this developed in 3 type-1 strains after 18-20 passages whereas 4 other type-1 strains retained the original round-cell CPE. Two type-2 strains showed no change in the relative proportions of syncytia and round cells. Schneweis examined these strains in HeLa, mouse, chick and rabbit cell cultures and commented that the CPE differed in these cells: this observation may well be relevant to the minor differences in CPE reported by various workers. The effect of the host cell on the CPE is also indicated by the work of Kohlage and Schieferstein (1965) who derived syncytium-forming virus from stocks grown from single plaques of round-cell type virus: a cell-fusing effect was demonstrable in rabbit kidney but not in HeLa cells. These observations also suggest that the variant strains arise



by mutation rather than by selection from a mixed population. Smith et al. (1971) concluded that plaque variants exist in the clinical lesion, and on this basis large-plaque variants would emerge by selection during passage.

Discrepancies are apparent when the evidence relating the cytopathic effects of isolates and antigenic type is examined. Schneeweis (1962b) tested his type 2 strains in a range of cells and found a mixed CPE consisting of round cells and small giant cells whereas Munk and Donner (1963) found that their genital strains gave rise to large syncytia in HeLa and HEp2 cells. In contrast to these reports, Plummer et al. (1968) failed to detect syncytium formation in either rabbit kidney or human lung fibroblasts, but type 2 strains did produce swollen globular cells. Similarly, Ejercito et al. (1968) recorded that type 2 strains gave rise to loose aggregates of rounded cells in HEp2 cells whereas the CPE of type 1 viruses consisted of clumps of rounded adherent cells. Two reports by Kleger and Prier (1968; 1969) illustrate the effect of the type of cell culture on the appearance of the CPE; they found that type 1 and 2 strains could not be distinguished in rabbit kidney cells, but that clear differences were apparent in human lung fibroblast cultures. Both sero-types produced rounding of cells, but syncytia were seen with type 2 viruses: these results are identical to those reported in this thesis.

There is no obvious explanation for the discrepancies described in the literature. Apart from differences in media and methods of handling cultures, the possibility exists that the widely used HeLa cells may differ between laboratories or that contamination of cell or virus stocks with arginine-utilising *Mycoplasma* species

could influence the growth of herpes viruses known to be dependent on arginine for replication, including the synthesis of membrane antigens. Most published reports make no mention of the state of cell cultures with regard to Mycoplasma contamination; this is most likely to occur with cell lines such as HeLa or HEp2 but should be rare with primary rabbit kidney or human embryo lung cultures. No evidence has been found concerning the influence of specific environmental conditions on the development of cell fusion, the absence of this effect being the major discrepancy in the results discussed. There is often a lack of information about the passage history of viruses, but this would not appear to be the sole explanation for the recorded differences.

Variation in the time of recording the CPE, or in the inoculation of different doses of virus could influence results. Infection of all the cells in a culture could preclude cell fusion, and early CPE changes could be missed by examining the cultures after even 48 hr incubation. Syncytia do not remain stretched out, but retract and become rounded globular cells as described by Kleger and Prier (1969). Epithelial cells could fail to show recognisable small syncytium formation. The plate illustrating the type of CPE described as consisting of loose foci of rounded cells in the paper of Ejercito et al. (1968) contains a large rounded cell that could be a small giant cell: this CPE was associated with type 2 viruses. On the evidence available it is impossible to identify any single factor to explain the differences in CPE that have been recorded in the reports discussed.

In summary it can be stated that many laboratory strains cause

extensive cell fusion in cell monolayers, but others are associated with cell rounding. IN BHK21 cells, freshly isolated viruses cause rounding of cells and in addition type 2 strains produce small syncytia. The method can be applied at the stage of virus isolation allowing growth and typing in one manoeuvre and the result should be available quickly. No discrepancies were detected in this study between serological and CPE type, and it was concluded to be a simple and rapid method.

#### Plaque morphology

Differences in the appearance of plaques under methylcellulose were noted, but they were small and variable. The method requires large numbers of cells to establish monolayers in petri dishes and for these reasons it was not applied on a large scale.

#### Electron-microscopical examination of infected cells

This test cannot be applied on a routine basis in most laboratories, but the present study has shown that it is a reliable method of distinguishing strains. The reports of Murphy et al. (1967), Couch and Nahmias (1969) and Schwartz and Roizman (1969b) described the presence of filaments or tubules within infected cells. Only single strains were examined by each of these groups: the present study is the largest recorded and establishes that this character is associated with type 2 strains.

Other workers have reported the presence of filamentous and tubular structures in herpes simplex virus-infected cells (Chitwood and Bracken, 1964). Replication of the virus was interrupted by fluorophenylalanine, and this led to the appearance of filamentous and granular structures, differing from those described above. The

filaments appeared to be related to the nuclear membrane and marginated chromatin; a similar association was seen with irregular, faintly-stained hollow filaments described by Murphy et al. (1967); these structures could be host cell chromosomes disrupted by the viral infection.

Filamentous structures have been reported in association with other members of the herpes virus group. Watrach (1962) noted 65 nm structures in the nuclei of cells infected with infectious laryngo-tracheitis virus; he concludes that they were aberrant forms of the virus. Cook and Sears (1970) described two types of filaments in cells infected with the Marek's disease herpesvirus: small irregular structures resembling some of the forms described by Murphy et al. (1967) and denser, banded filaments with a diameter of 70 - 75 nm. The latter resemble the aberrant forms of virus reported by Watrach (1962) and the large 100 nm filaments associated with herpes simplex virus nuclear capsids (Murphy et al., 1967).

The nature of the filamentous structures associated with herpes simplex virus is unknown: they could result from the assembly of capsomere protein into abnormal forms during the relatively inefficient replication of type 2 virus. Based on published micrographs their distribution within nuclei does not suggest any association with the modified nuclear membrane so readily detected in infected cells. Differences in the nuclear proteins have been reported between type 1 and 2 strains (Halliburton, 1972) and it is possible that extra type 2 nuclear proteins could form the filaments. This method has obvious limitations as regards routine application, but with one exception among 53 type-2 strains tested it gave results in good agreement with

other methods. The detection of filaments in infected cells was straightforward, and this method could be used in cases found to be ambiguous by other typing procedures.

#### Thermal stability

Because this technique was found to be laborious it was only applied to a few type 1 and type 2 strains. The survival of virus after incubation for 6 hr at 4°C allowed strains to be divided into two groups: these were in agreement with the antigenic typing results. The effect of the composition of the suspending fluid (Wallis et al., 1968) probably accounts for a number of discrepancies reported in the literature on the thermal stability of herpes simplex virus. Farnham and Newton (1959) reported a half-life of 3 hr for HFEM virus at 37°C when the virus was suspended in cell growth medium containing 5 per cent. calf serum and phosphate buffer. Hoggan and Roizman (1959b) reported that their MP strain had a half-life of 14 hr at 37°C in 5 per cent. serum containing medium. Ejercito et al. (1968) found a 45-70 per cent. survival of virus suspended in 0.01M phosphate buffered saline after 2 hr at 40°C. At this same temperature the two CPE-prototype strains were inactivated almost equally, although the half-life of the type 2 strain appeared to be about 100 min., it was greater than 2 hr for the type 1 strain. Results from this relatively short incubation period scarcely merit the conclusion that the two types are inactivated equally. The effect of the suspending fluid was emphasised by Smith et al. (1971) who recorded no difference at 45°C in the rate of inactivation of type 1 and 2 large-plaque clones suspended in growth medium. In contrast to Ejercito et al. (1968) differences were detected with viruses suspended in phosphate

buffered saline: type 1 virus was found to be more labile than the type 2 strains. Other reports have agreed that type 2 strains are more rapidly inactivated than type 1 strains at various temperatures of incubation. Figueroa and Rawls (1969) quote inactivation rates at 37°C of 0.07 log<sub>10</sub> plaque forming units per hr for type 1 and 0.27 log<sub>10</sub> units for type 2 strains. This leads to calculated half-lives of approximately 4 hr and 1 hr respectively at this temperature. Plummer et al. (1968; 1970) found half-lives of 15 hr and 5 hr for type 1 and 2 strains incubated at 37°C in 199 medium without serum.

Schneweis (1962b) reported that laboratory strain HPFM and two other type 1 strains were stable over a period of 4 months at 4°C whereas two type-2 strains were unstable at this temperature; type 1 strains diminished by only 1 log<sub>10</sub> plaque forming units, but type 2 strains lost 10<sup>5.0</sup> - 10<sup>6.0</sup> plaque forming units over this period.

Another variable factor could be the method of preparation of the virus suspension used for inactivation studies, as procedures such as sonication may damage virions (Smith, 1963). Differences could also be a reflection of whether virus was naturally released from cells or had been released artificially, as this has been shown to affect the degree of envelopment of the virus stock. Plummer et al. (1970) compared the thermal stability of extra- and intra-cellular virus but found that there was no difference between cell-associated and released virus at 37°C and that virus titres decreased to 0.1 per cent. by 9-12 hr. These authors did find a host cell-dependent difference in viral stability, although this was identical for both types. Virus released from human fibroblast cultures was slightly more heat labile than virus from rabbit kidney and mouse embryo cells. Conversely,

human fibroblast cell-associated virus was more stable than that of the other cells tested.

No absolute rates of inactivation can be deduced for the two virus types, but from the studies included in this thesis and those discussed above, it can be concluded that type 2 strains are inactivated more rapidly than type 1 strains. If the differences in titre between type 1 and type 2 strains could be increased without lengthening the duration of incubation, then a quantal assay, e.g. the microtitre system, rather than a focal assay would make this method more applicable. However the decrease in titre is only about one-half  $\log_{10}$  over a period of 6 hr at 4°C and is scarcely outwith titration error.

#### Correlation of characterisation methods

As listed in Table 50, good agreement was found between the main methods of typing virus isolates. Type 1 strains produce small pocks on the chorioallantoic membrane, no intranuclear filaments and a CPE consisting of round cells. Type 2 strains are associated with large pocks on the chorioallantoic membrane, intranuclear filaments, and a CPE including both round cells and small fusiform syncytia. Only two aberrant strains were detected. A strain (3668) classified as type 2 by serology, pock size and CPE did not show filament formation. Strain 4159 was antigenic type 1, but large pock-producing, perhaps because the virus population was a mixture of type 1 and type 2 strains. Both virus types could have been present in the patient's lesions or contamination could have occurred during handling in the laboratory. However this mixing occurred, type 1 virus would outgrow type 2 virus in cell cultures, and hence the virus suspension would appear to be

type 1. The fact that type 2 pocks are significantly larger than type 1 pocks could cause small pocks to be overlooked, although there did not appear to be many present (Fig. 13).

The finding that virus suspensions derived from single pocks of strain 4159 could be either type 1 or type 2 serologically, whereas the pock diameters could be large or small, may have arisen because the method of production of each pock-derived virus stock did not exclude cross-contamination. Proper plaque-purification of the original and pock-derived virus stocks would be necessary to investigate this problem. If these still showed mixed character, then recombination between type 1 and 2 strains could explain the findings: the origin of the two parental strains is impossible to assess, although with the subsequent isolation of a type 2 strain from the patient it is possible that he suffered from recurrent attacks of type 2 infection to which was added a type 1 virus - either in the penile lesions or in the laboratory. Recombination between strains of herpes simplex virus inoculated to the chorioallantoic membrane has been demonstrated by Wildy (1955) who used pock diameter as a strain marker. More recently, Timbury and Subak-Sharpe (1973) have recombined mutants of type 1 and 2 strains in cell culture. Recombination should lead to a stable hybrid with intermediate properties, and could explain the assortment of markers shown with some of the 10 derivatives of strain 4159. Serological studies showed that antibody reacting with both type 1 and 2 antigens was present in the patient's serum from the time of the initial isolation,

Strain 3668 was found to be antigenic type 2 but had other features more consistent with a type 1 strain. If mixed infection can



occur as proposed by Nahmias and Dowdle (1968) then recombination could explain the hybrid character of strains 3668 and 4159, as it is unlikely that all the diverse characters used to classify viruses are coded for by a single gene.

#### ASSOCIATION OF VIRUS TYPE WITH SITE OF INFECTION

The isolation of herpes simplex virus from young adults infected at different sites offered a good opportunity to test the postulated association of type 2 strains with the genital tract and related skin areas. Viruses isolated from oral, skin, finger, eye and genital infections were typed by the microneutralisation procedure as described. Of the 208 viruses studied, 61 per cent. were recovered from patients aged between 15 and 34 years.

