AN ANALYSIS OF THE TOXICITY FOR MICE OF INFLUENZA VIRUS

I. Intracerebral Toxicity

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Standard (non-neuro-adapted) strains of influenza virus are known to kill mice by a so-called toxic effect when large amounts are injected intracerebrally (Henle and Henle, 1944, 1946). Schlesinger (1950) showed that these strains underwent a single incomplete cycle of growth in the mouse brain, as detected by the production of haemagglutinin and complement fixing antigen. Cairns (1951*a*) however, showed that there was also an increase in infectivity titre during the cycle of growth, and that adapted strains differed by continuing to grow after this period.

The exact site of virus multiplication in tissues can now be demonstrated by the fluorescent antibody technique, and the intracerebral toxicity of standard influenza virus strains has therefore been analysed and compared with the behaviour of a neuro-adapted strain, using this technique.

MATERIALS AND METHODS

Viruses.—The MEL, PR8, and NWS strains of influenza A virus were used as allantoic fluid preparations. Red cell eluates, and concentrates obtained by centrifugation, were occasionally used.

Receptor Destroying Enzyme (RDE) (kindly provided by G. L. Ada) was used in the form of a *Vibrio cholerae* filtrate concentrated by adsorption-elution (Ada and French, 1950), and the principal experiments were repeated and confirmed using a pure, crystalline preparation (Ada and French, 1959). When 500 U RDE (French and Ada, 1954) were injected intracerebrally there was no protection against large doses of standard strains of virus given 1, 24, or 48 hr. later, and at least 1000 U were needed to give some degree of protection. Mice often showed hyperirritability and tremor after very large injections of impure RDE, and a cellular infiltration of the ventricles was detectable histologically.

Titrations.—Infectivity titrations were made in bits of allantois-on-shell as described by Fazekas de St. Groth and White (1958).

Haemagglutinin titrations were carried out in perspex trays, each cup containing 0·25 ml.; 0·05 ml. of 5 per cent fowl red cells were added, and partial agglutination taken as the end-point.

Mice.—Multicoloured outbred mice of the Walter and Eliza Hall Institute strain were used.

Fluorescent antibody technique.—Materials for examination were quick-frozen in tubes in a dry ice-alcohol bath, and thin sections cut on a Cambridge Rocker microtome in a $-20°$ refrigerator (Louis, 1957).

Serum from a chicken hyperimmune to the MEL strain of influenza was used to prepare a fluorescent conjugate. A crude γ-globulin fraction of the serum was conjugated with fluores-

cein isothiocyanate (Sylvana Chemical Corporation, Orange, New Jersey, U.S.A.), and fixation, staining, washing and mounting of sections carried out in the usual way.

Sections were examined under a Zeiss microscope equipped for fluorescent observations, with an Osram HBO 200 mercury vapour lamp as light source. Photographs were made on HP3 (Ilford) film.

RESULTS

Standard strains (MEL and PR8)

In a preliminary experiment tenfold dilutions of the MEL and PR8 strains were injected intracerebrally and general features of the toxic effect observed. Deaths occurred within the first 6–7 days, and sick mice were hunched and ruffled, trembled, and often died in the course of tonic flexor or extensor convulsions (Henle and Henle, 1944). Such convulsions could be induced by tail twirling and sometimes merely by holding mice upside down. Doses of less than about $10^{6.0}$ ID_{50} (about 1 AD) were not usually lethal, and mice given doses slightly less than $10^{6.0}$ ID_{50} sometimes became sick and then recovered. Mice were well for a short time after the injection of a toxic dose of virus, and although with the largest doses sickness was detectable within 2 hr, there were no deaths until at least 12 hr. The same lethal endpoint was obtained whether toxic preparations were titrated in 1–7 day old or in adult (35–45 day) mice. There is therefore no great difference in the susceptibility of suckling and adult mice.

Brains were removed and examined by fluorescent antibody staining at intervals after the intracerebral injection of large amounts of MEL and PR8 virus, with the following results: 5 min.—marked fluorescence of cytoplasm of many ependymal cells and also of cells lining subarachnoid spaces. 30 min.—considerable fading of fluorescence. 3 hr.—almost no specific fluorescence detectable. 5–6 hr.—moderate fluorescence of cytoplasm of most ependymal cells. Faint fluorescence of cytoplasm of many of cells lining subarachnoid spaces. 7 hr.—fluorescence brighter. 8 hr.—further increase in brightness. Ependymal cell fluorescence much brighter than that of cells lining subarachnoid spaces.

