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Genetic Determinants for Infectivity and Pathogenicity of Influenza Viruses

R. Rott

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Genetic determinants for infectivity and pathogenicity of influenza viruses

By R. Rott
Institut für Virologie, Justus-Liebig-Universität Giessen, Frankfurter Strasse 107,
6300 *Giessen, Germany*

[Plate 1]

The objective of the studies presented was to define a molecular basis for infectivity and pathogenicity of influenza virus. It is demonstrated that activation of the HA glycoprotein by post-translational proteolytic cleavage is indispensable for the formation of infectious influenza virus. There are two preconditions for influenza virus to be pathogenic: (1) the presence on the virus particle of a cleaved HA molecule essential for the infectivity, and (2) an optimal genome composition. In naturally occurring avian influenza viruses there is a direct correlation between the cleavability of the haemagglutinin, the potential of the virus to be produced in infectious form in a wide range of host cells, and the viruses' pathogenicity for chicken. It is concluded that Nature selects an optimal gene constellation for each individual field strain. In these viruses the structure of the haemagglutinin is the determining factor for pathogenicity.

Introduction

The present knowledge on structure and function of influenza virus components gives us a basis to tackle the problem of elucidating infectivity and pathogenicity of these viruses. There is no doubt that both viral properties are dependent on a functional viral genome. Any change of the genome by a process of random mutation might give rise to virus variants with altered biological properties. In my contribution I do not deal with this primary change of the viral genome, but rather I discuss the question of whether pathogenicity is a functional entity associated with any of the known properties of influenza virus components.

Viral haemagglutinin determines infectivity

Formation of highly infectious virus is a precondition for pathogenicity. It is fair to assume that the more cells of a given organism produce virus in infectious form, the greater is the chance of the virus reaching the target organ to exert pathogenic action. Recent investigations on influenza viruses revealed a direct correlation between infectivity and the structure of the haemagglutinin.

The haemagglutinin of influenza viruses consists of two glycoproteins, HA_1 and HA_2, which are derived from a common precursor HA by proteolytic cleavage that may occur either on smooth internal membranes or on the plasmalemma of the host cell (for references see Rott & Klenk 1977). It is generally assumed that the cleavage enzymes are host-cell specific. With influenza viruses this concept has been supported by the observation that for most influenza viruses, cleavage of the HA depends on both the host cell system and the virus strain used (Klenk *et al.* 1975; Lazarowitz & Choppin 1975). For example, the HA of virus N, an avian influenza virus, is cleaved in embryonated chicken eggs or in cultured cells of the chorioallantoic

membrane but not in a series of other cells such as chicken fibroblasts (figure 1). On the other hand, there are viruses, like fowl plague virus (FPV), that always are produced with cleaved HA regardless of the type of host cell. Double infection of chicken fibroblasts with these two viruses has proved that a potential activation of the cleavage by the sensitive strain cannot be used to cleave the resistant HA of the other strain (Klenk *et al.* 1977). This means that the individual structural characteristics of the HA, rather than activation of cellular enzymes by the infecting virus, determine whether cleavage takes place.

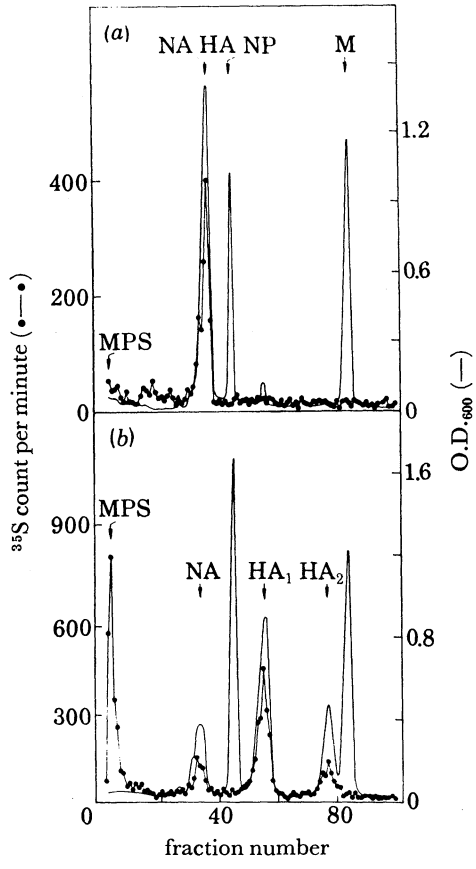

FIGURE 1. Comparative analysis of virus N (Hav2Neq1) grown (*a*) in chicken fibroblasts and (*b*) in chicken embryos. Virus labelled with [^{35}S]sulphate was purified and subjected to polyacrylamide gel electrophoresis. The gels were first stained with Coomassie brilliant blue and scanned at 600 nm in Gilford linear transport (——). Subsequently they were sliced for counting for radioactivity (•——•). The arrows indicate the positions of the virus proteins (Na, neuraminidase; HA, HA_1, HA_2, haemagglutinin; NP, nucleocapsid; M, membrane protein) and of mucopolysaccharide (MPS). For details see Klenk *et al.* (1977).

