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Variability in the characteristics of pocks produced on the chick chorioallantois by white pock mutants of cowpox and other poxviruses

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The 'pocks' produced on the chorioallantoic membrane (CAM) of the chick embryo by the variola-vaccinia group of viruses are usually sufficiently characteristic to make presumptive identification of these viruses very reliable. As well as being of use in confirming clinical diagnosis the pock character is used by those interested in the characteristics and inter-relationships of these viruses. Thus pock character has been used to study spontaneous mutants of cowpox (Haddock, 1952), rabbitpox (Fenner, 1958) and also in genetic recombination (Fenner & Comben, 1958), genetic hybridization (Woodroofe & Fenner, 1960) and non-genetic reactivation (Fenner & Woodroofe, 1960).

Stability of pock character is essential for the true interpretation of such studies and the results obtained by various workers appear to show such stability.

However, the work described here shows that some poxvirus pock mutants when grown on the CAM can 'mimic' those viruses from which they were derived, and also shows that environmental factors are important in determining pock character.

MATERIALS AND METHODS

Virus strains

Brighton strain of cowpox. Most of the work was done with the white pock mutant of this strain of cowpox. Suspensions with various passage histories on the CAM were available, including a suspension provided by Professor K. McCarthy which was made in 1956 and which had been stored at $-20°$C. since then. Dr C. R. Madeley kindly supplied a white pock mutant isolated on the rabbit skin by Professor K. R. Dumbell and passaged subsequently only on the rabbit skin.

Control experiments were done with parental red cowpox virus adapted to either CAM or rabbit skin.

Other strains of white cowpox. Professor Dumbell kindly made available the following strains of white cowpox. Austria, Larkin, Juffermans, Carmarthen, Maund and Ruthin.

Other poxviruses. Some experiments were done with white pock mutants isolated from the Utrecht strain of rabbitpox, Levaditi and ISM strains of vaccinia, Evans (British) and Wyeth (American) commercial vaccine strains, and the Merck strain of monkeypox. The Lister strain of vaccinia, Mill Hill strain of ectromelia, Harvey strain of variola and Butler strain of alastrim were also used.

Fertile hens' eggs

Fertile hens' eggs of various breeds were obtained from commercial suppliers. They were prepared for inoculation as described by McCarthy & Dumbell (1961). 12-day-old embryos from a particular breed of White Leghorn fowls (WL 4) were used unless otherwise stated. Virus dilutions were made in 10% nutrient broth saline and 0·1 ml. volumes were inoculated 1 hr. after the CAM had been prepared.

Incubation

The incubators used were controlled by 'Accuron' thermostats and heaters. Constant recording of temperature was made by 'Grant' recorders. Thermostats and recorders were accurate within $\pm 0{\cdot}1°C$.

General virological techniques

Techniques for measuring the virus content of single pocks, measuring pock diameters and for excision, passage and histology of single pocks were essentially those described by Fenner (1958).

Gel diffusion

Extracts of infected tissue were tested by gel diffusion for presence of 'd' antigen using reference materials already described (Baxby & Rondle, 1968). Antigen 'd' is present in red cowpox-infected tissues but not in white cowpox-infected tissues (Rondle & Dumbell, 1962).

RESULTS

When referring to cowpox viruses and the pocks they produce the following terms will be used.

Parental red cowpox virus (*PCV*). This produces haemorrhagic, ulcerated lesions (PCV pocks) on the CAM (Downie, 1939), and also produces a white pock mutant which is genetically stable (Downie & Haddock, 1952).

White cowpox virus (*WCV*). This is the white pock mutant. It is shown in this paper to produce two types of pock, one white (WCV pock) and one indistinguishable in appearance from PCV pocks, although it has the genotype of WCV. This second type of pock is described as an atypical WCV pock (AWCV pock).