There was no increase in the extent or brightness of fluorescence after 8 hr., and subependymal and subpial cells did not become fluorescent (Fig. 1).

Occasional fluorescent cells were seen in the Virchow Robin spaces as well as in the subarachnoid spaces. As a rule the ependymal cells covering the choroid plexuses showed a more intensive fluorescence than those in the ventricle wall, and there was often a brightly fluorescent cytoplasmic rim on the ventricular border of these cells. Most, but not all ependymal cells were fluorescent, and the few non-fluorescent cells were in groups involving, for instance, one side of a lateral ventricle.

The fact that there was no increase in the extent of fluorescence after 8 hr. suggested that there was a single cycle of growth. Most of the ependymal and subarachnoid cells, however, were fluorescent at this time after large doses of virus, and it was uncertain whether growth was limited in this experiment because only these cells were capable of supporting growth, or because growth was restricted to a single cycle. Accordingly 10- and 1000-fold dilutions of stock material were injected intracerebrally, and the brains were examined after 12, 17, 24 and 36 hr. A smaller proportion of the ependymal and meningeal cells became fluorescent with these doses, and there was no increase in the extent of fluorescence after 12 hr. Growth therefore is restricted to a single cycle. In this experiment ependymal cells were still brightly fluorescent at 4 days, although the fluorescence was

in denser, better defined cytoplasmic aggregates. By 7 days fluorescent cells were no longer visible.

A neuro-adapted strain (NWS)

The NWS allantoic fluid preparations used were lethal when diluted 10^{-5} (to contain about 1/1000 A.D.) before intracerebral injection. Deaths occurred from the 4th–7th day, depending on the dose, and although fatal convulsions occurred and could be induced by tail twirling they were less common than with unadapted strains of virus.

In a number of experiments undiluted allantoic fluid was injected intracerebrally and brains removed at different times and examined by fluorescent antibody staining (Fig. 2–5). By 8 hr. there were a moderate number of fluorescent ependymal cells, which were brighter and more numerous by 12 hr. At this time the fluorescence was identical in distribution to that seen with unadapted strains. By 24 hr., however, there were fluorescent cells extending away from the ependyma into the parenchyma. These extensions into the parenchyma were well marked by 36 hr., and even more pronounced at 48 hr. Collections of fluorescent cells were also seen below the meninges. It was not possible to decide whether the fluorescent parenchymal cells were glial or nerve cells.

NWS thus differs from unadapted strains in its ability to invade the parenchyma from the ependyma and meninges. In another experiment where smaller doses of NWS were injected and brains examined at 12, 24, 36, 60, and 83 hr., distinct increases in the extent of ependymal fluorescence were observed. This means that NWS is capable of successive cycles of growth in the ependymal sheet of cells.

Histological appearances

Henle and Henle (1946) reported that toxic doses of unadapted influenza virus strains produced destruction of the ependymal lining of the ventricles. This could be confirmed in sections of brain stained with haematoxylin and eosin. In addition there were large numbers of inflammatory cells in the ventricles, together with some blood, and there was an infiltration of cells into neighbouring areas of parenchyma. Choroid plexuses were often reduced to a few necrotic fragments. Ventricular profiles sometimes showed a slight hydrocephalic bulge and contained large amounts of fluid, as well as cells. In mice dying after 6–7 days the hydrocephalic tendency was more marked and the cellular reaction was more pronounced, for ventricles were often filled to capacity with inflammatory cells, and a more extensive infiltration of the parenchyma was seen.

The severe inflammatory response, originating in the ventricles, might result in an increase in intracranial pressure, as well as interfere with the production and circulation of cerebrospinal fluid. The signs and symptoms of cerebral oedema can sometimes be relieved by intravenous injections of hypertonic glucose, which reduce the intracranial pressure. The intraperitoneal or intravenous injection of 50 per cent glucose, however, had no detectable effect on the sickness of mice who had received toxic intracerebral injections of influenza virus.

Kitayama, Sunakawa and Fukumi (1953) showed that in NWS infected brains the principal pathological changes were in ependymal cells, and these are the cells which have been shown above to be primarily involved following intracerebral injections, even of small doses of virus.