Viruses formed with uncleaved HA are able to adsorb to receptors of the host cell containing neuraminic acid. They are, however, non-infectious. Such virus particles can be converted into infectious virions by treatment *in vitro* with trypsin or trypsin-like enzymes (table 1), which cleave the HA at specific sites (Klenk *et al.* 1975; Lazarowitz & Choppin 1975). These results clearly show the correlation between cleavage of HA and the infectivity of the virus. They further indicate that, in addition to its role in adsorption, haemagglutinin must have a decisive function for penetration. Comparative studies of nuclear magnetic resonance spectra of chicken fibroblasts exposed to influenza virus with either cleaved or uncleaved HA indicate that the

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virus containing the cleaved HA induces an alteration in the fluidity of the lipid bilayer of the plasma membrane early after infection. This altered fluidity is not seen when cells are infected with virus particles containing an uncleaved HA (Nicolau *et al.* 1978). Furthermore, primed T lymphocytes exert a cytotoxic effect only when the target cells are infected with influenza virus containing cleaved HA. Cytotoxicity cannot be observed, however, if virus particles containing uncleaved HA are merely adsorbed to the surface of a potential target cell (Kurrle *et al.* 1979). A possible interpretation of these data would be that cytotoxic T cells can only affect target cells after insertion of a fully functioning HA into their plasma membranes. Such a fusion process between the viral envelope and plasma membrane of the host cell, which depends on a cleaved HA, would also be necessary for penetration.

TABLE 1. EFFECT OF TRYPSIN TREATMENT ON HAEMAGGLUTINATING ACTIVITY AND INFECTIVITY OF DIFFERENT INFLUENZA A VIRUSES GROWN IN CHICKEN FIBROBLASTS

virus strain		rate of activation	
		haemagglutination	infectivity
A/PR/8/34	(H0N1)	1.0	27
A/Singapore/1/57	(H2N2)	1.0	193
A/swine/Shope/31	(Hav1N1)	1.0	200
A/equine/Miami/1/63	(Hav2Neq2)	1.0	111
A/chick/Germany/N/49	(Hav2Neq1)	1.0	150

Purified virus particles produced in chicken fibroblasts with uncleaved HA were incubated with trypsin (10 μg/ml) or without trypsin for 15 min at 37 °C. The activation rates are the ratios of the activities of trypsin-treated samples versus untreated controls. For details see Klenk *et al.* (1975).

TABLE 2. EFFECT OF TRYPSIN TREATMENT ON INFECTIVITY OF RECOMBINANTS CARRYING $HA_{(FPV)}$ $N_{(virus\ N)}$ AND $HA_{(virus\ N)}$ $N_{(FPV)}$ GROWN IN CHICKEN FIBROBLASTS

recombinant	trypsin treatment	infectivity (FPV/ml)
$HA_{(FPV)}$ $N_{(virus\ N)}$	−	1.5×10^8
	+	1.7×10^8
$HA_{(virus\ N)}$ $N_{(FPV)}$	−	5.7×10^5
	+	2.4×10^7

Purified virus particles were treated with trypsin as described in table 1 (Klenk *et al.* 1975).

That activation of infectivity depends on proteolytic modification of the haemagglutinin and not on other viral surface components was demonstrated by experiments employing recombinants that contained the HA of virus N and the neuraminidase of fowl plague virus or vice versa (table 2). Only recombinant viruses possessing virus N haemagglutinin exhibited host-specific variation of the HA cleavability, activation by trypsin and infectivity, features typical for virus N. These features are not found with the recombinants possessing the neuraminidase of virus N and the haemagglutinin of FPV (Klenk *et al.* 1975).

In conclusion, all present observations demonstrate that the structure of the HA as coded for by the viral genome determines whether a proteolytic enzyme of the host cell is capable of reacting with the precursor structure which ultimately results in a cleaved HA necessary for infectivity of the virus particle.