White cowpox virus

Attempts to show whether WCV pocks and AWCV pocks yield different viruses

These investigations were prompted by the presence of a high proportion (*ca.* 40%) of lesions resembling parental red cowpox pocks on CAM inoculated with the white pock mutant of Brighton cowpox. The unexpected presence of these 'atypical' pocks was thought to be due to accidental presence of PCV in the WCV stock, but various attempts to separate the two types failed. Passage of either single AWCV or single WCV pocks from these apparently mixed preparations through 10 single pock passages gave suspensions which both produced the same proportions of AWCV pocks. Ten serial passages of confluently infected CAM gave

a similar result (38 % AWCV pocks). After nine serial passages through RK 13 cells, which are 10 times more sensitive to PCV than to WCV (Baxby & Rondle, 1967) suspensions produced a similar proportion of AWCV pocks (36 %) when inoculated on CAM. Finally a new line of WCV was established by pock picking from the parental red strain. After 12 serial single pock passages it produced 38 % AWCV pocks, although parental red cowpox virus could no longer be recovered after the 2nd passage.

Of the various explanations possible, the one favoured was that white cowpox could produce two types of pock, one white and another indistinguishable from the parental type.

Rabbit inoculation

When inoculated on the rabbit skin various preparations of white cowpox gave no evidence of lesions characteristic of parental red cowpox. This suggested that the white cowpox suspensions were free of red cowpox. It also suggested that the factors responsible for production of 'atypical' white cowpox lesions on the CAM were not effective in the rabbit skin.

Gel diffusion

Further evidence that white cowpox virus preparations were uncontaminated with parental red cowpox strains was obtained by the failure of infected tissues to produce antigen 'd' and of infected rabbits to produce antibody to this antigen.

Characteristics of lesions produced by parental red cowpox and white cowpox viruses on the CAM

Fenner (1958) compared the characters of various poxviruses. Of the characters he used the only ones applicable to single pocks are the appearance, size and amount of virus extractable from single pocks.

Appearance of pocks

Macroscopically the atypical white cowpox lesions were indistinguishable from those produced by parental red cowpox. Stained sections of PCV pocks showed the characteristic inclusions and extensive haemorrhage first described by Downie (1939) (Pl. 1 A). Large cytoplasmic inclusions were present throughout the ectoderm and endoderm and could often be seen in the walls of blood vessels. WCV pocks showed no haemorrhage but were characterized by marked leucocyte infiltration and endodermal proliferation. Inclusions were smaller and were usually limited to cells at the edge of the pock (Haddock, 1952) (Pl. 1 C). AWCV pocks produced by white cowpox virus were indistinguishable from parental red cowpox (Pl. 1 B), haemorrhage being extensive and inclusions large and widespread.

Size of pock

As shown in Table 1 pocks produced by PCV were slightly larger than the AWCV pocks. However, the variation in size of both types was so great that they could not be distinguished with any certainty.

Amount of virus extracted from pocks

Fenner (1958) showed that about 10 times more virus could be extracted from disrupted pocks of PCV than from WCV. This was confirmed for PCV and WCV pocks. However, the same amount of virus could be extracted from both PCV pocks and the AWCV pocks (Table 1).

Factors affecting production of atypical pocks by white cowpox virus

The character of a pock is undoubtedly determined by the interplay of many complex physiological factors which are difficult to control in such a self-contained host as the chick embryo. Hence the effects of changing only simple environmental factors on the characteristics of the pocks were tested.

Table 1. *Size and virus content of pocks produced on chorioallantoic membrane by parental red cowpox and its white pock mutant*

	Pock size			Virus content		
	No. tested	Diam. (mm)*	S.D.† (mm)	No. tested	Log titre*	Log S.D.†
White cowpox virus pock	59	1·5	± 0·4	13	5·8	± 0·4
Atypical white cowpox virus	52	1·4	± 0·4	15	6·8	± 0·36
Parental red cowpox	84	1·7	± 0·5	10	6·8	± 0·38

* Pocks tested after 72 hr. incubation.
† S.D. = standard deviation.