Effect of RDE

Wagner (1952) reported that large intracerebral doses of RDE gave complete protection from the toxic effect of maximal intracerebral doses of standard influenza virus strains. This was readily confirmed, whether RDE was injected 1 hr. or 48 hr. before virus. When, however, brains of RDE-protected mice were examined by fluorescent antibody staining 24 hr. after a large intracerebral virus injection it was found that there had been growth in occasional ependymal and meningeal cells, although far fewer than in control mice. Protection by RDE was thus not absolute, at the histological level. In these experiments RDE and virus were injected into the same hemisphere, and although the injected material floods through the CSF spaces (Mims, 1960) it might not reach all the cells lining these spaces. It was pointed out earlier that even with the largest virus doses given to normal mice, there were occasional non-fluorescent ependymal regions at the time of death. Thus, virus might grow in ependymal and meningeal cells which had not received enough RDE as a result of slight differences in the distribution of virus and RDE.

When 1000-fold diluted stock virus was injected intracerebrally into RDE-protected mice fluorescent cells could not be detected at 24 hr. whereas there were moderate numbers of fluorescent ependymal cells in control non-protected mice. RDE therefore gives more effective protection against smaller doses of virus.

Cairns (1951*b*) showed that RDE treated mice were protected against moderate doses (up to 100 IC LD_{50}) of the NWS strain of virus ; this could readily be confirmed. It was shown in fluorescent antibody experiments that, as with standard strains of virus, protection against large doses of virus was not absolute at the histological level, for there were a number of fluorescent ependymal and meningeal cells 24 hr. after an RDE treated mouse had received a large intracerebral injection of NWS virus. These initially infected cells presumably infect neighbouring but unprotected cells, and a progressive spread of the infection through unprotected cells kills the mouse in the usual way.

The response of suckling mice

When 1–3 day old mice were injected intracerebrally with large doses of the MEL and PR8 strains they died within 24 hr. The heads were sectioned and stained with fluorescent antibody. The skin was removed from the heads because the stratum corneum has a brilliant yellow fluorescence, fragments of which tend to be dragged across the section by the microtome knife. Specific fluorescence was confined to the meninges and the ependyma, although occasionally more than one layer of cells were involved in ependymal regions (Figs. 6 and 7). Smaller doses of virus were then injected so that mice died after 3 or more days, and heads were examined at 12, 22, 27 and 46 hr. If mice were injected when less than 24 hr. old, it was clear that there was an increase in the number of fluorescent cells after 12 hr., and up to 6 layers of subependymal cells were often involved. There was less evidence for an increase in fluorescence in older suckling mice. In these sections brightly fluorescent cells lining the oronasal passages were often seen (Fig. 8), and this demonstration that there is growth of intracerebrally injected virus in these cells confirms the findings of Tyrrell and Cameron (1957), who described pathological changes in the nasal mucosa of suckling mice infected in this way. India ink was injected intracerebrally to infant mice, whose heads were

immediately removed, and haematoxylin and eosin stained sections prepared. Ink was present in submucosal lymphatics, in a few veins, and in the submucosal connective tissue spaces in some areas. Intracerebrally injected virus would be distributed to the submucosa in the same way and would therefore be able to grow in mucosal cells. There was a similar distribution of India ink in intracerebrally injected adult mice. The fluorescent cells lining the nasal passages were commoner when mice survived more than 2 days, and from the distribution of these cells it was clear that more than one cycle of growth was involved.

Thus, standard strains grow in the ependymal and meningeal cells of suckling mice, and at least in mice injected when less than 24 hr. old there are successive cycles of growth. The injected material also reaches the nasal mucosa, and growth here is not restricted to one cycle.

Similar experiments were done with the NWS strain, and there was extensive involvement of the brain parenchyma after preliminary growth in the meninges and parenchyma, as in adults.

DISCUSSION

Although the greater part of an intracerebral inoculum immediately spills over into the blood (Cairns, 1950), that part of the inoculum which stays in the brain is deposited in the cerebrospinal fluid spaces (Mims, 1960). The cells lining these spaces, therefore, are directly exposed to the injected virus, and may support virus growth. In the case of poxviruses growth occurs principally in these cells while there is a limited extension of the infection to the brain parenchyma (Mims, 1960). This extension into the parenchyma is perhaps a result of the migration of infected subependymal cells, for these cells are known to proliferate and then migrate into the parenchyma in the adult mouse (Messier, Lebland and Smart, 1958).