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Structure of haemagglutinin and pathogenicity

Since the tropism of a virus for host cells represents primarily an interaction between the surface components of the virus and cell receptors, it is tempting to postulate that surface structures of a virus might determine not only infectivity but also pathogenic properties of the virus, that is its ability to cause a disease in a given organism. Recent results might be interpreted in this sense because such an interrelation could be demonstrated with avian influenza viruses (Bosch *et al.* 1979). These viruses occur in Nature with at least nine different HA subtypes and in many haemagglutinin–neuraminidase constellations (Webster *et al.* 1976). Besides

Table 3. Correlation between haemagglutinin structure, infectivity and pathogenicity of avian influenza viruses

virus strain		HA present in cleaved form†	plaque formation without trypsin‡	pathogenicity for chickens
A/FPV/Dutch/27	(Hav1Neq1)	+	+	+
A/chick/Germany/N/49	(Hav2Neq1)	–	–	–
A/duck/England/56	(Hav3Nav1)	–	–	–
A/duck/Czechoslovakia/56	(Hav4Nav1)	–	–	–
A/turkey/Ontario/7732/66	(Hav5Nav6)	+	+	+
A/duck/Germany/1868/68	(Hav6N1)	–	–	–
A/duck/Ukraine/1/63	(Hav7Neq2)	–	–	–
A/turkey/Ontario/6118/68	(Hav8Nav4)	–	–	–
A/turkey/Wisconin/66	(Hav9Neq1)	–	–	–

† Viruses were grown in MDCK cells, chicken or turkey fibroblasts.
‡ Plaque test was performed in MDCK cells, chicken, turkey, duck or quail fibroblasts. For details see Bosch *et al.* (1979).

the genes coding for haemagglutinin and neuraminidase there are considerable differences in base sequence homologies of the other genes (Scholtissek, personal communication). The results of investigation on the cleavability of the HA glycoprotein in MDCK cells as well as chicken, duck, turkey and quail fibroblasts, plague formation in these cells as indicator of viral infectivity, and pathogenicity for chickens of nine different avain influenza subtypes are summarized in table 3. Indeed, a strict correlation exists between structure of the HA and pathogenicity for chicken. Only those viruses that are produced in an infectious form in a broad spectrum of host cells are pathogenic. It should be emphasized that there are not only differences in cleavability of the HA glycoprotein and pathogenicity between the different subtypes but even between a single subtype. Although all strains in the subtype Hav1 have a serologically closely related haemagglutinin, they differ in cleavability and pathogenicity (table 4). Analysis of the genetic relatedness of the HA gene of these viruses show significant differences in their base sequence homology (Scholtissek, unpublished results).

The narrow host range of avian influenza viruses non-pathogenic for chicken was clearly demonstrated after infection of the chorioallantoic membrane of chicken embryos (figure 2, plate 1). After inoculation of the ectodermal layer with non-pathogenic viruses, only ectodermal cells produce viral material, while with pathogenic strains all cell layers become infected under the same conditions. The infection remains localized in the endodermal layer after inoculation with the non-pathogenic virus into the allantoic cavity, but dissemination occurs with pathogenic strains (unpublished results). There is evidently a correlation between the structure of