Table 2. *Passage histories and strains of white cowpox in which atypical pocks have been seen*

Strains	Passage history before test*	Atypical pocks(%)	Strains	Passage history before test*	Atypical pocks(%)
Brighton	R 6	48	Austria	E 2	36
Brighton	E 40 RK 9†	36	Larkin	E 4	39
Brighton	E 20	38	Ruthin	E 18	43
Brighton	E 25‡	39	Juffermans	E 8	43
Brighton	E 40	42	Carmarthen	E 8	49
Brighton	E 50	38	Maund	E 11	36
Brighton	E 40 W 10§	38			
Brighton	E 40 R 10‖	41			
Brighton	Red/W 12¶	38			

* R = passaged on rabbit skin, E = on CAM.
† E 40 RK 9 = 40 confluent CAM passages then 9 passages in RK 13 cells.
‡ This suspension was made in 1956 and stored at −20° C., until tested in 1969.
§ E 40 W 10 = 40 confluent CAM passages then 10 single white pock passages.
‖ E 40 R 10 = 40 confluent CAM passages then 10 single atypical pock passages.
¶ Red/W 12 = New white cowpox strain after 12 single pock passages.
All suspensions were tested on White Leghorn (type 4) embryos at 35° C. (see Table 3).

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Passage history of virus

As indicated previously serial passage of various suspensions did not alter the proportion of red pocks, suggesting that the phenomenon was independent of passage history. These figures are shown in Table 2. It is interesting that atypical pocks were produced both by a suspension of WCV which had been prepared in 1956 and stored at $-20°$C. since then, and also by the white pock mutant isolated on the rabbit skin on its first passage on the CAM.

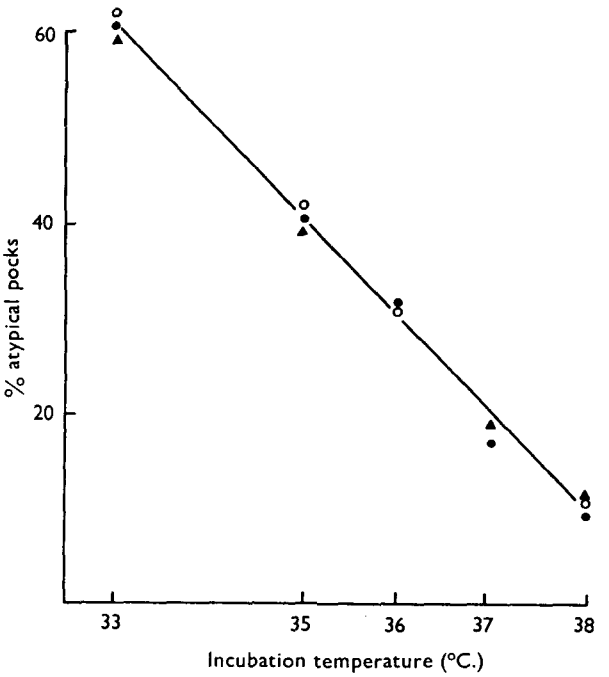

Fig. 1. Effect of incubation temperature on production of atypical pocks by white cowpox. ●, ○, ▲, three separate experiments.

Virus strains

The original observations on Brighton white cowpox were also repeated with all of six other strains (Table 2).

Inoculation technique

Most of the variables tested by Westwood, Phipps & Boulter (1957) in their study of poxvirus titration techniques were tested for their effect on pock character of white cowpox. The only factor found to alter the proportion of atypical pocks was the age of the embryos. Embryos 10, 11 and 14, 15 days old produced only half as many AWCV pocks as embryos 12 and 13 days old.

Dose response

With various poxviruses it has not been uncommon to find a certain degree of haemorrhage on CAM bearing confluent infections. With CAM bearing semi-

confluent and confluent infection with white cowpox virus the degree of haemorrhage was so marked that many membranes were indistinguishable from those infected with parental red cowpox. On CAM inoculated with up to *ca.* 150 pocks/membrane the percentage of atypical pocks remained constant at about 36–42 %.

Incubation temperature

The effect of incubation temperature on the pock character of white cowpox virus is shown in Fig. 1. The proportion of AWCV pocks was reduced by *ca.* 10 % for each 1°C. rise in temperature over the range 33–38°C. Above 38°C. atypical pocks were infrequent and Pl. 2 B shows a membrane incubated at 39°C. which