The results of the present study are in broad agreement with the reports of Dowdle et al. (1967) and Nahmias and Roizman (1973b), although some divergence was noted with strains of virus isolated from female genital tract infections. Sixty strains isolated from the oral cavity were tested by the author and found to be type 1. The isolation of type 2 virus from sites other than the genital tract has been recorded on rare occasions. Nahmias et al. (1968a) report that they isolated type 2 virus from both the mouth and vulva of a 9-year-old girl who was apparently experiencing a primary infection. Further cases have been recorded by Kaufman and Rawls (1972) and Nahmias et al. (1971d), although it is possible that the patient described in this last report is the same as that referred to in Nahmias et al. (1968a). Juel-Jensen and MacCallum (1972) describe a

patient of 24 years who developed ulceration of the labia, and lesions in the mouth associated with malaise and adenopathy; type 2 virus was isolated from both sites, but the authors state that infection could not have been acquired venereally although no evidence is given to support this conclusion and no other mode of transfer is suggested. The patient described by Kaufman and Rawls (1972) had sexual intercourse and oro-genital contact with an infected partner and developed cervical, labial and pharyngeal ulcers that yielded type 2 virus. These are isolated cases; type 2 oral infections would appear to be uncommon, but could occur in the sexually active age groups.

Ten isolates from lip infections, 13 from facial and 10 from finger lesions were all of type 1. The findings with the lip and facial strains are in agreement with published series, but Nahmias and Roizman (1973b) examined 19 hand and arm strains and found that 9 were of type 2; these were probably acquired by contact with infected genital tract secretions. The finger strains examined in Edinburgh were isolated from children or nurses: the results suggest that oral secretions were the source of these viruses.

The ten isolates from skin lesions could be divided into sites located either on the trunk, neck and limbs or in the lower lumbar, perianal and buttock regions. The type of virus isolated was found to differ; all the isolates from the first group of sites were of type 1, but 3 of 4 strains isolated from buttock and perianal regions were of type 2. This agrees with the classification proposed originally by Dowdle et al. (1967) and subsequently confirmed by further reports from the same group (Nahmias and Roizman, 1973b): this lists only 4 of 57 strains from the skin below the waist as belonging

to type 1. The Edinburgh type-1 isolate from the buttock was from a recurrent lesion of a 5-year-old girl. This strain, 1657, was used to make the antigens employed in the serological survey discussed (Smith et al., 1967).

All 39 strains of virus recovered from eye infections were of type 1: the eye seems only to be colonised by this type, presumably reflecting the oral origin of the viruses. Nahmias and Roizman (1973b) record that all their 22 strains were of type 1.

The major difference from other published results was the isolation in Edinburgh of significant numbers of type 1 strains from genital infections. Eleven of the 66 strains tested were allocated to type 1, a rate of 16.7 per cent. Analysis of the results according to the sex of the source patients gave rates of 5.6 per cent. (2 of 36) for males and 30 per cent. (9 of 30) for females. In other studies, information is not given or genital isolates are all assumed to be of type 2. Rawls et al. (1971) record two type-1 genital isolations from adult female patients; an exact total of type 2 isolates was not given, although this must have been at least 30 and therefore, at the highest estimate this is a rate of 6.7 per cent. The reports by Hutfield et al. (1967) stated that most strains produced large pocks on the chorioallantoic membrane: the study of Hutfield and Longson (1968) described the type 2 features of a single isolate. The description of large-pock production by genital strains (Parker and Banatvala, 1967) did not include any small pock strains among 23 genital isolates.

Other small series do not record the isolation of any type 1 strains. Kleger and Prier (1969) studied 8 genital strains and found

they were of uniform type as judged by CPE production. A similar approach was used by Amstey and Balduzzi (1970) in a study of 18 isolates: all were found to be "genital type". More recently, Centifanto et al. (1972) in reporting the isolation of 31 strains from asymptomatic infections of the male genital tract stated that only a proportion were typed by immunofluorescence and confirmed as type 2.

The report of Longson (1971) concerning the use of a temperature-marker test to differentiate strains of herpes simplex virus included observations with 9 genital isolates, 4 of which were apparently of type 1. These strains may have been selected from a larger study but no information could be found concerning the true frequency of type 1 strains. The large survey of Nahmias and Roizman (1973b) indicated that, over the age of 1 year, 3.4 per cent. (5 of 146) of isolates from male patients were type 1, whereas 9.1 per cent. (23 of 252) infections of female patients were with this type. The actual frequency of isolation of type 1 strains from adult patients is impossible to assess as no age distribution of the patients is given, although it is known that some were simultaneous isolations from oral and genital infections in young children (Nahmias et al., 1968a).

In the Oxford area, Dr. A. H. Tomlinson (personal communication) found that 16 per cent. (6 of 37) of the genital isolates were classified as type 1 in 1971 and that in 1972 the proportion had risen to 22 per cent. (12 of 54). This last figure is in good agreement with the present Edinburgh results. In contrast, Juel-Jensen and MacCallum (1972) reported that 11.5 per cent. (6 of 52) genital isolates were of type 1. This study was also performed in the Oxford

area, but the figures relate to an earlier period than the results supplied by Dr. Tomlinson. Kaufman et al. (1973) isolated 13.4 per cent. (9 of 67) type 1 strains in a study of genital infections in women attending a private practice. This study and the report of Rawls et al. (1971) were from the same group of workers.

When these results are compared, it can be concluded that the proportion of type 1 genital infections is related to the socio-economic background of the populations studied. The occurrence of type 1 strains is low in Atlanta in a predominantly negro population (Nahmias and Roizman, 1973b) whereas it is higher in private practice patients in Houston (Kaufman et al., 1973). In clinic attenders in Oxford and Edinburgh more type 1 strains were isolated from genital infections than in Houston. Apart from the presence of a susceptible population, one other important factor could be the extent of genital type 2 infection in the respective communities. If type 2 strains are widespread in any section of the community, they will cause the great majority of new infections even in private practice patients. On the other hand, if a community has a relatively small reservoir of type 2 infection then exposure to the virus may not occur and the oral strains of virus will assume a greater importance.

Analysis of the Oxford results according to the sex of the source patients was not available; the study of Nahmias and Roizman (1973b) and the present work both found that more isolations of type 1 strains were made from female genital infections. This trend was most obvious in the Edinburgh study in which type 1 strains were isolated 5 times more frequently from female patients.

The finding that two of the 6 Edinburgh isolates from the

cervix were of type 2 suggests that the 30 per cent. isolation rate of type 1 strains was not associated solely with superficial infections. Kaufman et al. (1973) also isolated similar strains from the cervix.

Oral secretions or the lesions of herpes labialis are the most probable source of these viruses: infection of the genitalia could occur either in infancy or in later life. The detection of 4 primary type 1 genital infections among the 7 adult female patients tested in the present study supports the concept that infected lesions of the genitalia, lip or oral cavity of a male consort are the usual source of virus. The small number of type 1 infections of the male genitalia suggests that the virus is usually acquired from the mouth. Oro-genital contact or the use of saliva as a penile lubricant during intercourse could lead to the transfer of the virus. Male genital infections could be acquired in the same manner or during coitus with an infected partner. In both sexes auto-inoculation could occur.

Thus, the 3 main factors that are postulated to be related to the frequency of genital infection with type 1 virus are (i) the proportion of the population without antibody to either type of virus (ii) the reservoir of type 2 infection and (iii) sexual practices that facilitate contact between saliva and the female genitalia.

The isolation of a significant number of genital type 1 strains of virus is relevant to the problem of the association between carcinoma of the cervix and previous type 2 infection.

#### Genital herpes simplex virus infection and carcinoma of the cervix

Investigations performed over many years have established that carcinoma of the cervix has many epidemiological features suggesting

that an aetiological agent is transmitted by sexual intercourse. Before any studies to implicate a particular virus had been undertaken, Gagnon (1950) reported that he had not found a single case of carcinoma of the cervix among a large population of nuns in the Quebec region. Subsequent studies have confirmed that this is indeed a very rare disease in celibate women, although a few cases have been discovered (Towne, 1955). Martin (1967) stated that the other basic feature was the near absence of virgins among women with the disease. Martin (1967) also discussed various factors associated with a greater risk of developing carcinoma, and found that in American cities the disease was associated with various immigrant groups, e.g. Puerto-Rican, Mexican and Negro women, and low economic status in general; the disease was commoner among prison inmates, prostitutes and patients attending venereal disease clinics, compared with church-going women of high socio-economic background living in rural areas.

From an analysis of the histories of individual patients, Martin (1967) and Rotkin (1967) found that there was a positive correlation between carcinoma of the cervix and early marriage, early first coitus, early first pregnancy and multiple sexual partners. Apart from the first pregnancy degrees of parity were not found to be correlated with carcinoma, nor was intercourse with uncircumcised partners. Several of these associations could be related, and Coppleson and Reid (1967) and Coppleson (1969) postulated that the cervical epithelium undergoes a benign metaplastic change in adolescence and during first pregnancy; infection at this stage could initiate the reaction leading to atypia, carcinoma-in-situ

and invasive carcinoma. Only cytomegalovirus (Alexander, 1973) and herpes virus type 2 have been isolated with any regularity from the cervix, and most attention has been focussed on herpes simplex virus. The epidemiological studies of the Atlanta group of Nahmias, Josey, Naib and colleagues have reaffirmed that genital herpes virus infection is a sexually transmitted disease, and that it is more prevalent in low socio-economic groups.

Attempts to correlate evidence of type 2 virus infection and carcinoma of the cervix have met with success on several occasions, although the reliability and interpretation of these findings is debatable. The evidence has been accumulated from three main approaches; the detection of an increased rate of abnormal cytology findings in patients with genital herpes; the demonstration of antibodies to type 2 virus in the sera of women with carcinoma of the cervix and finally in the demonstration of herpes virus antigens or nucleic acid in cervical tumour cells.

Cytology surveys. The early report of Naib et al. (1966) of an association between genital herpes virus infection and all types of cervical atypia has been followed by further progress reports. The review article of Nahmias et al. (1971d) includes results from the routine examination of 88,393 cervical smears; 357 were positive for herpetic infection and 3,452 atypical smears were detected. The overall proportion of atypical smears was 3.9 per cent., but among patients with cytological evidence of herpes infection the rate was found to be 24.9 per cent. - a 6-fold increase. Ng et al. (1970) detected the cellular changes of herpes simplex virus in 212 of 239 patients with herpetic infection; of these, 3 showed atypical



squamous cells and one was classified as demonstrating slight dysplasia. This rate of 1.9 per cent. is very different from the Atlanta results and may emphasise differences in the interpretation of smears of exfoliated cells. With regard to the progression of the disease, Ng et al.(1970) found that the 3 patients referred to remained normal during a 3-year follow up period. Wolinska and Melamed (1970) studied 43,331 women attending family planning clinics and detected herpetic infection in 37, an overall rate of 0.09 per cent.; there was no evidence of cervical carcinoma or carcinoma in-situ in these patients although two women had been treated for dysplastic changes before the herpetic infection was detected. Ng et al. (1970) followed 90 women with diagnosed herpetic infection for up to 5 years, and in this time 6 developed cellular evidence of slight dysplasia; this regressed spontaneously and the patients remained normal during a 2-year follow-up. In contrast to these findings are the results reported by Nahmias, Naib and Josey (1972) from a prospective study of the risk of developing carcinoma-in-situ following the diagnosis of herpes genitalis; the cumulative risk at 5 years was 0.6 per cent. in women without genital herpes and this rose to 1.3 per cent. in the non-pregnant and 6.2 per cent. in the pregnant groups with herpetic infection.

Serological studies. Initial serological studies of control populations and patients with diagnosed carcinoma of the cervix showed a good correlation between the disease and antibodies to type 2 virus, although the results depended to a great extent on the interpretation given to intermediate antibodies. The studies discussed in the Introduction concerning the occurrence of antibodies in different age groups suggested that the intermediate values reflected dual

infection with both types of virus and this assumption has been made by most workers.

Significant results were obtained with invasive carcinoma patients of whom 83 per cent. were found to have type 2 antibodies (Nahmias et al., 1970c), 78 per cent. (Rawls et al., 1969), 97-100 per cent. (Royston and Aurelian, 1970) and 83 per cent. (Sprecher-Goldberger et al., 1970) compared with rates of 35, 22, 61 and 33 per cent. in the respective control groups. The only discordant report was from a study in New Zealand women (Rawls et al., 1970b); this found that 26-33 per cent. of both patient and control groups were positive for type 2 antibodies. Subsequent studies in Colombia, a country with a high carcinoma rate, also failed to demonstrate a correlation between carcinoma and antibodies to type 2 virus (Rawls, Adam and Melnick, 1972), and these authors, in reviewing their own and other studies concluded that the only association established was that there was a high occurrence of type 2 antibodies in negro women (68-96 per cent.) and that a lower rate was found (27-60 per cent.) in other races. The most extensive survey reported from Britain is that of Skinner et al. (1971); groups of patients with abnormal cytological or histological findings were compared with a negative control group apparently unmatched in any respect other than sex. In this study, a higher mean type 2 neutralisation index was found in the carcinoma group. The effect of careful selection of controls was studied by Adam et al. (1971) who matched the carcinoma patients with controls selected for age at first intercourse, age at first pregnancy and number of live births, marriages and sexual partners. Studies in matched groups from 3 separate populations in Uganda, Texas and

Colombia showed that, although the findings in Colombia were similar to those of the Ugandan and Texan studies, the occurrence of type 2 antibodies in the control groups was higher than the previous studies and indeed the differences between cancer and control patients were not significant in any region. Nahmias et al. (1972) also extended the criteria for the selection of controls, but still found that carcinoma patients differed from their controls in respect of antibodies to herpes simplex virus type 2 and age of first pregnancy. In a review of the current status of serology surveys, Rawls, Adam and Melnick (1973) re-examined their data with respect to the mean antibody titres of patients and controls, and established that women with carcinoma of the cervix had higher mean titres to herpes virus type 2 than the accurately matched control groups.