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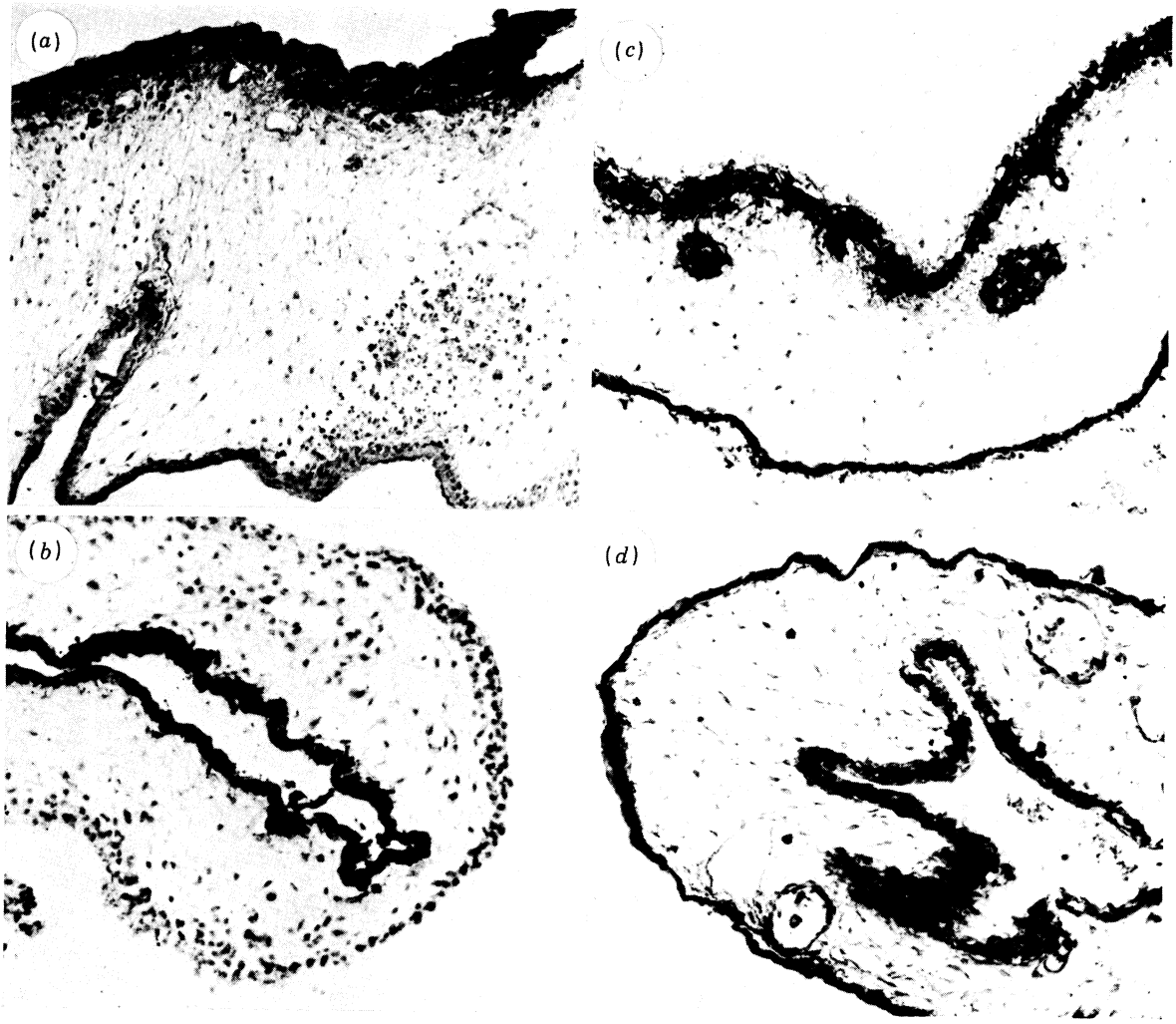

FIGURE 2. Spread of avian influenza viruses in the chorioallantoic membrane of chicken embryos. Chicken embryos were inoculated with the non-pathogenic virus N (*a*, *b*) or the pathogenic fowl plague virus (*c*, *d*) onto the ectodermal layer (*a*, *c*) or into the allantoic cavity (*b*, *d*). After incubation for 24–48 h after infection, virus specific antigens were demonstrated in the membranes by the peroxidase-antiperoxidase method.

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the HA glycoproteins and the capacity to spread in the infected host, which is so important for pathogenicity.

Non-pathogenic avian influenza viruses with a limited host cell range multiply in infectious form and with cleaved HA in cells of at least the upper respiratory and the intestinal tract of birds without inducing signs of disease (Bosch *et al.* 1979; Webster *et al.* 1978). This is of epidemiological importance since clinically normal, infected birds shed virus which can be spread to other hosts for perpetuation of the infection in the population.

TABLE 4. DEPENDENCE OF INFECTIVITY AND PATHOGENICITY ON CLEAVABILITY OF HAEMAGGLUTININ OF HAV1 SUBTYPE AVIAN INFLUENZA VIRUSES

strain		HA present in cleaved form†	plaque formation without trypsin‡	pathogenicity for chickens
A/FPV/Rostock	(Hav1N1)	+	+	+
A/FPV/Dutch/27	(Hav1Neq1)	+	+	+
A/fowl/Victoria/75	(Hav1Neq1)	+	+	+
A/turkey/England/63	(Hav1Nav3)	+	+	+
A/parrot/Ulster/73	(Hav1N1)	−	−	−
A/turkey/England/77	(Hav1Neq1)	−	−	−
A/turkey/Oregon/71	(Hav1Nav2)	−	−	−

†, ‡, See table 3.