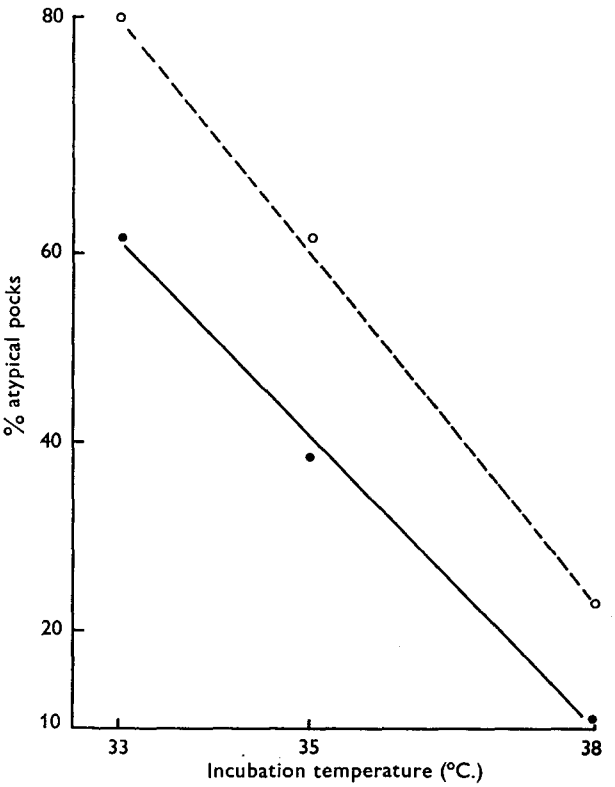

Fig. 2. Effect of position of chorioallantoic membrane during incubation on production of atypical pocks by white cowpox virus. ●——●, Embryos incubated with CAM lowered; ○– – –○, embryos incubated with CAM raised up against vitelline membrane 4 hr. after infection.

gives the 'classical' appearance of white cowpox. Plate 2 C shows a membrane similarly inoculated but incubated at 33°C., showing a very high proportion of atypical pocks. Plate 2 A shows parental red cowpox virus pocks on a membrane incubated at 35°C for comparison.

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Position of CAM during incubation

Initially it was noticed that atypical pocks were often more frequent round the periphery of the inoculated area and that such pocks were often on a part of the CAM which had risen back up against the vitelline membrane. Subsequently the outline of the inoculated area was marked on the shell and counts from membranes which had risen were rejected. Figure 2 shows the effect of incubating embryos with the CAM replaced in its original position by suction after incubation; the proportion of atypical pocks is considerably increased. Controls showed that the effect was caused by the actual position of the CAM during incubation rather than the trauma involved in manipulation.

Table 3. *Production of atypical white cowpox lesions on the chorioallantoic membrane of chick embryos from different sources*

Embryo*	Source. County and Dealer	Percentage atypical pocks at 33°	35°	38°	Slope of graph (%)†	Efficiency of plating‡	Mortality %§
WL 1	Kent (A)	33	18	3	7	0·7	30
WL 2	Cheshire (B)	41	27	0·7	8	0·8	30
WL 3	Cheshire (C)	52	32	4	10	1·4	80
WL 4	Glos. (D)	61	42	9	10	1·0	100
WR	Cheshire (B)	50	25	4	9	1·3	60
WC	Cheshire (B)	30	19	2	5	0·7	20
BPR	Cheshire (E)	36	18	0·2	8	1·0	0
RIR	Cheshire (B)	44	25	3	10	1·0	50
RIR × LS	Kent (A)	66	43	4	10	0·8	100
RIR × LS 2	Cheshire (F)	47	30	10	9	0·8	60
WL × RIR	Cambs (G)	59	40	6	10	1·2	90

* WL = White Leghorn; WR = White Rock; WC = White Cornish; BPR = Buff Plymouth Rock; RIR = Rhode Island red; LS = Light Sussex.

† Increase in white pocks per 1°C. rise in temperature.

‡ Ratio of infective titre in WL 4 embryos to titre in embryos tested.

§ Percentage of embryos killed in 3 days by 10^3 infective doses.

Breed of embryo

Tests on embryos from hens of different breeds are shown in Table 3. Although the percentage of atypical pocks produced at a given temperature in various breeds sometimes differed, in all breeds a 1°C. rise in incubation temperature gave *ca.* 8% decrease in atypical pocks. Although extensive tests of embryo mortality were not made there was some correlation between the proportion of atypical pocks and embryo mortality. Embryos of a breed which produced a high proportion of atypical pocks tended to be killed by virus doses which did not kill embryos producing fewer atypical pocks. The extremes were best demonstrated with a dose of *ca.* 10^3 infectious units. This killed 90–100% of White Leghorn (type 4), Rhode Island Red × Light Sussex and White Leghorn × Rhode Island Red, but killed only 0–20% of Buff Plymouth Rock and White Cornish embryos. The differences in mortality were not due to differences in efficiency of plating

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(Table 3), but could be due to the more invasive nature of the atypical lesion and the greater amount of virus it contained.