More recent studies by Tarro and Sabin (1973) have demonstrated reactions between an early virus-infected cell antigen and sera from patients with several types of tumour including cervical carcinoma. Hollinshead et al. (1972) and Hollinshead and Tarro (1973) have found that antigens extracted from cervical and lip tumour cells react with antisera to a similar viral antigen. Aurelian (1973) has claimed that an early infected-cell complement fixing antigen reacted only with sera from patients with tumours. These results await confirmation before their significance can be assessed.

Demonstration of herpes simplex virus, antigens or nucleic acid. Only one isolation of infectious herpes simplex virus from cervical tumour tissue has been published (Aurelian et al., 1971). This virus was recovered from spontaneously degenerating cultures derived from an intra-epithelial cervical tumour: degeneration was apparent between

the 10th to 18th subcultures, and the virus isolated was found to be of antigenic type 2. Exposure of the cultures to high pH could induce the virus from further surviving cell cultures. Two per cent. of the cells of another culture established from an invasive tumour contained virus membrane antigens, but virus was not isolated (Aurelian, 1973). Royston and Aurelian (1970) reported that exfoliated cervical cells reacted with antisera to type 2 herpes virus in immunofluorescence tests: the degree of staining observed with the control patients was high (10 per cent.) and casts some doubt on the validity of this observation although it was claimed that these control patients suffered from herpetic cervicitis. Nahmias et al. (1972) record that they have made similar observations with cultured cervical cells, but no details are given, and this observation does not seem to have been confirmed. The presence of herpes simplex virus type-2-specific viral DNA and RNA in a cervical tumour has been reported by Frenkel et al. (1972). RNA specific for 5 per cent. of viral DNA was detected in the tumour cells and corresponded to a fraction of viral RNA transcribed early in the productive infection of HEp2 cells. One-half of the DNA on which this RNA was transcribed is common to both virus types. No virus was isolated and no virus antigens were detected in the tumour cells and it was deduced that only 39 per cent. of the viral DNA was present. This was thought to be linked by covalent bonds to cell DNA and one fragment of viral DNA was estimated to be present in each tumour cell. The authors speculate on the origin of this DNA and suggest that these findings could arise either because herpes virus can infect non-permissive cells of unknown type, or that infection with defective

virus could cause an abortive infection and allow integration of the viral DNA fragment, a situation analogous to the transformation of various rodent cells by inactivated herpes simplex virus type 1 and 2.

The ability of inactivated herpes simplex virus type 1 and 2 to transform hamster embryo fibroblasts has been reported; animals inoculated with some of these transformed cell lines develop tumours. Their sera contain neutralising antibody to the virus and therefore structural antigens must be synthesised in these cells.

Most of the evidence linking herpes simplex type 2 and carcinoma of the cervix is circumstantial and could be explained if the virus and a transmissible factor associated with the carcinoma were both spread by sexual contact. Constitutional and hormonal factors must be important in determining if carcinomatous changes develop or survive, as many studies have emphasised how widespread type 2 infection is in certain social groups. A lytic growth cycle normally results from infection with the virus, and this would have to be modified either as a result of the type of cell infected or by inactivation of the virus by some unknown means.

The conflicting evidence obtained from sero-epidemiological surveys and the problem of interpretation of these results are the main obstacles to acceptance of the theory of a direct causal relationship between genital infection with type 2 virus and the development of carcinoma of the cervix.

Much impetus to these studies derived from the finding that genital strains of herpes simplex virus differed from isolates from other sites. The failure to demonstrate an association between antibodies to type 2 virus and carcinoma of the cervix in a population

in New Zealand (Rawls et al., 1970b) could arise if the frequency of type 1 genital strains was similar to that described in Edinburgh. This could suggest that the other studies referred to show an association solely because the genital type 2 herpes virus and the postulated agent related to carcinoma are both transmitted by sexual intercourse.

A further possibility is that either type 1 or 2 infection could result in subsequent metaplasia and carcinoma-in-situ; this would not be revealed by sero-epidemiological studies due to the relatively high proportion of patients with antibodies to type 1 virus in most populations. The oncogenic potential of type 1 strains has not been studied extensively. A report by Wyburn-Mason (1957) suggests a relationship between herpes labialis and squamous carcinoma of the same site: lip infection is so common that a genuine causal relationship should have been established more clearly. Recent studies by Hollinshead and Tarro (1973) of reactions between membrane antigens extracted from lip tumours and non-virion antigens of herpes virus-infected cells require further clarification of the nature of these viral antigens. The demonstration of cell transformation by inactivated type 1 virus (Duff and Rapp, 1973) has been reported.

Whatever the association between carcinoma of the cervix and genital herpetic infection, it is of interest to speculate on the origin of the two virus types. The use of the term type has been criticised by Roizman et al. (1970b) and Hampar et al. (1971), who claim that the recorded differences between strains are not sufficient to justify the use of this term, and that subtype is a more accurate

description. Indeed the International Committee for the nomenclature of viruses has avoided the use of both terms and has recommended that the viruses should be referred to as human herpes viruses 1 and 2.

Studies of antigenic and biological variation among virus isolates are relevant to this problem, and information has been published in a number of reports.

Differences in the antigenic type of established laboratory strains have been discussed. Other studies to establish the stability of virus serotypes have also shown some discrepancies. Variation in the cytopathic effects of herpes simplex virus has been recorded on numerous occasions and many of these variant strains have been compared with the parental virus. The syncytial MP strain of Hoggan and Roizman (1959a) could be separated from the related mP strain by kinetic neutralisation tests. Plummer (1964) and Dowdle et al. (1967) examined both these strains, and found mP to belong to type 1 whereas MP was closer to type 2 although it was still within the type 1 range. Further studies by Ejercito et al. (1968) have classified MP as an intermediate type, an interesting observation in view of the report that this strain lacks a type-specific envelope glycoprotein.

Variants of this same sort were examined by Hinze and Walker (1961), Nii and Kamahora (1961), Kohlage and Siegert (1962) and Rapp (1963) and were not found to differ antigenically from the parental strains.

Ashe and Scherp (1963) examined 4 of their strains after 23 passages in cell culture and failed to detect any change in antigenic type by their sensitive system. Viruses passaged 30 times in cell culture and other strains passaged 10 times in mice by the intra-

cerebral route were examined by Plummer et al. (1970) and found to be identical to the original strains. This report included both type 1 and type 2 strains, as did the study of Smith et al. (1971) who also failed to show antigenic change during 20 passages in cell culture. The feasibility of genetic studies with temperature-sensitive mutants of both types supports the concept that type 1 and 2 strains are antigenically stable.

The studies of Hampar and his colleagues have shown that a persistent infection of a chinese hamster cell line can be established in the presence of antibody and that the viruses recovered from such a culture show gradual small antigenic changes over the period in culture (Hampar and Keehn, 1967). This is a rather specialised laboratory exercise but it has certain parallels in the natural association between the virus and man in whom the presence of high-titre neutralising antibody does not prevent either a latent or a low-grade chronic infection. A number of strains isolated from successive recurrences in patients have been examined: Gross, Ortmann and Reissmann (1957) failed to detect any difference between two strains isolated from the same skin site at an interval of 6 months. However, Garabedian and Syverton (1955) examined 4 strains of virus isolated from 4 successive attacks in a patient and found some antigenic differences by cross-neutralisation tests. Ashe and Scherp (1965) made similar observations.

It appears therefore that ordinary laboratory passage has little effect on the antigenic type of the virus, unless some selection pressure is applied by including antiserum in the culture medium. A similar situation may arise in man, although the changes noted in the



laboratory experiments do not seem to be of sufficient magnitude to cause the conversion of a type 1 strain to type 2. Nahmias and Roizman (1973a) report that laboratory strains may show variation in their glycoprotein composition. Obviously the differences in cytopathic effect that can be detected with some laboratory strains are not necessarily associated with major type-antigen changes, although there is good agreement between the antigenic and cytopathogenic types of freshly isolated strains from different clinical sites.

It would seem possible that some form of host- or site-induced modification of virus infecting the female genital tract could lead to the spectrum of properties shown, perhaps related to the acid and mucoprotein environment of the cervix. However, no firm evidence is available to support this and the degree of the changes detected in laboratory studies does not suggest that this mechanism alone could account for the observed differences between strains. Furthermore, as reported in this thesis the isolation of apparently typical type 1 strains from the female genital tract, including the cervix, strongly suggests that environmental selection does not occur to any great extent. In the present work, the failure to detect any variation of antigenic or pox character of type 1 strains when analysed according to the site of infection or age of the source patient might result from the lack of sensitivity of the laboratory methods, but could be interpreted to indicate that two virus types exist.

The spectrum of properties commented on by Roizman et al. (1970b) and Hampar et al. (1971) might have arisen by recombination between strains. The genital tract could be a melting-pot as far as the identity of strains is concerned, especially if significant

numbers of patients experience infection with type 1 strains at this site. Therefore, the discovery of two aberrant strains from the genital tract in the Edinburgh study may not be entirely fortuitous.

To investigate this possibility, it will be important to continue to characterise isolates from the genital tract and not to assume that all are typical type 2 strains. In this way it will be possible to assess if genetic recombination between the virus types occurs in the female genital tract and also if the relative frequency of infection with the two types is changing with time. A defined population with established patterns of infection at each of the main clinical sites is available in Edinburgh to investigate these problems.

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APPENDIX A

### Expression of dilutions and titres

The convention adopted in this thesis for the expression of dilutions and titres is based on the advice to authors of the Journal of Medical Microbiology and an expanded account provided by Professor J. P. Duguid, an editor of the Journal. Similar conventions have been adopted for the expression of titres in the Communicable Diseases, Scotland (CDS) Report series (CDS 72/32).

Dilutions are expressed as "a dilution of 1 in 2" or as a single number denoting the measurement of the extent of the dilution e.g. "a dilution of 2", or "a two-fold dilution".

It is incorrect to express dilutions as fractions, as "a dilution of  $1/2$ " is not equivalent to "a dilution of 1 in 2". In this example, it is the concentration and not the dilution that is halved; correspondingly the dilution is doubled.

Titres expressed in terms of "limiting" or "end-point" dilutions are stated as "a titre of 2" when the limiting or optimal dilution is 2. This convention avoids the contradiction involved in representing increasing titres of antibody by decreasing numerical values: a serum with a neutralising antibody titre of 64 is 8 times more potent than a serum with a titre of 8. Representing these titres as  $1/64$  and  $1/8$  does not express the relative potency of the sera.

According to this convention, titres are expressed directly as the limiting dilution, provided this is stated as a measurement as described above.

Dilutions are stated in terms of the initial serum dilution



irrespective of any extra dilution arising from the addition of other reagents to the test system. It is accepted that to allow comparison between different laboratories, expression in terms of the final dilution is the more suitable method.

Comparison with published results only arises in the case of the pN values calculated according to the formula described in Appendix B: this calculation does allow for the volumes of the reagents used in neutralisation tests.

APPENDIX B

Derivation of formula for calculation of neutralising potency or pN value for the reaction between a serum and virus. Method is taken from Pauls and Dowdle (1967).

$$\text{pN} = \log \log \text{virus neutralised (V)} + \log \text{serum end-point (A)} + \log \text{test volume (B)}.$$

- Let
- c =  $\log_{10}$  (TCID<sub>50</sub> of virus control)
  - a =  $\log_{10}$  (virus dilution used in neutralisation reaction)
  - d = volume of serum dilution = 0.90 ml
  - g = volume of virus suspension added to each serum dilution = 0.10 ml
  - h = volume of cell suspension added to each well = 0.02 ml
  - i = inoculum volume of virus-serum mixture = 0.04 ml
  - t =  $\log_{10}$  (50 per cent. serum end-point in terms of initial serum dilution)

i) Calculation of  $\log \log V$

$$\begin{aligned} \log V &= C - 0.16^* - a + \log \text{dilution in test} \\ &= C - 0.16 - a + \log \frac{g}{d + g} \end{aligned}$$

$$\begin{aligned} \log \log V &= \log (C - 0.16 - a + \log 0.1) \\ &= \log (C - 0.16 - a - 1.0) \\ &= \underline{\log (c - 1.16 - a)} \end{aligned}$$

ii) Calculation of  $\log A$ .

$$\begin{aligned} \log A &= t - \log \text{dilution in test} - \log \text{inoculum} \\ &= t - \log \frac{d}{d + g} - \log i \\ &= t - \log 0.9 - \log 0.04 \\ &= t - (-0.05) - (-1.40) \\ &= t + 1.45 \end{aligned}$$

\* = constant converting TCID<sub>50</sub> to infectious units (St. Groth, 1961).

iii) Calculation of log test volume

$$\log B = \log (h + i) = \log 0.06$$

$$= \underline{-1.22}$$

Basic formula when standard volumes used in test

$$pH = \log (C - 0.16 - a) + t + 1.45 - 1.22$$

$$= \underline{\log (C - 1.16 - a) + t + 0.23}$$

APPENDIX C

In the following lists, the patients from whom viruses were isolated are grouped according to the clinical site of infection, age and sex of the source patients and the observed antigenic and biological characters of the isolates.