GENE CONSTELLATION AND PATHOGENICITY

The prime significance of the HA for pathogenicity of avian influenza viruses seems to be in contrast to genetic studies on influenza virus recombinants obtained *in vitro*. From these studies, evidence was found for the polygenic nature of influenza virus pathogenicity (for references see Burnet 1959; Kilbourne 1963). Although all recombinants tested for pathogenicity in the chicken possessed an HA cleavable in a broad range of host cells, they were non-pathogenic. It emerged from these investigations that the HA alone did not determine pathogenicity (Rott *et al.* 1976). Genetic analysis of a large number of recombinants obtained from fowl plague virus, highly pathogenic for chicken, and another influenza virus prototype strain which is not pathogenic for chicken, proved that an exchange of any RNA segment of the pathogenic strain can modify pathogenicity. In any case of a single gene exchange the pathogenic properties of a recombinant are determined by the gene that was exchanged and by the virus strain from which this corresponding gene was derived (Scholtissek *et al.* 1977). For example, if the RNA segment 1 is taken from the influenza virus strain PR8, pathogenicity is not achieved, whereas RNA segment 1 transferred from swine influenza virus yielded recombinants as pathogenic as the wild-type fowl plague virus. On the other hand, if RNA segment 2 originates from swine influenza virus, no pathogenicity is recovered, whereas RNA segment 2 from an A2 strain or virus N produces highly pathogenic virus (table 5). As a rule, recombinants of influenza viruses with defined multiple gene exchanges produce less severe signs of clinical illness with increased gene exchanges (Florent *et al.* 1977; Oxford *et al.* 1978; Rott *et al.* 1978). These data indicate that an optimal constellation of all RNA segments is required for the genome of a highly pathogenic virus strain.

This assumption is supported by the finding that recombinants obtained from non-pathogenic viruses can assume pathogenic properties (Rott *et al.* 1978; Vallbracht *et al.* 1978; Scholtissek *et al.* 1979) or, in contrast, that reassortment between highly pathogenic parent strains may lead

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to pathogenic as well as non-pathogenic viruses (Rott *et al.* 1979). Increase or loss of pathogenicity seems to be dependent not only on the haemagglutinin, but also on the influenza virus genes involved in viral RNA synthesis.

The implications of these observations with recombinant influenza viruses obtained *in vitro* for pathogenicity are: (1) the pathogenic virus must possess an HA that is cleavable in a broad range of cells; (2) there is no single gene responsible for pathogenicity; (3) it is impossible to establish a rule for the combination of the different genes indicative of pathogenicity of all influenza viruses; (4) in each reassortment an optimal genome composition might be achieved which depends on the parent virus strains used.

TABLE 5. INFLUENCE OF SINGLE GENE EXCHANGES ON PATHOGENICITY OF FOWL PLAGUE VIRUS FOR CHICKEN

FPV RNA segment exchanged	RNA segment derived from			
	PR8	A2 Singapore	swine influenza	virus N
1	○	◑	●	◑
2	◑	●	○	●
3	◑	◑	◑	●

Chickens were inoculated intramuscularly with 1 ml of infectious allantoic fluid containing 128 haemagglutinating units. ●, Highly pathogenic as fowl plague virus; ◑, weakly pathogenic; ○, non-pathogenic. For details see Scholtissek *et al.* (1977).

Comparing the results obtained with influenza virus recombinants isolated *in vitro* and those obtained from naturally occurring avian influenza viruses, it can be assumed that Nature selects an optimal gene constellation for each individual field strain and that naturally occurring viruses with a suboptimal gene constellation will not survive in Nature. The requirement for an optimal genome composition seems to be met by all avian influenza viruses. If, in addition to the optimally functioning genome, the viruses possess a haemagglutinin that is cleaved in many different host cells and thereby becomes activated, they are always pathogenic.

We are not able at present to define optimal gene constellation. There is evidence, however, that not all of the 254 (2^8-2) possible gene constellations between two virus strains can be isolated from a given host (Rott *et al.* 1976). Furthermore, it was observed that certain groups of genes appear to be transferred together during reassortment (Scholtissek *et al.* 1976). For example, the transfer of the FPV haemagglutinin was accompanied in most cases by the transfer of the gene coding for the M protein. It was also observed that a concomitant transfer of the genes coding for viral polymerase activity is critical for host range (Scholtissek *et al.* 1978) and pathogenicity (Rott *et al.* 1979; Scholtissek *et al.* 1979). This could mean that an interrelation of the genes or gene products may exist, the role and extent of which we do not comprehend.