The slight differences that were detected in embryos of different breeds were reproducible using different batches of such embryos. However, it was found that embryos of the same breed from one supplier did not necessarily give the same result as embryos of the same named breed from another supplier. Also, different results were obtained from different named breeds from the same supplier, which had received the same treatment as regards housing, feeding and vaccination. Hence with the evidence available it is difficult to say whether the slight differences are truly genetic, or due to some obscure environmental factor.

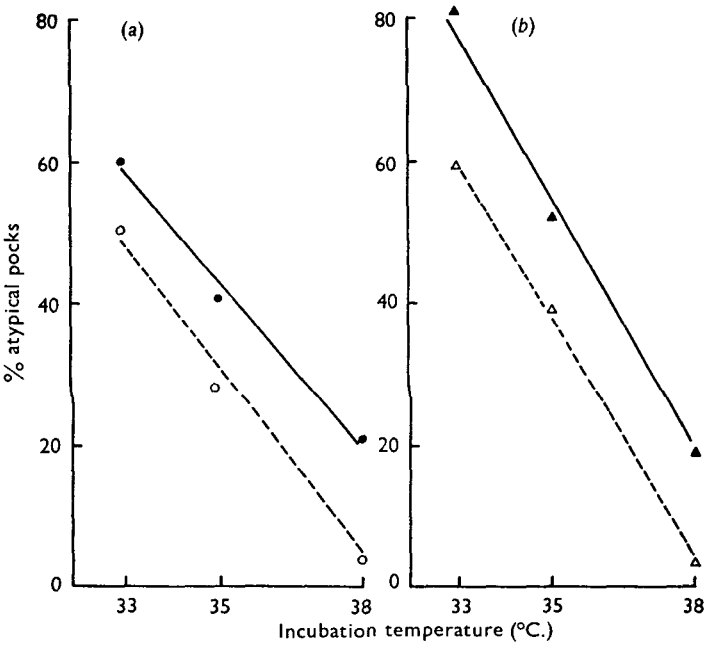

Fig. 3. Effect of incubation temperature on production of atypical pocks by (A) white pock mutant of rabbitpox virus (Utrecht), and (B) white pock mutant of vaccinia (Levaditi) in White Leghorn (type 4) and Buff Plymouth Rock embryos. Rabbitpox in WL4 (●——●), and BPR (○– – –○). Vaccinia in WL4 (▲——▲), and BPR (△– – –△).

Other poxviruses

Parental red cowpox

As white cowpox produces pocks indistinguishable from those of parental red cowpox, some of the red pocks on CAM inoculated with parental red cowpox should be of white cowpox genotype. This was tested by estimating the proportion of white pocks produced by parental red cowpox at different temperatures. At 33°C. this was 1·2 %, at 35°C., 4·8 %, and at 38°C., 15 %. However, at 38°C. some pocks which contained parental red cowpox were classed as white and so it seems that elevated temperatures can also affect the pock character of the parental virus to some extent. Cloning experiments indicated that only *ca.* two-thirds of the white pocks produced by parental red cowpox at 38°C. were white pock mutants.

White variants of rabbitpox, monkeypox and vaccinia

The pocks produced by rabbitpox virus, the Merck strain of monkeypox and various strains of vaccinia are naturally red owing to haemorrhage, although this is not usually as marked as it is with parental red cowpox. These viruses also produce white pock mutants, and some experiments were done with white pock mutants isolated from rabbitpox (Utrecht), monkeypox (Merck) and the Levaditi, ISM, Evans and Wyeth strains of vaccinia. All such variants produced some pocks resembling those produced by the parent virus. The effect of altering incubation temperature on the pock character of rabbitpox (Utrecht) and vaccinia (Levaditi) is shown in Fig. 3, whilst Pl. 2 D and E illustrates pocks produced by the Levaditi mutant at 39 and 33°C. respectively.