The abbreviations used in the column headings are:

N = patient number

VN = laboratory number allocated to a virus isolate

Age is given in years

Sex: M = male patient; F = female patient

AS = reference antisera. Two sets of reference antisera were used in this study; they were designated 1st and 2nd.

HFT =  $\log_{10}$  (type 1 reference antiserum 50 per cent. end-point dilution with the virus indicated)

MST =  $\log_{10}$  (type 2 reference antiserum 50 per cent. end-point dilution with the virus indicated)

pN1 = neutralising potency value of virus with type 1 reference antiserum

pN2 = neutralising potency value of virus with type 2 reference antiserum.

Both pN1 and pN2 values were calculated according to the formula described in Appendix B.

In some instances the values listed are the means of replicate determinations; the individual values are given in the tables in the Results section.

pND = pN difference = pN1 - pN2

P = mean diameter of pock on the chorioallantoic membrane: derived from the measurement of at least 6 pocks.

- C = cytopathic effect (CPE) in BHK21 cell cultures: classified as
  - R = round cell type of CPE
  - FS = fusiform syncytia are a feature of the CPE
- EM = result of electron-microscopical examination of infected BHK21 cell: classified as
  - F = filaments or microtubules detected in infected cells
  - NF = no filaments detected

Patients with oral infections

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
01	3709	1	F	1st	1.85	≤1.00	2.27	≤1.42	≥0.85	0.30		
02	3075	2	F	"	2.07	≤1.00	2.67	≤1.60	≥1.07			
03	0848	2	M	"	1.60	≤1.00	2.05	≤1.45	≥0.60			
04	5237	4	F	"	1.71	≤1.00	2.23	≤1.52	≥0.71	0.53		
05	5810	4	F	"	1.60	≤1.00	2.11	≤1.51	≥0.60			
06	1339	4	M	"	1.73	≤1.00	2.17	≤1.44	≥0.73			
07	3330	4	F	"	1.85	≤1.00	2.56	≤1.71	≥0.85			
08	3254	4	F	"	1.99	≤1.00	2.70	≤1.71	≥0.99			
09	2771	6	F	"	1.67	≤1.00	2.24	≤1.57	≥0.67			
010	4810	7	M	"	2.09	≤1.00	2.49	≤1.40	≥1.09			
011	5477	8	F	"	1.70	≤1.00	2.28	≤1.58	≥0.70			
012	0497	9	F	"	1.77	≤1.00	2.25	≤1.48	≥0.77			
013	3166	9	F	"	1.78	≤1.00	2.20	≤1.42	≥0.78			
014	4941	10	F	"	2.31	≤1.00	2.88	≤1.57	≥1.31	0.63	R	
015	3848	11	M	"	2.30	≤1.00	2.86	≤1.56	≥1.30			
016	6096	15	F	"	1.69	≤1.00	2.39	≤1.70	≥0.69			
017	6246	17	F	"	2.07	≤1.00	2.50	≤1.43	≥1.07	0.50	R	
018	5356	19	M	"	1.48	≤1.00	2.11	≤1.63	≥0.48	0.80		
019	6084	19	F	"	1.71	≤1.00	2.20	≤1.49	≥0.71			
020	6020	19	M	"	2.07	≤1.00	2.48	≤1.41	≥1.07			
021	3883	21	F	"	2.03	≤1.00	2.45	≤1.42	≥1.03			
022	6222	21	F	"	2.22	≤1.00	2.64	≤1.42	≥1.22	0.50		
023	4918	22	M	"	2.33	≤1.00	2.86	≤1.53	≥1.33		R	
024	5100	24	F	"	1.77	≤1.00	2.35	≤1.58	≥0.77	0.45		
025	3060	54	F	"	1.70	≤1.00	2.30	≤1.60	≥0.70			
026	1108	2	F	"	2.96	2.02	2.98	2.04	+0.94			
027	5128	4	F	"	2.96	1.47	3.19	1.70	+1.49	0.45		
028	5392	7	M	"	2.32	1.13	2.73	1.54	+1.19	0.55		
029	3191	7	F	"	2.58	1.20	2.87	1.47	+1.38			
030	4545	12	F	"	2.36	1.11	2.56	1.31	+1.25		R	
031	5099	17	F	"	2.10	1.05	2.34	1.29	+1.05	0.42	R	
032	4210	18	M	"	2.00	1.10	2.73	1.83	+0.90	0.78	R	
033	7332	18	M	"	2.55	1.47	3.07	1.99	+1.08	0.33		
034	4214	19	M	"	2.18	1.00	2.54	1.36	+1.18			
035	7221	20	F	"	2.43	1.73	3.00	2.30	+0.70	0.60		
036	4457	22	M	"	2.12	1.15	2.55	1.70	+0.85	0.43		
037	6986	24	F	"	2.63	1.79	2.97	2.13	+0.84	0.36		
038	8907	2	M	2nd	2.48	1.64	3.23	2.39	+0.84			
039	7082	7	M	"	1.73	1.39	2.13	1.79	+0.34			
040	7614	14	F	"	2.53	2.03	2.68	2.18	+0.50	0.51		



N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
041	4454	16	F	2nd	2.60	2.20	3.04	2.64	+0.40			
042	8502	17	F	"	2.38	1.87	2.78	2.27	+0.51			
043	8181	17	F	"	2.06	1.83	2.53	2.30	+0.23			
044	9090	18	M	"	2.85	2.25	3.00	2.40	+0.60			
045	2808	18	F	"	3.10	2.57	3.67	3.14	+0.53			
046	5622	18	M	"	2.81	2.25	3.14	2.58	+0.56			
047	9631	19	F	"	1.83	1.15	2.31	1.63	+0.68			
048	8778	20	M	"	2.31	1.90	2.89	2.48	+0.41			
049	8040	22	M	"	2.03	1.60	2.64	2.21	+0.43			
050	6000	22	M	"	2.35	2.12	3.17	2.94	+0.23			
051	9183	23	M	"	1.68	1.10	2.30	1.72	+0.58			
052	8637	23	F	"	2.67	2.13	3.09	2.55	+0.54			
053	8534	23	F	"	2.00	1.67	2.56	2.23	+0.33			
054	8539	23	M	"	2.62	1.98	2.84	2.20	+0.64			
055	8072	24	F	"	2.08	1.48	2.45	1.85	+0.60			
056	9119	27	F	"	1.85	1.33	2.50	1.98	+0.42			
057	8211	27	M	"	2.09	1.71	2.56	2.18	+0.38			
058	8903	28	F	"	2.06	1.63	2.67	2.24	+0.43			
059	8290	34	F	"	2.08	1.75	2.60	2.27	+0.33			
060	4788a	55	F	"	2.24	1.95	2.92	2.63	+0.29			

Patients with skin infections

(a) Skin of trunk etc.

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
S1	1907	2	M	1st	1.85	≤ 1.00	2.25	≤ 1.40	≥ 0.85			
S2	0913	5	M	"	1.79	≤ 1.00	2.50	≤ 1.71	≥ 0.79	0.40	R	
S3	1657	5	F	"	1.72	≤ 1.00	2.34	≤ 1.62	≥ 0.72	0.25		
S4	6359	7	F	"	2.01	≤ 1.00	2.54	≤ 1.53	≥ 1.01		R	
S5	1648	12	M	"	1.73	≤ 1.00	2.18	≤ 1.45	≥ 0.73			
S6	8852	18	F	"	1.00	1.13	1.57	1.70	-0.13	2.58	FS	
S7	2482	21	F	"	1.18	1.03	1.79	1.64	+0.15	1.75	FS	
S8	5518	60	F	"	1.56	1.51	1.93	1.88	+0.05	1.75	FS	
S9	7634	18	F	2nd	1.39	1.19	2.05	1.85	+0.20	0.58		
S10	8640	21	F	"	1.98	1.75	2.49	2.26	+0.23			

(b) Herpetic whitlows

S11	2113	4	M	1st	2.07	≤ 1.00	2.59	≤ 1.52	≥ 1.07			
S12	6428	20	F	"	2.30	≤ 1.00	2.75	≤ 1.45	≥ 1.30		R	
S13	2827	20	F	"	1.96	1.09	2.52	1.65	+0.87	0.78		
S14	1068	21	F	"	2.23	1.00	2.65	1.42	+1.23			
S15	4697	3	M	2nd	2.13	1.85	2.63	2.25	+0.38			
S16	8795	10	F	"	2.25	1.87	2.67	2.29	+0.38			
S17	8744	14	M	"	1.90	1.55	2.32	1.97	+0.35			
S18	8233	18	F	"	2.38	2.05	3.00	2.67	+0.33			
S19	5756	20	F	"	2.41	2.07	2.74	2.40	+0.34			
S20	7986	21	F	"	1.53	1.07	2.14	1.68	+0.46			

(c) Herpes labialis

S21	3512	5	F	1st	1.82	≤ 1.00	2.22	≤ 1.40	≥ 0.82	0.42		
S22	2018	52	M	"	1.77	≤ 1.00	2.31	≤ 1.54	≥ 0.77			
S23	8209	14	F	2nd	1.90	1.60	2.52	2.22	+0.30			
S24	4092	17	M	"	2.58	2.23	3.07	2.72	+0.35			
S25	4610	19	F	"	3.05	2.75	3.22	2.92	+0.30			
S26	9165	21	M	"	2.30	1.92	2.90	2.52	+0.38	0.75		
S27	7953	23	F	"	1.67	1.30	2.26	1.89	+0.37	0.45		
S28	9130	32	M	"	2.03	1.35	2.76	2.08	+0.68			
S29	8231	35	M	"	2.39	1.99	2.82	2.42	+0.40			
S30	9430	45	M	"	2.02	1.69	2.38	2.05	+0.33			

(d) Herpes facialis

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
S31	5424	5	M	1st	2.40	1.07	2.80	1.47	+1.33			
S32	4922	20	M	"	2.07	≤ 1.00	2.47	≤ 1.40	≥ 1.07	0.50		
S33	0371	21	M	"	2.02	≤ 1.00	2.33	≤ 1.31	≥ 1.02			
S34	5400	29	M	"	1.79	≤ 1.00	2.36	≤ 1.57	≥ 0.79	0.53		
S35	7288	1	F	2nd	2.00	1.60	2.48	2.08	+0.40			
S36	7412	5	M	"	2.10	1.98	2.39	2.27	+0.12			
S37	9184	10	F	"	2.10	1.68	2.68	2.26	+0.42			
S38	8075	19	M	"	2.00	1.74	2.50	2.24	+0.26			
S39	9319	22	M	"	1.96	1.42	2.56	2.02	+0.54	0.67		
S40	9043	25	M	"	2.08	1.53	2.52	1.97	+0.55			
S41	7552	45	F	"	2.05	1.53	2.63	2.11	+0.52	0.60		
S42	-	47	F	"	2.53	2.15	3.05	2.67	+0.38			
S43	3662	60	F	"	2.75	2.33	3.20	2.78	+0.42			