Both standard and neuro-adapted strains of influenza virus, evidently, grow in the ependymal and meningeal cells immediately available to them after intracerebral injections. Standard strains are only capable of a single cycle of growth, so that there is no spread of the infection to underlying cells, nor to neighbouring uninfected cells. If enough virus is injected there is a lethal amount of growth in this single cycle, and the so-called toxic effect is produced. At least 10^6 ID_{50} have to be injected to produce this effect, and if it is assumed that at the most 10 per cent of the inoculum stays in the brain, then 10^5 ID_{50} infect enough cells to kill the mouse.

In spite of the fact that there is no further growth of virus after about 8 hr., mice often survive for several days. Mice probably die as a result of the cellular reaction to infection, rather than to the mere growth of virus in ependymal cells, although a disturbance in cerebrospinal fluid production must result from the heavy infection of the ependymal cells covering the choroid plexuses. Perhaps one way in which the intense cellular reaction contributes to death is by obstructing the flow of cerebrospinal fluid, eventually producing the frankly hydrocephalic condition seen in mice dying after 6–7 days.

As was pointed out by Cairns (1954) the inability of standard strains to undergo more than one cycle of growth might be due to a defect in the virus produced in this cycle, to its retention in the cell, or to some other inability to spread to other cells. At least part of the virus produced is infective for the egg (Cairns, 1951*a*), although non-infective virus is produced when large inocula are given (Schlesinger,

1950), and it may be that there is an inability to spread to other cells. The non-infectious haemagglutinin produced by influenza virus in HeLa cells is retained in cells until they are destroyed (Henle, Girardi and Henle, 1955). The fact that antigen often accumulates at the ventricular edge of ependymal cells suggests defective release, but similar appearances were seen by Watson and Coons (1954) in infected cells of the chick embryo. It is known that fowl plague antigen accumulates at the cell membrane before virus is released (Breitenfeld and Schäfer, 1957).

Standard strains behave differently when injected into mice less than 24 hr. old for there are then successive cycles of growth in the brain.

Neuro-adapted strains of influenza virus undergo a primary period of growth in the ependymal and meningeal cells immediately available, and their adaptedness lies in their ability to undergo successive cycles of multiplication, for they then invade underlying parenchymatous tissues, as well as neighbouring uninfected cells. There is a similar spread of infection in infant mouse brain. Whether virus grows in neurones or only in neuroglial cells, death might result from the damage to these parenchymal cells. Alternatively after there has been a lethal extent of growth in the ependyma and meninges, death could occur in the same way as with unadapted strains.

The results of RDE pretreatment are understandable in the light of the different growth patterns of standard and neuro-adapted strains. It was shown that RDE protection against large doses of virus is not absolute at the histological level, perhaps because of minor differences in the distribution of intracerebrally injected RDE and virus. Most ependymal and meningeal cells, however, are protected, whether virus is given 1 hr. or 48 hr. after RDE. Standard strains, which are lethal only if they grow in most of these cells, are then no longer lethal. The neuro-adapted strain, on the other hand, since it also can infect a few of these cells and is capable of successive cycles of growth, is still lethal. The lethal endpoint is reduced about a hundredfold (Cairns, 1951*b*), because with small doses of virus RDE protection is complete.

A similar investigation of the growth of intracerebrally injected influenza viruses has recently been made by Fraser, Nairn, McEntegart and Chadwick (1959). Their findings are substantially the same as those reported here.

SUMMARY

Standard strains of influenza virus undergo a single cycle of multiplication in the ependymal and meningeal cells immediately available to them after intracerebral injection. If enough cells support virus growth mice die and the so-called toxic effect is produced. RDE prevents the infection of most cells and thereby protects against toxicity.

Standard strains undergo successive cycles of growth when injected into the brains of mice less than 24 hr. old. Some of the injected virus is deposited below the nasal mucosa, and there is then growth in mucosal cells.

Neuro-adapted strains of influenza virus, which undergo successive cycles of multiplication in adult mice, grow first in ependymal and meningeal cells, and then in underlying parenchymal cells. Although RDE prevents the infection of most ependymal and meningeal cells a small number of these cells support virus growth, which then slowly progresses, and may kill the mouse.

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EXPLANATION OF PLATES

FIG. 1.—Fluorescent antibody stained section of adult mouse brain 17 hr. after the intracerebral injection of a large dose of MEL virus. Ventricular profile with fluorescence limited to ependymal cells. ×124.

FIG. 2.—Fluorescent antibody stained section of adult mouse brain 17 hr. after the intracerebral injection of a moderate dose of NWS virus. Ventricular profile with fluorescent ependymal cells limited to the tips of the choroid plexuses. ×93.