Lister strain of vaccinia, and variola, alastrim and ectromelia

The Lister strain of vaccinia has always been recorded as producing white pocks, and suspensions did so under all conditions tested.

In view of the variability of almost all the other poxviruses tested it was reassuring to find that the pocks produced by variola and alastrim appeared typical on the membranes of all the embryos tested. Whilst embryos of different breeds were available the opportunity was taken to test the ceiling temperatures of variola and alastrim in them. At 38·25°C., the temperature recommended by Dumbell, Bedson & Rossier (1961) for distinguishing between the two viruses, variola virus produced pocks in all breeds of embryo, alastrim in none.

Pocks produced by the Mill Hill strain of ectromelia remained constant under all conditions tested.

DISCUSSION

The pock character of white mutants has been influenced by incubation temperature, position of CAM during incubation and source and age of the chick embryo. What we do not know is the precise physiological mechanism being affected by these environmental changes. The fact that there is a particular age of embryo which produces a high proportion of atypical pocks suggests the need for a particular developmental stage in the embryo. The effect of changing the position of the CAM may affect gaseous exchange. This, and many other factors, would be influenced by temperature. As white pocks are characterized by leucocyte infiltration, and red pocks by haemorrhage and damage to blood vessels, it is possible that the environmental changes are affecting the haematopoietic and vascular systems.

These results emphasize the way in which virus and host *together* are responsible for determining pock character. Thus, parental red cowpox produces very haemorrhagic lesions on the CAM and the rabbit skin, whereas white cowpox can produce red lesions on the CAM in some circumstances, but not on the rabbit skin. Although with white cowpox the same stimulus was given to both hosts, the response by different hosts varied.

The practical significance of these results for those working with poxviruses is that pock character is not as reliable for use in the identification of certain pox-

viruses as is generally thought. Fortunately the pock character and ceiling temperatures of variola and alastrim appear to be stable and so no confusion is likely to be caused in the laboratory diagnosis of smallpox, and confusion between the other viruses and their white pock mutants is unlikely to have serious consequences in human or veterinary medicine. However, the variability in pock character described here is likely to cause confusion and to increase greatly the technical difficulties involved in doing genetic and other work in which pock character has been used in presumptive identification of poxviruses. It would be most useful if tissue culture plaque assays were available which distinguished between parental and mutant types, but McClain's results (1965) indicate that perhaps too many factors need careful control to make such methods reliable routinely. The most reliable method for distinguishing between white pock mutants and their parental types is still intradermal rabbit inoculation (Haddock, 1952; Fenner, 1958).

The fact that the phenomenon described here has not been reported before may be explained by failure of previous workers to recognize it, or because it is of recent origin brought about by changes in the virus or chick embryo. Such evidence as is available favours the last alternative. White pock mutants of cowpox have been studied extensively since their initial isolation in 1952, and nothing unusual was seen in their pock character as late as 1966 (Baxby & Rondle, 1967). In particular, attempts have been made to obtain parental red cowpox by recombination between strains of white cowpox (Bedson & Dumbell, 1964; Fenner & Greenland, 1964) and in these circumstances production of red pocks would surely have been noticed and investigated. These factors, plus the fact that atypical pocks have now been seen elsewhere (C. R. Madeley, 1969, personal communication), leads one to suggest that the phenomenon has not been missed previously but is of recent origin. That it is not due to any change in the virus is indicated by the fact that virus suspensions made in 1956 and stored at $-20°$C. until 1969 behaved in the same way as suspensions which have been passaged continually. Hence it is possible that the phenomenon may be due to some recent changes in the chick embryo.

SUMMARY

White pock mutants of cowpox virus produce pocks in the CAM which 'mimic' those produced by the parent virus, in macroscopic and microscopic appearance, and in the amount of virus they contain. The proportion of such 'atypical' pocks was influenced by the age and source of embryo, incubation temperature and position of the CAM during incubation. Similar results were obtained with white pock mutants isolated from strains of vaccinia, rabbitpox and monkeypox. The appearance of pocks produced by variola, alastrim, ectromelia and vaccinia (Lister) was unaffected by changes in environmental conditions.