Patients with eye infections

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
E1	2862	8	F	1st	2.00	≤ 1.00	2.59	≤ 1.59	≥ 1.00			
E2	3447	9	M	"	2.07	≤ 1.00	2.64	≤ 1.57	≥ 1.07			
E3	2669	16	M	"	1.51	≤ 1.00	2.15	≤ 1.64	≥ 0.51			
E4	2939	17	F	"	1.90	≤ 1.00	2.53	≤ 1.63	≥ 0.90			
E5	0165	20	F	"	1.53	≤ 1.00	2.20	≤ 1.67	≥ 0.53			
E6	3292	22	M	"	2.03	≤ 1.00	2.64	≤ 1.61	≥ 1.03			R
E7	3168	23	M	"	2.47	≤ 1.00	2.68	≤ 1.21	≥ 1.47			
E8	3940	27	M	"	1.75	≤ 1.00	2.46	≤ 1.71	≥ 0.75			
E9	0082	30	M	"	2.02	≤ 1.00	2.42	≤ 1.40	≥ 1.02			
E10	3224	31	M	"	1.99	≤ 1.00	2.42	≤ 1.43	≥ 0.99			
E11	0383	32	F	"	1.73	≤ 1.00	2.43	≤ 1.70	≥ 0.73			
E12	0549	34	F	"	2.04	≤ 1.00	2.46	≤ 1.42	≥ 1.04			
E13	3909	36	M	"	1.73	≤ 1.00	2.13	≤ 1.40	≥ 0.73			
E14	4000	44	F	"	1.77	≤ 1.00	2.32	≤ 1.55	≥ 0.77			
E15	0379	44	F	"	1.90	≤ 1.00	2.30	≤ 1.40	≥ 0.90			
E16	0612	46	M	"	2.20	≤ 1.00	2.46	≤ 1.26	≥ 1.20			
E17	2865	47	M	"	2.05	≤ 1.00	2.20	≤ 1.15	≥ 1.05			
E18	2944	50	M	"	1.61	≤ 1.00	2.28	≤ 1.67	≥ 0.61			
E19	3081	50	M	"	1.95	≤ 1.00	2.35	≤ 1.40	≥ 0.95			
E20	2879	54	M	"	1.81	≤ 1.00	2.32	≤ 1.51	≥ 0.81			
E21	1151	54	F	"	1.71	≤ 1.00	2.28	≤ 1.57	≥ 0.71			
E22	2623	54	F	"	2.17	≤ 1.00	2.36	≤ 1.19	≥ 1.17	0.38		
E23	0523	54	F	"	2.38	≤ 1.00	2.53	≤ 1.15	≥ 1.38			
E24	3256	55	M	"	1.79	≤ 1.00	2.35	≤ 1.56	≥ 0.79			
E25	0829	59	M	"	1.80	≤ 1.00	2.13	≤ 1.33	≥ 0.80			
E26	0845	68	F	"	2.08	≤ 1.00	2.53	≤ 1.45	≥ 1.08			
E27	2712	69	F	"	1.80	≤ 1.00	2.44	≤ 1.64	≥ 0.80			
E28	2828	70	F	"	1.95	≤ 1.00	2.59	≤ 1.64	≥ 0.95			
E29	1592	75	M	"	1.85	≤ 1.00	2.08	≤ 1.23	≥ 0.85			
E30	2755	80	M	"	1.89	≤ 1.00	2.34	≤ 1.45	≥ 0.89			
E31	1348	18	M	"	2.17	1.10	2.55	1.54	+1.07			
E32	3366	19	F	"	2.16	1.25	2.64	1.73	+0.91			
E33	1891	24	F	"	2.15	1.08	2.51	1.48	+1.07			
E34	3182	29	M	"	2.57	1.57	2.85	1.85	+1.00			
E35	2703	48	M	"	2.13	1.03	2.55	1.45	+1.10			
E36	0044	50	F	"	2.11	1.03	2.43	1.35	+1.08	0.45		
E37	0689	51	M	"	1.83	1.00	2.25	1.42	+0.83	0.50		
E38	1320	51	F	"	2.31	1.32	2.57	1.58	+0.99			
E39	3152	53	M	"	1.99	1.00	2.37	1.38	+0.99	0.30		

Patients with genital tract infections

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
G1	5760	17	F	1st	1.67	1.73	2.07	2.13	-0.06	1.62	FS	F
G2	3274	21	F	"	1.05	1.66	1.48	2.09	-0.61	1.67	FS	F
G3	6587	22	F	"	1.75	1.53	2.11	1.89	+0.22	1.19	FS	F
G4	6653	22	F	"	2.43	2.29	2.33	2.19	+0.14	2.00	FS	F
G5	4035	23	F	"	1.66	1.66	1.97	1.97	0.00	1.67	FS	F
G6	6977	23	F	"	1.73	≤ 1.00	2.34	≤ 1.61	≥ 0.73	0.67	R	NF
G7	5285	24	F	"	1.62	≤ 1.00	2.14	≤ 1.53	≥ 0.61	0.51	R	NF
G8	5423	26	F	"	1.43	1.50	1.67	1.74	-0.07	2.00	FS	F
G9	2107	28	F	"	1.05	1.30	1.64	1.89	-0.25	1.62	FS	F
G10	3668	19	M	"	1.20	1.75	1.61	2.16	-0.55	1.01	FS	NF
G11	5179	21	M	"	2.05	1.84	2.41	2.20	+0.21	1.63	FS	F
G12	6749	23	M	"	1.32	1.17	1.70	1.55	+0.15	1.10	FS	F
G13	1718	24	M	"	1.73	1.65	2.22	2.14	+0.08	1.42	FS	F
G14	5102	30	M	"	1.96	2.50	2.12	2.66	-0.54	1.58	FS	F
G15	5009	33	M	"	1.21	1.71	1.69	2.19	-0.50	1.83	FS	F
G16	4090	34	M	"	1.70	1.70	2.08	2.08	0.00	1.75	FS	F
G17	6248	39	M	"	2.16	2.09	2.49	2.42	+0.07	1.10	FS	F
G18	6173	41	M	"	1.45	1.49	1.89	1.93	-0.04	1.50	FS	F
G19	8790	17	F	2nd	1.90	2.25	2.22	2.57	-0.35	1.37	FS	F
G20	9541	18	F	"	2.45	2.75	2.52	2.82	-0.30	1.33	FS	F
G21	7927	19	F	"	2.40	2.03	2.66	2.29	+0.37	0.49	R	NF
G22	9468	19	F	"	1.25	2.05	1.63	2.43	-0.80	1.31	FS	F
G23	9007	21	F	"	2.35	2.55	2.59	2.79	-0.20	1.88	FS	F
G24	8730	22	F	"	1.47	1.71	2.09	2.33	-0.24	2.45	FS	F
G25	8742	22	F	"	1.82	2.15	2.26	2.59	-0.33	1.53	FS	F
G26	8682	23	F	"	2.34	2.03	2.79	2.48	+0.31	0.58	R	NF
G27	7464	24	F	"	1.59	1.69	1.59	1.69	-0.10	2.50	FS	F
G28	8755	24	F	"	2.65	2.98	2.47	2.80	-0.33	1.28	FS	F
G29	9291	24	F	"	2.44	2.02	2.84	2.42	+0.42	0.57	R	NF
G30	9794	24	F	"	1.42	1.95	2.68	3.21	-0.53	1.50	FS	F
G31	9810	24	F	"	1.00	1.05	1.73	1.78	-0.05	2.04	FS	F
G32	7637	25	F	"	2.20	1.83	2.58	2.21	+0.37	0.54	R	NF
G33	8236	25	F	"	1.33	1.40	1.75	1.82	-0.07	1.46	FS	F
G34	9712	25	F	"	1.83	1.42	2.42	2.01	+0.41	0.64	R	NF
G35	9431	26	F	"	2.88	2.20	3.18	2.50	+0.68	0.61	R	NF
G36	8983	29	F	"	2.16	2.82	2.51	3.17	-0.66	2.00	FS	F
G37	9907	34	F	"	2.08	2.24	2.60	2.70	-0.16	1.23	FS	F
G38	7951	34	F	"	2.73	2.53	2.91	2.71	+0.20	0.45	R	NF
G39	7365	38	F	"	2.09	2.37	2.18	2.46	-0.28	1.55	FS	F
G40	7842	18	M	"	2.05	2.35	1.95	2.25	-0.30	1.69	FS	F

N	VN	Age	Sex	AS	HFT	MST	pN1	pN2	pND	P	C	EM
G41	9770	18	M	2nd	1.67	1.65	2.40	2.38	+0.02	1.70	FS	F
G42	8928	20	M	"	1.90	2.17	2.12	2.39	-0.27	1.90	FS	F
G43	9009	21	M	"	2.09	2.65	2.51	3.07	-0.56	1.97	FS	F
G44	9696	21	M	"	1.46	1.72	1.77	2.03	-0.26	2.10	FS	F
G45	9840	22	M	"	1.42	1.49	1.96	2.03	-0.07	1.18	FS	F
G46	9296	22	M	"	1.76	2.01	2.04	2.29	-0.25	1.12	FS	F
G47	9869	24	M	"	1.12	1.35	1.80	2.03	-0.23	1.75	FS	F
G48	9855	24	M	"	2.17	2.34	2.67	2.84	-0.17	1.18	FS	F
G49	9161	25	M	"	2.26	1.73	2.81	2.28	+0.53	0.50	R	NF
G50	9579	25	M	"	1.52	1.94	1.98	2.40	-0.42	1.40	FS	F
G51	9758	25	M	"	2.57	2.05	2.83	2.31	+0.52	0.73	R	NF
G52	9771	25	M	"	1.95	2.29	2.52	2.86	-0.34	2.10	FS	F
G53	9796	25	M	"	2.30	2.77	2.69	3.16	-0.47	1.46	FS	F
G54	7991	28	M	"	1.22	1.92	1.60	2.30	-0.70	1.75	FS	F
G55	8732	29	M	"	1.79	2.00	2.39	2.60	-0.21	1.04	FS	F
G56	9930	29	M	"	1.05	1.43	1.65	2.03	-0.38	1.39	FS	F
G57	9746	30	M	"	1.28	1.72	2.01	2.45	-0.44	1.18	FS	F
G58	9854	30	M	"	1.72	1.90	2.08	2.26	-0.18	1.15	FS	F
G59	7992	31	M	"	1.10	2.02	1.36	2.28	-0.92	1.38	FS	F
G60	8208	33	M	"	1.78	2.20	2.27	2.69	-0.42	1.63	FS	F
G61	9663	36	M	"	1.15	1.56	1.76	2.17	-0.41	1.67	FS	F
G62	9864	40	M	"	2.11	2.09	2.71	2.69	+0.02	2.04	FS	F
G63	9816	45	M	"	2.08	2.07	2.60	2.59	+0.01	1.60	FS	F
G64	9853	47	M	"	2.06	2.40	2.59	2.93	-0.34	1.40	FS	F
G65	7967	68	M	"	2.74	3.02	2.73	3.01	-0.28	1.00	FS	F
G66	8984	67	M	"	2.31	2.69	2.69	3.07	-0.38			

APPENDIX D

MATERIALS AND SOLUTIONS

Dulbecco's Phosphate Buffered Saline

Solution A

NaCl	8.0 g
KCl	0.2 g
Na <sub>2</sub> HPO <sub>4</sub>	1.15 g
KH <sub>2</sub> PO <sub>4</sub>	0.2 g
Glucose	0.2 g
Distilled water	1000 ml

1% aqueous phenol red - 1 or 2 drops

Distributed in 100-ml. amounts, autoclaved at 115°C for 20 min.  
and stored at 4°C.

Solution B

CaCl <sub>2</sub>	2.0 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2.0 g
Distilled water	100 ml

Dispensed in 2.5-ml amounts, sterilised at 121°C for 20 min.  
Stored at 4°C.

To make the complete salt solution 0.5 ml solution B added to 100 ml  
solution A.



Hanks' salt solution

Solution A

(1) NaCl	160 g
KCl	8 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2 g
Distilled water	800 ml
(2) CaCl <sub>2</sub>	2.8 g
Distilled water	100 ml

(1) and (2) mixed slowly and volume made up to 1000 ml with distilled water, 2 ml CHCl<sub>3</sub> added and stored at 4°C.

Solution B

Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	3.04 g
KH <sub>2</sub> PO <sub>4</sub>	12 g
Glucose	20 g
Distilled water	800 ml

100 ml of 0.4% phenol red in 0.1N NaOH added, made up to 1000 ml with distilled water, 2 ml CHCl<sub>3</sub> added and stored at 4°C.

For use: 100 ml stock solution A  
100 ml stock solution B  
Distilled water 800 ml

Dispensed in 100-ml volumes, sterilised at 121°C for 20 min. and stored at 4°C.

Phosphate buffered saline

NaCl	8.00 g
K <sub>2</sub> HPO <sub>4</sub>	1.21 g
KH <sub>2</sub> PO <sub>4</sub>	0.34 g
Distilled water to 1000 ml	

Dispensed in 100-ml volumes and stored at 4°C.

Formal saline

NaCl	5.0 g
Na <sub>2</sub> SO <sub>4</sub>	15.0 g
Distilled water to 900 ml	

100 ml 40 per cent. formaldehyde added. Dispensed in 100-ml volumes.

Skim milk

1 per cent. solution in distilled water. Dispensed in 100-ml volumes. Sterilised by heating to 100°C for 1 hr on 3 successive days. Stored at 4°C.

Calf serum

Whole calf blood collected at the slaughterhouse from calves less than 1 week old and allowed to clot. Serum removed and clarified by centrifugation at 3,000 G for 20 min., and sterilised by either of 2 methods.

- a) serum filtered twice through a Seitz sterilising grade disc, dispensed in 100-ml volumes and stored at 4°C.
- b) serum filtered through membrane filters of porosity 0.5 to 0.22 $\mu$ . The final grade was repeated with a sterilised membrane, and the serum dispensed in 20-ml volumes and stored at -20°C.