FIG. 3.—Fluorescent antibody stained section of adult mouse brain 36 hr. after the intracerebral injection of NWS virus. Fluorescence is beginning to extend from the ependymal to the subependymal cells. × 93.

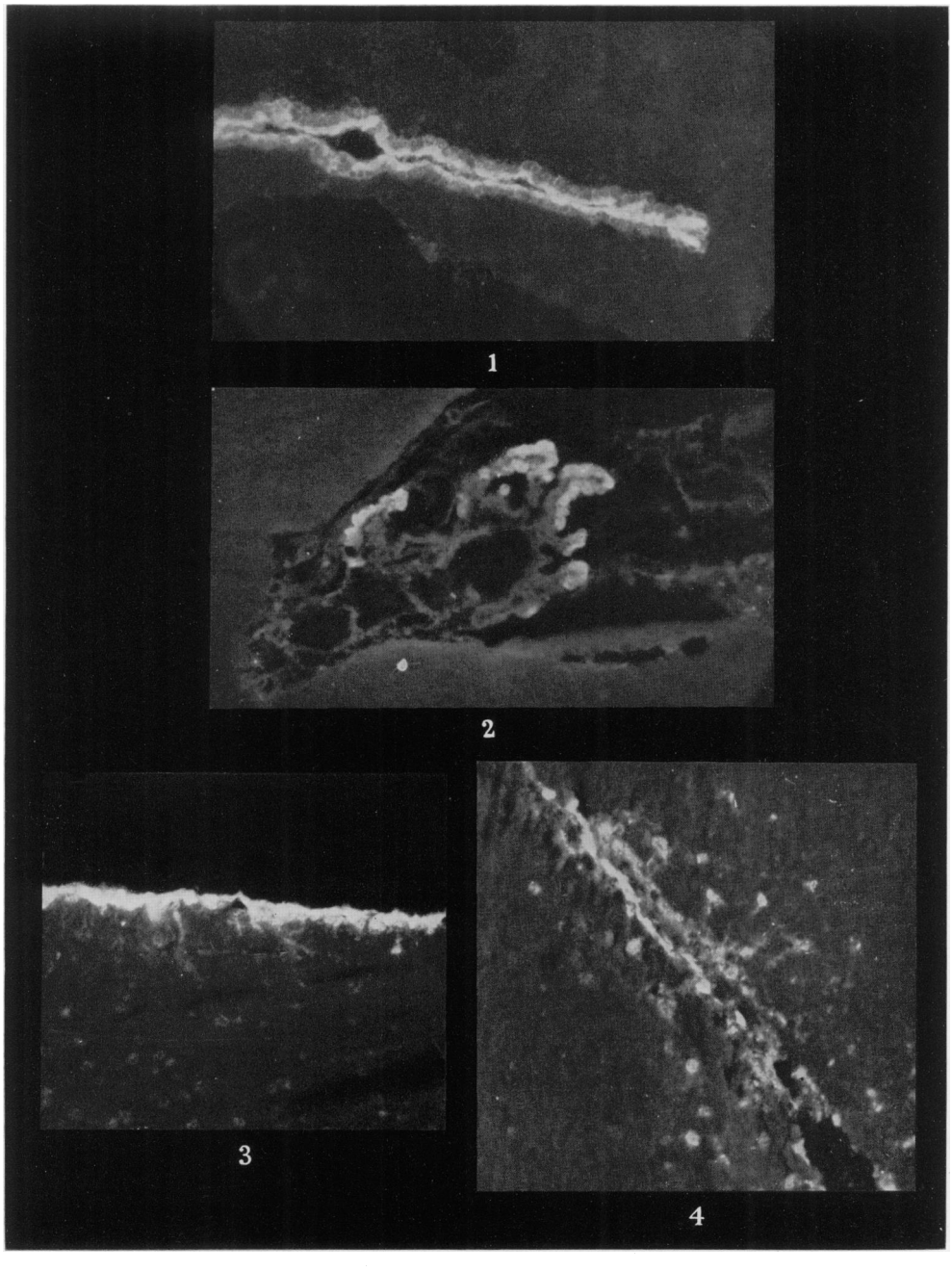
FIG. 4.—Fluorescent antibody stained section of adult mouse brain 3½ days after the intracerebral injection of NWS virus. A sulcus, with fluorescent cells lining the subarachnoid spaces, and numerous fluorescent cells in the neighbouring parenchyma. ×124.

FIG. 5.—The same section (NWS 3½ days), showing fluorescent parenchymal cells. ×243.