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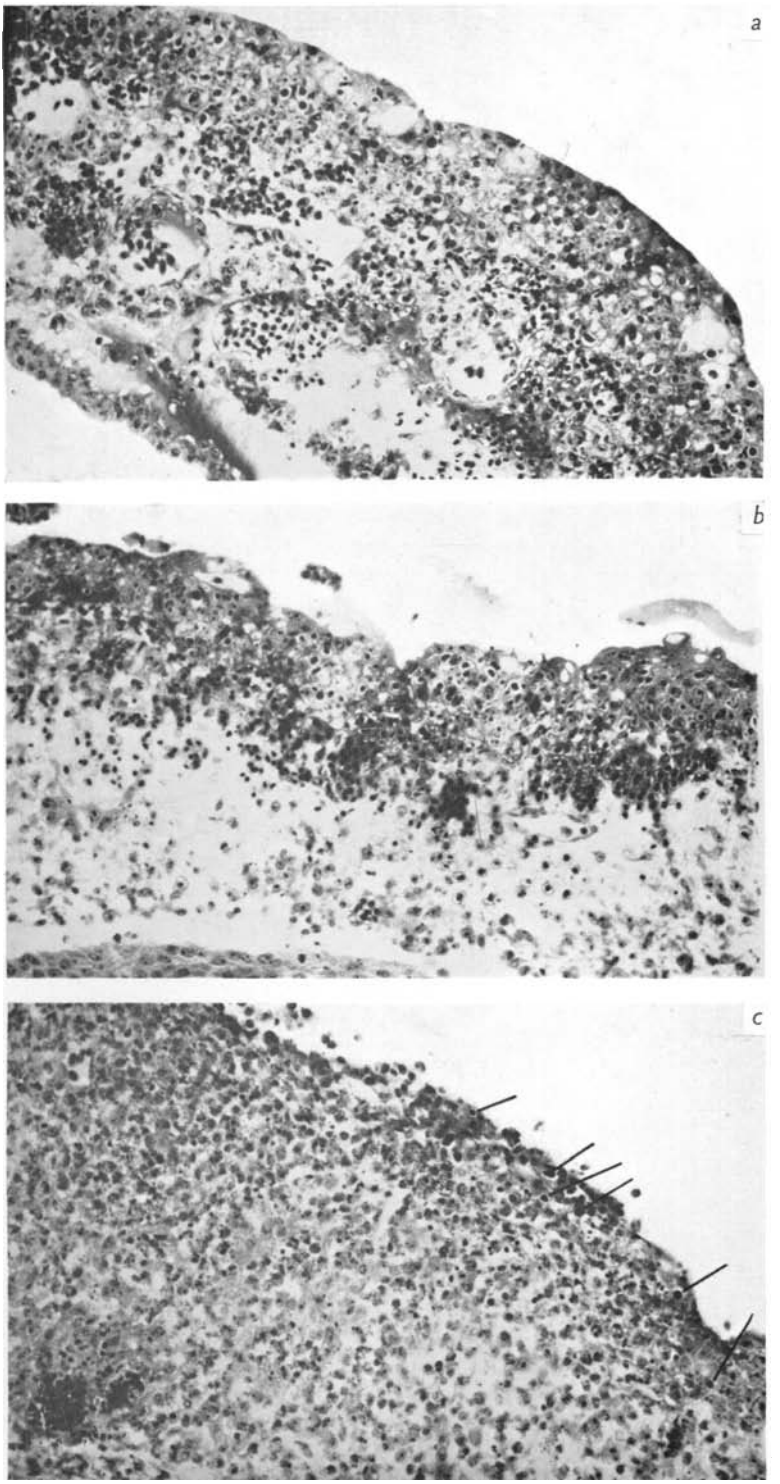