Tryptose Phosphate Broth (TPB)(Oxoid)

29.5 g dissolved in 1000 ml distilled water.

Mixed well and dispensed in 100-ml amounts, sterilised at 121°C for 20 min. and stored at 4°C.

Sodium bicarbonate solutions: 1.4 per cent. or 4.4 per cent.

14 g or 44 g NaHCO<sub>3</sub> dissolved in 1000 ml distilled water. A few drops of 1% phenol red added and CO<sub>2</sub> bubbled through the solution until colour is pale pink.

Distributed to bottles with as little air space as possible and caps screwed on tightly. Autoclaved at 115°C for 20 min. and stored at 4°C.

Antibiotic solution

5 vials crystalline benzyl penicillin (10<sup>6</sup> units per vial) and 5 vials streptomycin (1g/vial) dissolved in 30 ml distilled water, and made up to 50 ml with distilled water. Distributed in 5 ml volumes and stored at -30°C.

Trypsin

"Difco" Trypsin (1:250)      10 g  
Distilled water              1000 ml

Left for 2-3 hr at 37°C to dissolve. Dispensed in 10-ml volumes, sterilised by filtration, and distributed in 10-ml amounts and stored at -20°C.

Versene

Sequestric acid, di-sodium salt    10 g  
(British Drug Houses)  
Distilled water                      1000 ml

Distributed in 1.5-ml amounts, autoclaved for 20 min. at 121°C and stored at 4°C.

Trypsin-Versene

Trypsin solution	10 ml
Versene "	2.0 ml
Dulbecco solution A	90 ml

4.4% sodium bicarbonate added to bring to alkaline pH, and stored at 4°C.

Methocel Carboxymethylcellulose (Koch-Light)

500 ml distilled water cooled to 4°C

500 ml distilled water heated to 90°C and 50 g methocel added (to wet methocel). This mixture cooled then cold 500 ml distilled water added. Dispensed in 50-ml amounts, sterilised by autoclaving at 121°C for 20 min. and stored at 4°C.

To use:	Eagle's MEM (10x concentrated)	8 ml
	TPB	10 ml
	Calf serum	10 ml
	Methocel	19 ml
	1.4 per cent sodium bicarbonate	3 ml
	Penicillin and streptomycin solution	0.2 ml
	Distilled water to	100 ml

Trypan Blue

0.5 per cent. w/v solution in saline.

Dispensed in 2-ml volumes, sterilised by autoclaving at 121°C for 20 min.

Methyl Violet

Methyl violet 6B 10 g

Distilled water 1000 ml

For use 10 ml added to 90 ml formal saline.

APPENDIX E

# The Specificity of Rabbit Antisera to *Herpesvirus hominis* and its Dependence on the Dose of Virus Inoculated

BY

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# THE SPECIFICITY OF RABBIT ANTISERA TO *HERPESVIRUS HOMINIS* AND ITS DEPENDENCE ON THE DOSE OF VIRUS INOCULATED

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THE quantal neutralisation test of Fazekas de St. Groth (1961) was applied by Pauls and Dowdle (1967) to distinguish between strains of *Herpesvirus hominis*. The application depended on the development of tissue culture micro-techniques and utilised primary rabbit kidney tissue cells. The present paper demonstrates that BHK21 cells (Macpherson and Stoker, 1962) can also be used in this procedure. During the study it was found, in contrast to the findings of Pauls and Dowdle, that rabbit antisera to type-1 virus cross-reacted strongly with type-2 virus, whereas antisera to type-2 virus showed a less marked cross-reaction. An investigation into the effect of varying the dose of virus given to the rabbits to produce the antisera provided a possible explanation for the discrepancy.

## MATERIALS AND METHODS

Virus strain MS obtained from Dr G. Plummer was taken as a representative type-2 virus (Plummer, 1964; Pauls and Dowdle); strain HF obtained from the Institute of Virology, Glasgow, was taken as a representative type-1 virus (Pauls and Dowdle); strain 1657 isolated in Edinburgh has been used in the preparation of complement-fixing and neutralising antigens in this laboratory (Smith, Peutherer and MacCallum, 1967). In addition to these three strains, the Watson and Dawson strains of *Herpesvirus hominis*, previously reported as being type-1 and type-2 strains respectively (Plummer, Waner and Bowling, 1968), were obtained from Dr Plummer. Rabbits were given inoculations of virus strains MS, HF and 1657 produced in RK13 cells grown at 35°C in 1-litre Roux flasks containing 100 ml of medium 199 supplemented with 5 per cent. rabbit serum. Cells showing an almost complete cytopathic effect (CPE) after 1-2 days were harvested with glass beads in 40 ml medium per Roux flask. Before use, these cell suspensions were frozen and thawed three times and treated for 5 min. at full power in a Kerry ultrasonic bath. These stocks were titrated for infectivity as described below in BHK21 cells. Extracts from uninfected cells were prepared in the same way. Antisera were produced in rabbits of the department stock according to the schedule of Plummer. Intramuscular and intraperitoneal injections of 3 ml of virus suspension without adjuvant were given initially and repeated 3 wk later. One week after the second inoculation, the rabbits were bled out. Pairs of rabbits were immunised with suspensions of each of the three viruses HF, MS and 1657 containing  $10^{3.5}$ - $10^{5.8}$  TCID<sub>50</sub> per 0.02 ml to produce "high-dose" (H) sera. In addition, pairs of rabbits were given inoculations of the above virus stocks diluted to contain  $10^{1.5}$ - $10^{2.5}$  TCID<sub>50</sub> per 0.02 ml to produce "low-dose" (L) sera.

Stock virus for neutralisation tests was grown in BHK21 cells in diploid cell medium (Grand Island Biological Corporation, Grand Island, New York) supplemented as described by Peutherer and Smith (1966), in rolling 2-litre culture bottles (House and Wildy, 1965). Cells were harvested as above in 20 ml medium per bottle and treated at full power for

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5 min. in an MSE ultrasonicator. After centrifugation at 400*g* for 10 min., the supernate was stored in small volumes at  $-65^{\circ}\text{C}$ .

Both infectivity and neutralising antibody titrations were performed according to the methods of Pauls and Dowdle in "Linbro" plastic disposable plates (ISFB96), with BHK21 cells at a concentration of 500,000 cells per ml. Pipettes were drawn from glass tubing and calibrated with a wire gauge to a diameter that delivered drops of 0.02 ml. A separate dropping pipette was used for each serum dilution in the neutralising antibody estimation. Plates were sealed with clear adhesive tape and incubated for 3 days at  $35^{\circ}\text{C}$ , after which each well was examined with an inverted microscope. Wells showing 5 per cent. or more of CPE were scored as positive and 50 per cent. end-point dilutions were calculated for both infectivity and neutralising antibody titrations by the method of Reed and Muench (1938). In these calculations, for example, a dilution of 1 in 10 was expressed as 1/10, or  $10^{-1}$ , and the 50 per cent. end-point dilutions in the same manner.

TABLE I

*Serum neutralisation titres and pN values of an HF-H serum against HF virus used at different concentrations in the neutralisation test*

Titre of challenge virus used in test ( $\log_{10}$ TCID <sub>50</sub> per 0.02 ml)	$\log_{10}$ titre of neutralising antibody in serum	pN value
2.0	2.74	2.89
2.7	2.52	2.94
3.0	2.31	2.80
3.7	2.09	2.72
4.0	2.03	2.71

The reciprocal of the end-point dilution of the virus suspension was taken as the infectivity titre (TCID<sub>50</sub>) and that of the 50 per cent. end-point dilution of the serum as the neutralising antibody titre. The neutralising potency of a serum against a test strain was determined in terms of the serum pN value calculated from the formula of Pauls and Dowdle. Allowing for the different size of standard drop employed,  $\text{pN} = \log(V - d - 1.16) + t + 0.23$ , when  $V = \log_{10}$  virus control titre,  $d = -\log_{10}$  dilution of virus used in neutralisation reaction, and  $t = \log_{10}$  serum titre. In some instances, no neutralisation of a virus could be demonstrated even at the highest concentration of serum tested (1 in 10 dilution). Based on a neutralisation end-point dilution of 1 in 10, in fact, the minimum calculable value of pN is 1.49. To allow average values and pN differences to be calculated, the pN values of these sera were scored as "0", instead of " $<1.49$ ".

## RESULTS

The reproducibility of the values for infectivity titres (TCID<sub>50</sub>) was tested by performing six replicate titrations of a single preparation of MS virus grown in BHK21 cells. The values obtained all fell within the range  $10^{5.50}$ – $10^{6.00}$  TCID<sub>50</sub> per 0.02 ml, with a mean titre and standard deviation of  $10^{5.58 \pm 0.20}$  TCID<sub>50</sub> per 0.02 ml. The effect of varying the dose of virus for use in neutralisation tests on the titre of neutralising antibody and on the resultant pN value of an anti-HF-H (high dose) serum is shown in table I. Thus, whilst variation of the virus concentration by  $\pm 1 \log_{10}$  from the recommended dose of  $10^{3.0}$  TCID<sub>50</sub> per 0.02 ml gave serum titres ranging from  $10^{2.03}$  to  $10^{2.74}$ , the corresponding pN values varied only within the narrow range of 2.71–2.94.



A similar experiment with an HF-L serum showed almost identical degrees of variation in the serum titres and pN values. The reproducibility of the pN values determined for three sera with the homologous viruses is shown in table II. The standard deviations are comparable to those previously reported (Dowdle *et al.*, 1967). Antisera produced against uninfected cell extracts showed no detectable neutralisation of any of the viruses tested. Table III compares the pN values obtained for the sera of rabbits given inoculations of high (H) and low (L) concentrations of virus. With 1657-L and MS-L sera the values of less

TABLE II

*Reproducibility of pN values obtained in four replicate neutralisation tests made between each of three sera and their homologous viruses*

Serum	pN values in four tests	Mean pN value	Standard deviation of pN values
1657-H	2.68 2.57 2.60 2.72	2.64	±0.07
HF-H	2.62 2.32 2.84 2.95	2.68	±0.28
MS-L	2.87 2.55 2.41 2.49	2.58	±0.20

than 1.49 arose because the serum of one of the pair of rabbits did not neutralise the virus tested. Sera produced with the higher immunising doses of virus cross-reacted to a considerable degree with heterologous viruses, although in each case the homologous pN value was greater than the heterologous value. The sera produced with the lower concentrations of immunising virus showed greater specificity and in some cases reacted with homologous virus only. This effect of the concentration of immunising virus may account for the different cross-reactions observed initially in this laboratory compared with the reported findings of Pauls and Dowdle. With the higher doses of virus inoculum, viral antigens will persist in the tissues for a longer period of time, and a hyperimmune antibody response will occur. Under these circumstances, it is accepted that the antisera obtained will show less specificity than those produced with lower concentrations of immunising virus (Boyd, 1956). However, the response of rabbits may be unpredictable because of the ability of *Herpesvirus hominis* to multiply in the rabbit. Apart from the effect of variation of the dose of immunising virus, one other difference existed between the experimental systems of this report and that of Pauls and Dowdle. These authors used primary rabbit kidney cell cultures both for the production of virus for immunisation of rabbits

and for the neutralisation reactions. In my study the virus for immunisation was grown in RK13 cells and the virus for neutralising antibody titrations in BHK21 cells. A control antiserum, produced by the inoculation of uninfected RK13 cells, showed no detectable neutralisation of virus grown in BHK21 cells. It cannot be excluded, however, that there may be differences in antibody production according to whether the immunising virus is grown in primary rabbit kidney cells or RK13 cells.

TABLE III

*Effect of the size of the dose of virus inoculated into rabbits on the pN value of the resulting antiserum measured against different viruses*

Serum	Dose of virus inoculated into rabbit	pN value * of serum in neutralisation test with virus of strain		
		HF (type 1)	MS (type 2)	1657 (type 1)
HF-H . .	High	2.70	2.43	2.51
HF-L . .	Low	1.73	0	0
MS-H . .	High	2.61	2.66	2.64
MS-L . .	Low	1.08	2.67	0.90
1657-H . .	High	2.62	2.42	2.71
1657-L . .	Low	0.73	0	1.25

\* Value shown is the average of the pN values obtained in two tests made with each of two sera prepared in different rabbits. pN values obtained in these tests that were less than 1.49 were scored as "0" (see Materials and methods). Average values of less than 1.49 shown in the table indicate that one or both of the sera tested gave a value of 0.

Virus 1657 gave pN values similar to those of HF and MS viruses when titrated against H sera. With L sera, however, it gave results similar to those obtained with HF virus, indicating that 1657 is HF-like, or type 1.