Just like the phenomenon described for avain influenza viruses, a similar strict correlation between the structure of the viral glycoproteins and pathogenicity has been found for the paramyxovirus of NDV (Nagai *et al.* 1976). Our results support the concept that a relatively simple molecular mechanism, namely the susceptibility of the viral glycoproteins to proteolytic cleavage, is of high importance for the complex phenomenon of pathogenicity, at least of avian myxoviruses.

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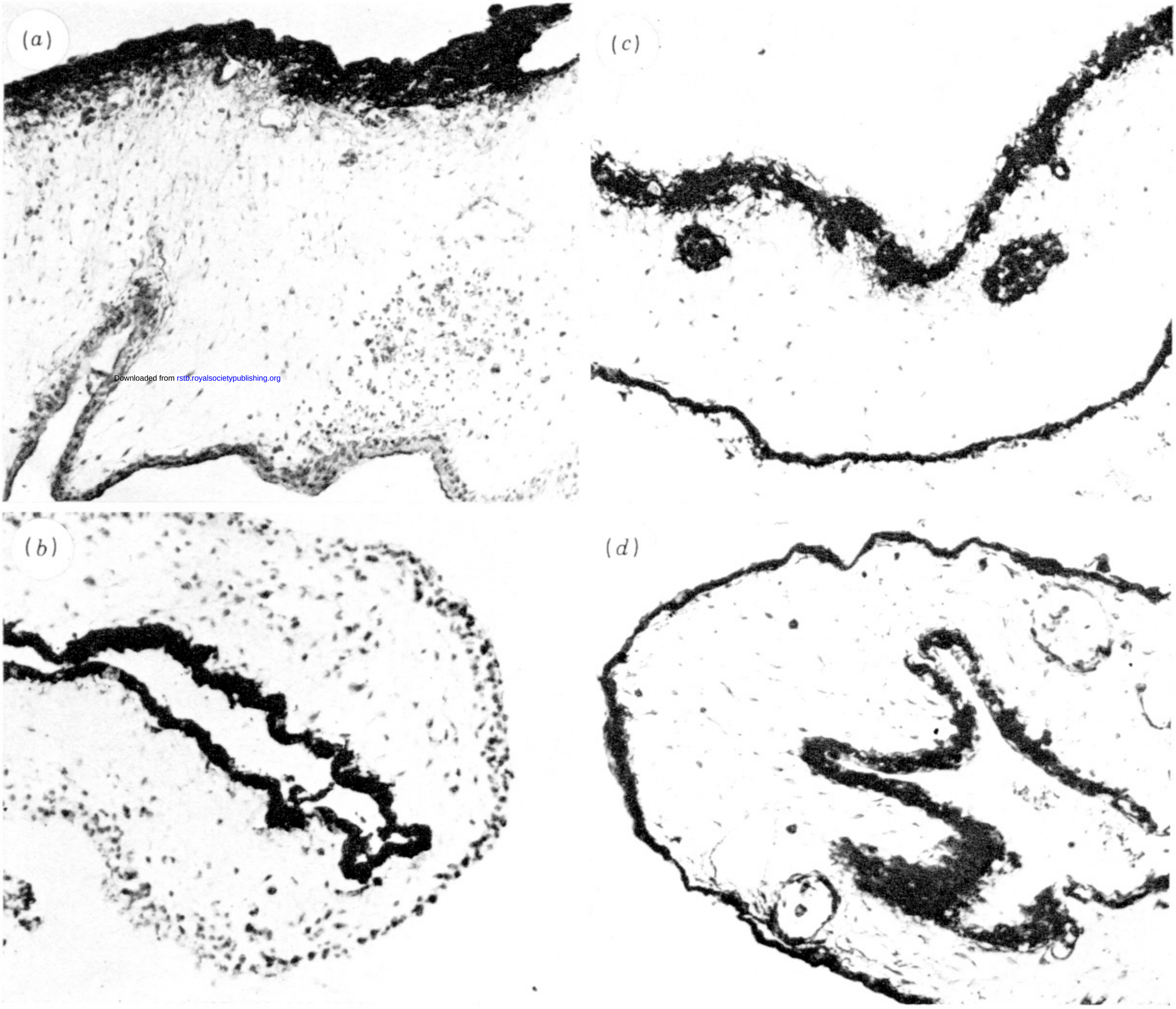

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