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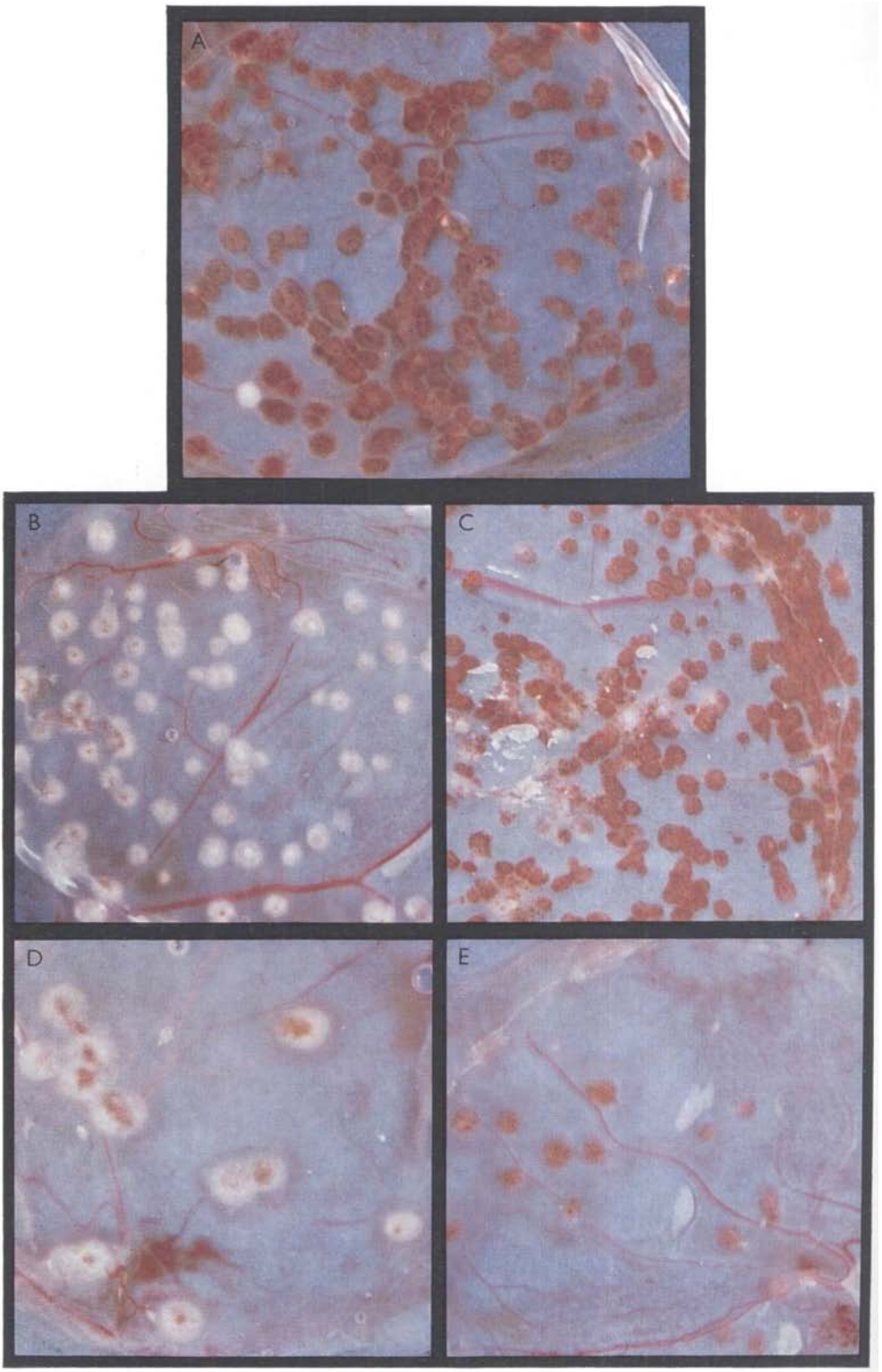

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EXPLANATION OF PLATES

PLATE 1

Sections of single pocks taken from CAM of White Leghorn embryos after 72 hr. incubation at 35°C. Stained with haematoxylin and eosin, × 260.

A. Parental red cowpox.

B. Atypical white cowpox.

C. White cowpox. Inclusions arrowed.

PLATE 2

CAM of White Leghorn embryos 72 hr. after inoculation with the stated virus, × 2. Photographed on Kodachrome 11A using a blue tile as background.

A. Parental red cowpox incubated at 35°C. Note one white mutant pock.

B. White cowpox incubated at 39°C., showing classical appearance of white cowpox.

C. White cowpox incubated at 33°C., showing a very high proportion of atypical pocks.

D. White pock mutant of Levaditi vaccinia incubated at 39°C., showing typical white, ulcerated pocks.

E. White pock mutant of Levatidi vaccinia incubated at 33°C., showing atypical pocks.