Mixtures prepared for all the neutralisation experiments were kept at room temperature for  $\frac{1}{2}$  hr before inoculation on plates. Incubation of the virus-serum mixture at 37° or 4°C for  $\frac{1}{2}$  hr had no appreciable effect on the cross-reactivity of the sera. Incubation for longer periods at room temperature, or at 37° or 4°C, did not enhance the specificity of the pN values; in some cases it increased the degree of cross-reaction.

#### DISCUSSION

The modification described above to the method of Pauls and Dowdle (1967) is a reproducible procedure for estimating the infectivity of preparations of *Herpesvirus hominis* and the virus-neutralising (pN) values of antisera. Compared with the original method, it has the following advantages: (a) the use of a semicontinuous cell system, BHK21, is less laborious than the preparation of a primary rabbit kidney cell culture, (b) the use of calibrated pasteur pipettes

avoids the need to purchase or manufacture a large number of permanent standard-drop pipettes, and (c) the incubation time is reduced from 5 to 3 days.

Strains of *Herpesvirus hominis* can be typed by determining the pN values with type-1 and type-2 antisera (Dowdle *et al.*, 1967). Values of the difference,  $pN_1 - pN_2$ , fall into two groups, although the range of values depends on the potency and cross-reactivity of each of the typing sera used. The evidence of the present work indicates that if both of the typing sera show a marked degree of cross-reactivity it may prove difficult to allocate an unknown virus to either type 1 or type 2. To distinguish between strains of *Herpesvirus hominis* a pair of antisera, one of greater and one of lower specificity, are used in this laboratory. Table IV illustrates how such a pair of sera can separate a number of strains into

TABLE IV

*pN values, pN difference and type of virus determined in neutralisation tests of five strains of virus with an HF-H and an MS-L antiserum*

Virus strain	pN value for virus determined in serum		pN difference (pN <sub>1</sub> -pN <sub>2</sub> )	Type of virus indicated by result
	HF-H (pN <sub>1</sub> )	MS-L (pN <sub>2</sub> )		
HF . . .	2.68	0	+2.68	1
MS . . .	2.32	2.58	-0.26	2
1657 . . .	2.41	0	+2.41	1
Watson . . .	2.40	0	+2.40	1
Dawson . . .	1.95	2.10	-0.15	2

two antigenic types. The type-1 viruses reacted only with HF antisera and gave a positive value for the difference  $pN_1 - pN_2$ . The type-2 viruses reacted with both antisera and gave a negative value for the difference  $pN_1 - pN_2$ . The antigenic typing of strains Dawson and Watson agrees with the reported results of Plummer *et al.* (1968).

## SUMMARY

Typing of strains of *Herpesvirus hominis* according to the method of Pauls and Dowdle can be performed in BHK cells instead of in primary rabbit kidney cells. The modified method has the advantage of a reduction in incubation time and the convenience of working with a semi-continuous cell line. The concentration of virus antigen inoculated into rabbits to produce typing antisera has an effect on the cross-reactivity of the sera obtained. Higher immunising doses of virus produce antisera with higher degrees of cross-reactivity than is found in antisera produced in response to low immunising doses of virus.

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# Characterization of genital strains of *Herpesvirus hominis*

BY

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# Characterization of genital strains of *Herpesvirus hominis*

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Slavin and Gavett (1946) reported antigenic differences between strains of *Herpesvirus hominis* (HVH) and subsequent serological studies have allowed strains of HVH to be allocated to one of two types (Pauls and Dowdle, 1967). In addition, an association between the clinical site of infection and the antigenic type of virus recovered has been established (Dowdle, Nahmias, Harwell, and Pauls, 1967; Schneeweis, 1967), genital isolates being designated Type 2, while the typical oral, eye, and central nervous system strains are classified as Type 1. Further studies have shown that these two types of HVH may be distinguished biologically, by the cytopathic effect (CPE) they produce in tissue culture (Munk and Donner, 1963), by the size of pock that develops on the chorioallantoic membrane (Parker and Banatvala, 1967), and by the electron microscopical demonstration of filaments within the nuclei of infected cells (Couch and Nahmias, 1969). Other factors, such as the base composition of the viral nucleic acid and the pathogenicity in animals, can also be used to differentiate the two types of virus (Nahmias and Dowdle, 1968).

Josey, Nahmias, and Naib (1968) drew attention to a possible link between HVH infection of the cervix and cervical neoplasia. Evidence of the association was obtained in two ways. Firstly, from the results of cytological surveys (Naib, Nahmias, and Josey, 1966), it was apparent that infection of the cervix with HVH was associated with an increased incidence of cellular anaplasia. Secondly, serological studies indicated that sera from women with cervical cancer were more likely to contain antibody to HVH Type 2 than sera from controls matched for age and race (Royston and Aurelian, 1970; Rawls, Tompkins, and Melnick, 1969; Nahmias, Josey, Naib, Luce, and Guest, 1970; Skinner, Thouless, and Jordan, 1971). However, when additional factors such as parity and age at first pregnancy were considered in the selection of control patients, the difference between the two groups became less apparent (Rawls, Adam, and Melnick, 1972). Indeed, in studies of New Zealand (Rawls, Iwamoto, Adam, Melnick, and Green, 1970) and American cancer populations (Smith,

Lowry, Melnick, and Rawls, 1972), no difference could be detected between the carcinoma and control patients.

Further knowledge of the general ecology and particularly the anatomical sites of infection with the two types of HVH in different populations is obviously relevant to this problem. A previous report (Smith, Peutherer, and MacCallum, 1967) established that in the Edinburgh area a significant proportion (49.5 per cent.) of the population in the sexually active range 15 to 25 years does not have detectable antibody to HVH. These individuals are fully susceptible to infection with either type of HVH, and it was therefore considered worthwhile to study the viruses isolated from genital infections. This paper reports observations on the characterization of the viruses isolated from the genital tract of 65 patients.

## Material and methods

### CELL CULTURE

BHK21 cells (MacPherson and Stoker, 1962) and RK cells were propagated and maintained as described previously (Peutherer and Smith, 1966).

### VIRUS ISOLATION

Over the 5-year period ending October 31, 1971, swabs were submitted for virus isolation from patients attending the venereal disease clinic of the Royal Infirmary, Edinburgh, and some private patients referred to the above department by the gynaecologists. The swabs were transferred to the laboratory in Hanks's balanced salt solution containing antibiotics; isolation was attempted in BHK and RK cells and the viruses identified by neutralization with specific antisera.

### ANTIGENIC TYPING

Virus isolates were typed by a modification (Peutherer, 1970) of the microneutralization test (Pauls and Dowdle, 1967), using rabbit antisera to HVH Types 1 and 2. The HF strain was used as the Type 1 reference virus and the MS virus as the Type 2 strain. The neutralizing potency (pN) values for the reference virus strains were calculated for both types of antisera, and the pN differences (pN<sub>1</sub>-pN<sub>2</sub>) established for each virus isolate allowed these to be allocated to either Type 1 or Type 2 (HF-like or MS-like).

## EGG INOCULATION

Ten-fold dilutions of each virus isolate were prepared in 1 per cent. skim milk and 0.2 ml. inoculated on to the chorioallantoic membrane of 10 to 12-day-old embryonated eggs. The eggs were incubated at 35°C. for 7 days when the chorioallantoic membranes were excised and fixed in formol saline, and the diameter of at least six discrete pocks was measured with a Vernier hand lens.

## ELECTRON MICROSCOPY

Monolayers of BHK cells were inoculated with sufficient virus to give complete CPE in 24 hours. The cells were harvested using glass beads, fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite (Glauert, 1965). Ultrathin sections were cut on an LKB 1 microtome, stained with uranyl acetate and lead citrate, and viewed in an AEI EM6 electron microscope.

## CYTOPATHIC EFFECT

A low dose of virus was inoculated on to BHK cells. Unstained preparations were examined in the light microscope at daily intervals and the CPE was recorded.

## SEROLOGICAL TESTS

Complement-fixation tests (CFT) and neutralization tests (NT) were performed as described previously (Smith and others, 1967).

## SPECIMENS

During the 5-year period swabs or scrapings were received from 78 female and 91 male patients, all of whom were

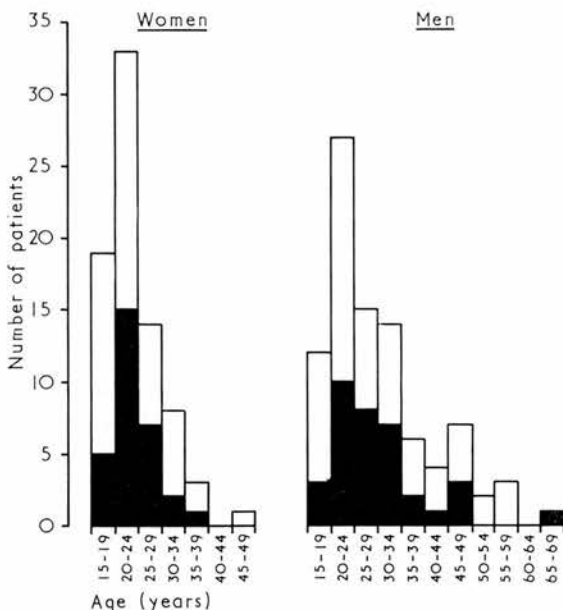


FIG. 1 Age distribution of male and female patients ■ HVH isolated

diagnosed as having herpes-like lesions on the external genitalia or the cervix. The age distribution of the patients is shown in Fig. 1. The average age of the female patients was 24 years (15 to 49) and that of the male patients 30 years (17 to 68). Two-thirds of the female patients were less than 25 years of age while only one-third of the male patients were in this age group.

Specimens from thirty female and 35 male patients yielded HVH in cell culture, *i.e.* an isolation rate of 38.5 per cent. in both series. Second isolations were made in eight instances at intervals ranging from 2 days to 4 years. The average ages of the patients from whom HVH was isolated coincided with those of the total sample, *i.e.* 24 and 30 years respectively. Cervical scrapings were received from thirteen patients and four of these yielded HVH. From a further four patients specimens were received from both the cervix and the external genitalia. HVH was isolated from both specimens in three cases but from neither specimen in the fourth.

## Results

## ANTIGENIC TYPING

Eleven of the virus strains were found to be Type 1 and 54 were Type 2.

## POCK SIZE

The viruses isolated could be divided into two groups on the basis of the size of pock produced. The average diameter of the pocks of eleven strains was less than 0.75 mm. (0.45-0.73) while that of the remaining 54 strains was 1.00 mm. or greater (1.0-2.5).

## ELECTRON MICROSCOPY

Ultrathin sections of cells infected with 64 of the isolates were examined (one strain became contaminated with fungus). In all cases virus particles were observed in both the nucleus and the cytoplasm, but additional structures were found in the nuclei of cells infected with 52 of the strains. These structures have been described as filaments or microtubules and appear either in palisade formation or as 'chain-mail' (Fig. 2). These structures were never observed with any of the remaining twelve strains.

## CYTOPATHIC EFFECT

The virus strains could again be divided into two groups on the basis of the CPE produced in BHK cells. One group of eleven isolates produced discrete foci of infection in contrast to the loose aggregates of cells which developed with the remaining 54 strains. These differences were found to be a reflection of the CPE in that, while both groups gave rise to round refractile cells, the second group additionally produced long fusiform syncytia (Fig. 3).