FIG. 6.—Fluorescent antibody stained section of suckling mouse head 24 hr. after the intracerebral injection of the PR8 strain of influenza virus. Ventricular profile with fluorescent ependymal cells covering tips of choroid plexuses, and extension of fluorescence into subependymal cells. × 90.

FIG. 7.—Same slide showing fluorescent cells on meningeal surface of brain. × 60.

FIG. 8.—Fluorescent antibody stained section of infant mouse nasal mucosa 6 days after intracerebral injection of the PR8 strain of influenza virus. A focus of fluorescent mucosal cells. ×60.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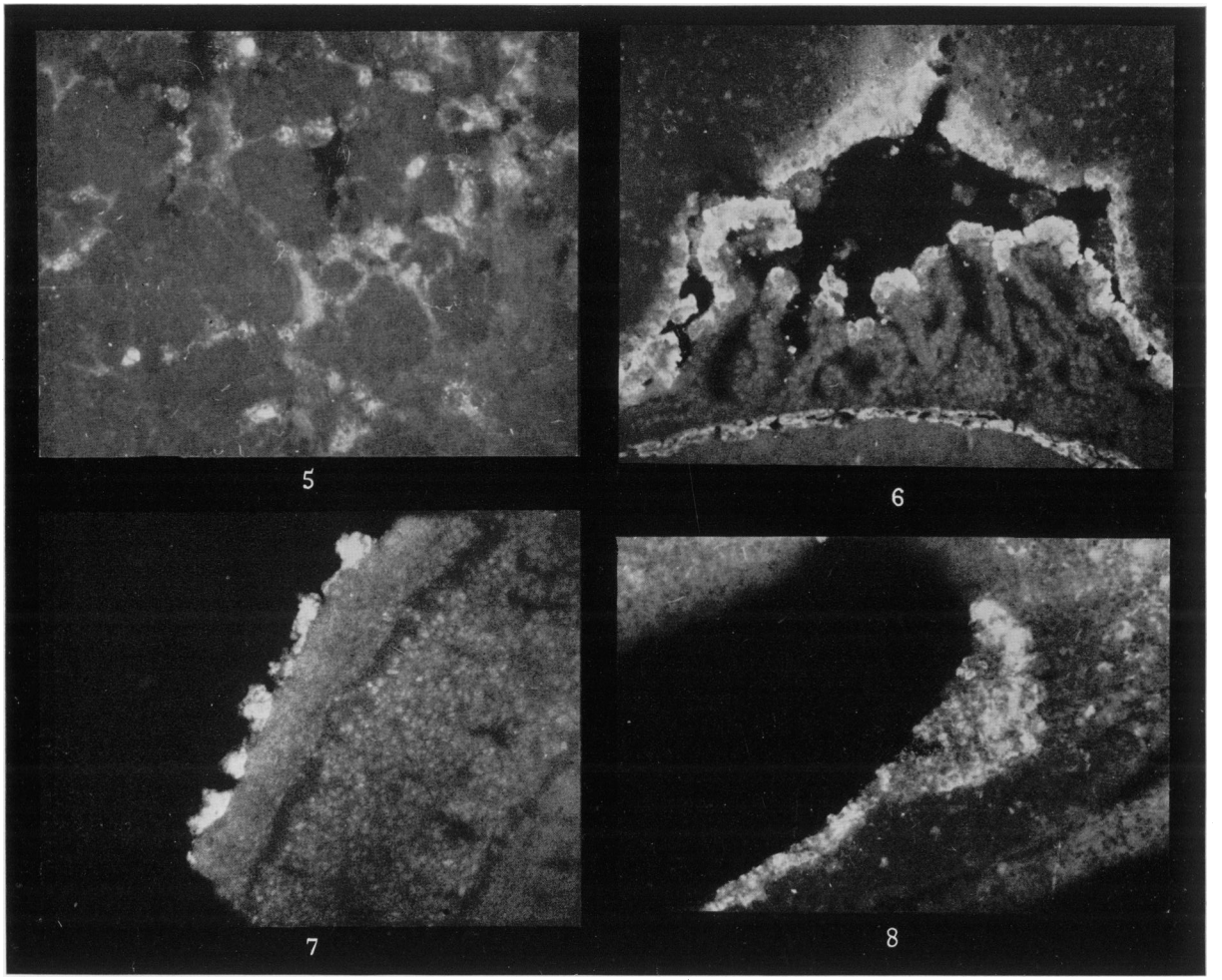

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