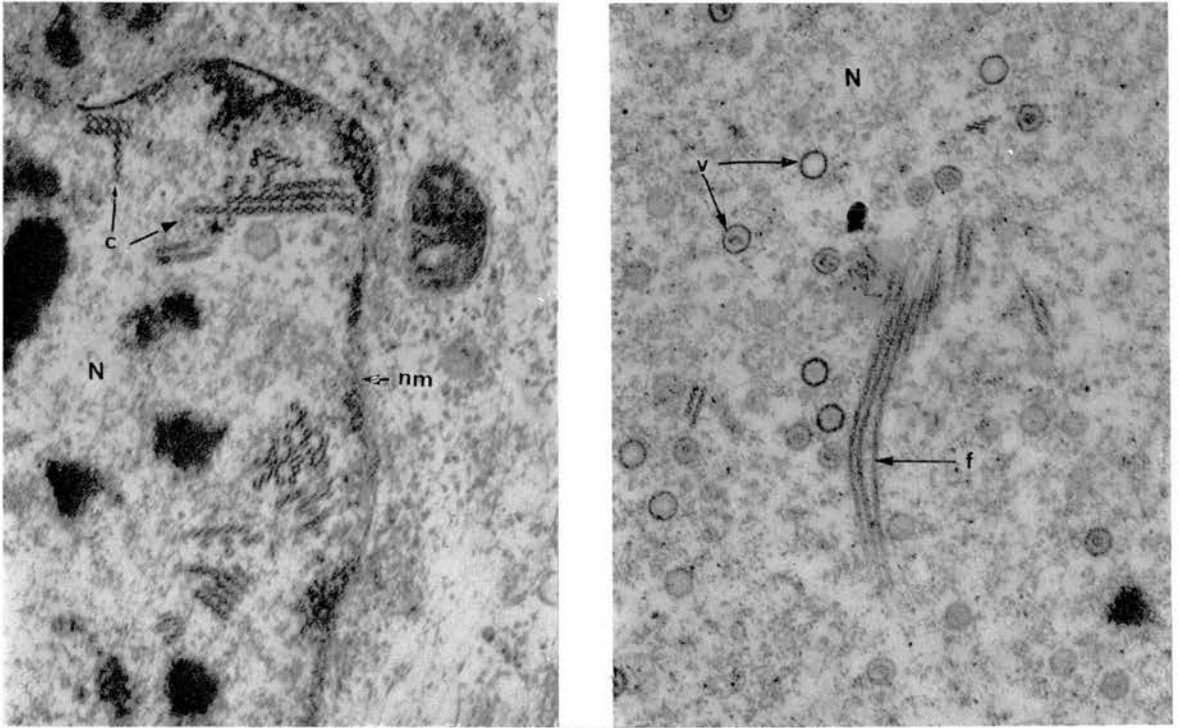


FIG. 2 Electronmicrographs of BHK cells infected with Type 2 HVH. N=nucleus; nm=nuclear membrane; c='chain-mail' structures; v=virus particles; f=filaments. Lead citrate, uranyl acetate(a)  $\times 60,000$  (b)  $\times 40,000$

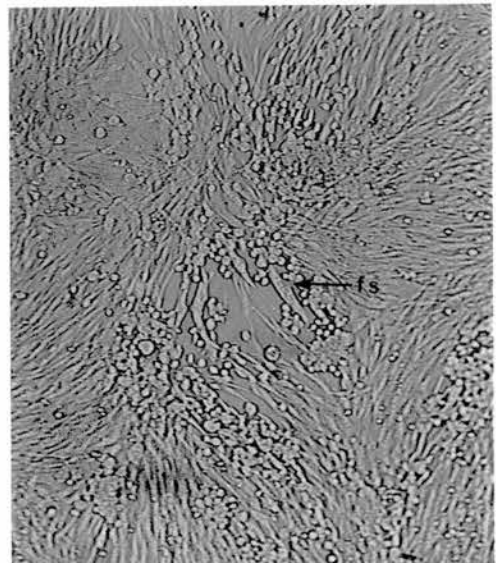
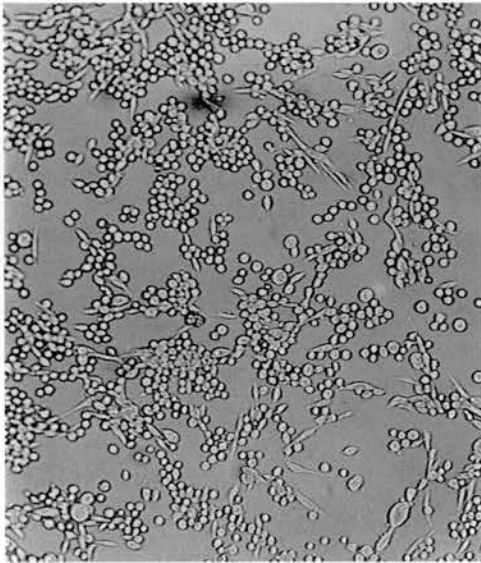


FIG. 3 Unstained light micrographs, showing the cytopathic effect produced by (a) HVH Type 1 and (b) HVH Type 2 in BHK cells. fs=fusiform syncytium.  $\times 70$



The relationship of these biological attributes to serotype is summarized in Table I, where it can be seen that eleven (9 female and 2 male) isolates which were found to be Type 1 serologically produced small pocks on the chorioallantoic membrane, no filaments in the nuclei of infected cells, and a CPE composed of round refractile cells. The remaining 54 strains (21 female and 33 male), which were found serologically to be Type 2, all produced large pocks on the chorioallantoic membrane and long fusiform syncytia. Electron microscopical examination of infected cells showed that 52 produced filaments and one did not (one strain was lost before examination).

TABLE I *Summary of serological and biological properties of 65 strains of Herpesvirus hominis isolated from patients with genital infections*

No. of isolates	Antigenic type	Size of pock*	Presence of filaments in nucleus	Cytopathic effect
11	1	Small	Absent	Round cells
1	2	Large	Absent	Fusiform syncytia
1	2	Large	N.D.	Fusiform syncytia
52	2	Large	Present	Fusiform syncytia

N.D. = Not done

\* = Small < 0.75 mm.  
Large  $\geq$  1.0 mm.

#### SEROLOGICAL STUDIES

First specimens of serum from 159 patients were examined for the presence of complement-fixing antibodies to herpes simplex virus, and 118 (74 per cent.) were found to have such antibodies. Second specimens of serum were only available in 42 instances, but when these paired sera were examined five (1 male and 4 female) of the eight patients from whom a Type 1 virus was isolated and eight (3 male and 5 female) of the 34 from whom a Type 2 virus was isolated had developed antibody during the course of infection (Table II).

TABLE II *Proportion of patients developing antibody during infection with Herpesvirus hominis Type 1 or Type 2*

Type 1 virus isolated		Type 2 virus isolated	
Male	Female	Male	Female
1/1	4/7	3/20	5/14

#### Discussion

The age distribution of the patients sampled is a reflection of the population attending venereal disease clinics in that the female patients are younger than the male patients and the peak attendance is in

the 20 to 30-year-old age group. This sample, therefore, does not seem to be unusual in its age distribution.

Before discussing the implications of the isolation of HVH Type 1 from genital sites, the methods used to establish the type of virus will be considered.

The serological differentiation of the two types of HVH has been established (Pauls and Dowdle, 1967), but this distinction depends on the production of suitable antisera. Here the rabbit antisera produced by the injection of the HF (Type 1) and MS (Type 2) viruses could be used to distinguish these viruses and other established Type 1 and 2 herpesviruses (Peutherer, 1970), so the results reported in this paper are valid. In general, this serological method is quite easy to perform, but it might prove too time-consuming for a diagnostic laboratory.

The differentiation of the two serotypes by the inoculation of the chorioallantoic membrane is again a simple technique, but it may be invalidated by failure of the virus to grow (Nahmias and Dowdle, 1968). We have experienced no difficulty in growing the viruses. Death of the embryo during the 7-day post-inoculation period, confluent growth, and secondary pock production can complicate the interpretation of the results. To overcome some of these difficulties, other workers (Nahmias, Dowdle, Naib, Highsmith, Harwell, and Josey, 1968) harvest the membrane 3 days after inoculation and consider that pocks less than 0.5 mm. in diameter are due to Type 1 viruses while those larger than 0.5 mm. are Type 2. The increase in the incubation time to 7 days allowed a clearer distinction of the two types of virus, since the Type 1 serotypes produce pocks less than 0.75 mm. and Type 2 produce pocks of 1.0 mm. or greater. With care, this test can be useful in typing virus isolations.

Electron microscopical examination of infected cells proved a very good method for the distinction of the two types of virus. 52 strains gave rise to filaments in the nuclei of infected cells in addition to the normal virions. These strains all appeared to be Type 2 HVH on the basis of antigenic typing. The eleven strains which were thought to be Type 1 did not produce such filaments, nor did one strain which was thought to be antigenically Type 2. This strain was examined in more detail but filaments could not be found in cells at 6, 12, 18, 24, and 48 hrs after infection. It was therefore concluded that this strain was atypical. This test, while possible in some laboratories, is time-consuming and is unlikely to be applied routinely.

Distinguishing the two serotypes of virus by CPE is rapid, 48 hrs being sufficient in most cases, and observations can be made on unstained preparations

in the light microscope. Provided the inoculum is not so great that all the cells show CPE in 24 hrs, long fusiform syncytia are produced in BHK cells by Type 2 viruses. These syncytia are not so obvious in RK, VERO, or HEP-2 cells and we could not distinguish the two types of HVH by the effect on chick embryo cells (Figueroa and Rawls, 1969). The type of CPE can be seen on initial isolation, so that no adaptation to tissue culture is necessary. In some cases, continued passage in tissue culture resulted in the production of large flat syncytia such as those produced by MP strains (Hoggan and Roizman, 1959). The production of these large syncytia is not confined to either type of virus but is found in both Types 1 and 2. If this variation occurs, it is more difficult to distinguish the two types of CPE, but the majority of strains breed true and have retained their cytopathogenic character through many passages.

The four tests used in this laboratory gave a good correlation in 63 of the 64 strains fully tested. It would appear, therefore, that as the tests applied have previously been shown to be of value in distinguishing the two types of HVH (Nahmias, Naib, and Josey, 1971), the identification of eleven of 65 strains (16.9 per cent.) as Type 1 HVH in this series is a valid observation.

From the results of this study, it appears that the close correlation reported previously between virus serotype and site of infection does not hold in Edinburgh. Eleven of the 65 isolates (16.9 per cent.) were found to be Type 1 in contrast to an earlier American report (Nahmias and others, 1971) of nine out of 265 (3.4 per cent.); Type 1 HVH was also recovered from the mouths of four of the latter, two of whom were aged 3 and 8 years.

Nine of the Type 1 strains of HVH were isolated from female patients. Only specimens from lesions of the external genitalia were received from seven of these patients, but in the other two instances specimens from the cervix yielded Type 1 virus. Normal precautions were taken during the collection of the cervical specimens, but it is obviously impossible to exclude completely the possibility that the virus was picked up from external lesions. The association of Type 1 infection of the lower genital tract and cervical infection with the same type is worthy of further study.

One explanation of this difference might be in the proportion of people who are 'at risk' in the sexually active age group. In a previous survey (Smith and others, 1967) in Edinburgh, it was found that 27.5 per cent. of the 15 to 39-year age group had no antibody to HVH. A similar figure (26 per cent.) was found in this series. Thus, a quarter of the people in this study would be fully susceptible to

infection with either type of virus. Type 1 virus is widely distributed in the community, and both primary oral and recurrent herpes occur in the young adult age group. Contact with a partner suffering from oral infection or the common herpes labialis or facialis could lead to transfer of the virus to the genitalia by direct oro-genital contact or *via* contaminated fingers. In one instance, there was circumstantial evidence that a partner of one of the female patients from whom a Type 1 HVH was isolated had suffered from a cold sore shortly before the patient developed genital herpes. This patient had no neutralizing antibodies to HVH at the onset of infection but as she subsequently developed antibody this was considered to be a primary infection.

Paired sera were collected from only 42 patients, but by use of both complement-fixation and neutralization tests it was possible to show that five of the patients from whom a Type 1 virus had been isolated and eight of those from whom a Type 2 virus was isolated developed antibody during the course of infection. It was necessary to employ both tests, since complement-fixing antibody can be detected before neutralizing antibody (unpublished observations) and it was therefore possible to confirm the change in antibody status by the use of this latter test. Thus 13 of these 42 genital infections were primary in nature. These thirteen patients were all between 18 and 25 years of age, except for one 39-year-old male. In Edinburgh, therefore, primary genital herpetic infections can occur with either type of virus.

The early American reports (Royston and Aurelian, 1970; Rawls and others, 1969; Nahmias and others, 1970) were based on predominantly Negroid populations, but the latest survey (Rawls, Gardner, Flanders, Lowry, Kaufman, and Melnick, 1971) investigated populations of different socio-economic groups. It was found that, of the higher socio-economic group, 40 out of 90 (44 per cent.) of the patients had no antibody to either type of HVH while 16 out of 85 (18.8 per cent.) of the lower socio-economic group had no antibody. These groups, therefore, contained a reasonable proportion of people who were at risk to infection with either type of virus. Some primary infections are recorded but, of an unspecified number of viruses (more than 30), only two were Type 1. Thus a population with a fair proportion of persons 'at risk' does not necessarily give rise to Type 1 genital infections. The proportion of genital Type 1 infections in Edinburgh may be a reflection of the size of the population 'at risk', as well as of sexual practices and hygiene. The possibility of the oro-genital spread of HVH infection is worthy of further study.

## Summary

During a 5-year period *Herpesvirus hominis* was isolated from the external genitalia or cervix of 65 (30 female and 35 male) patients. The virus isolates were characterized by antigenic typing, pock size, appearance of infected cells in the electron microscope, and cytopathic effect in tissue culture cells. Eleven of these viruses were found to be Type 1. Reasons for this high incidence of Type 1 infection of the genital tract are discussed.

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## Caractérisation des souches génitales d'*Herpes virus hominis*

### SOMMAIRE

Pendant une période de cinq ans, le virus de l'*Herpes virus hominis* (NVS) a été isolé des voies génitales externes et du col de l'utérus chez 65 malades (30 femmes et 35 hommes). Les isoléments viraux furent caractérisés par le typage antigénique, la dimension des pustules, l'aspect des cellules infectées au microscope électronique et l'effet cytopathique sur les cellules de culture cellulaire. Onze de ces virus furent trouvés de type I et l'on discute de la haute incidence de l'infection de type I dans les voies génitales.

