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## *VIRUS PARTICLES AND VIRAL ANTIGENS IN CHICKEN TISSUES FREE OF INFECTIOUS AVIAN LEUKOSIS VIRUS**

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Two situations in which avian leukosis viral antigens and viral particles were found in chicken tissues that do not contain detectable infectious virus have been reported from this laboratory.[1–3] The first report described noninfectious virus particles associated with "nonproducer" Rous sarcoma cells.[1, 2] This finding was recently confirmed.[4] Work reported at this symposium showed that these particles had the same buoyant density as infectious avian leukosis/sarcoma viruses and that they contained an RNA component with the same molecular weight and base ratios as their infectious counterpart.[5] Further, Vogt[6] reported that the particles, which he termed "helper-independent virus," were infectious for certain specific avian tissues. It appears from this that nonproducer Rous sarcoma cells release Rous virus particles whose defectiveness is reflected in a restricted host range.

Recently, we observed viral particles in chick embryos that were free of infectious virus.[3] This report deals with an extension of this work to determine the nature and mode of transmission of these apparently noninfectious particles.

*Materials and Methods.*—Fertile hens' eggs were obtained from six different flocks, four of which are maintained in isolation specifically to exclude infection with avian leukosis virus (ALV). These include experimental flocks maintained by Dr. Roy Luginbuhl at the University of Connecticut; Dr. F. B. Bang at The Johns Hopkins University; Dr. B. R. Burmester at the Regional Poultry Research Laboratory in East Lansing, Michigan, and a commercial flock maintained by

SPAFAS, Inc., Norwich, Connecticut. The two remaining flocks are not strictly isolated, but we have found them to be largely free of infection with ALV. These include a flock from Shamrock Farms, Milltown, New Jersey, and a flock maintained by Mr. R. Sachs, Marcellus, New York.

Cell cultures were prepared from single chick embryos and cell stocks were stored frozen in liquid nitrogen as described previously.[1] A sample of each frozen cell stock was examined to confirm the absence of congenitally transmitted ALV and to determine susceptibility to infection with three strains of Rous sarcoma virus (RSV) representing each of the antigenic subgroups described by Ishizaki and Vogt.[7] In this study only cell stocks with the genotype C/O, susceptible to all known strains of ALV and RSV, were used.

Tissues were tested for infectious virus by the complement-fixation avian leukosis (COFAL) test described by Sarma *et al.*[8] Crude 20 per cent tissue extracts (0.1 ml) were placed in 60-mm Petri dishes with $8 \times 10^5$ C/O cells and 4 ml of tissue culture medium. The cells were passaged four times and the final passage cells were tested for avian leukosis group-specific (*gs*) antigen by complement fixation. Each test included a positive control culture, infected with the F-42 strain of ALV, and an uninfected, negative control culture.

Serological tests for avian leukosis *gs* antigen were carried out with antisera from Syrian hamsters infected subcutaneously within 24 hours of birth with a cell-free preparation of Schmidt-Ruppin RSV derived from chick cell tissue culture.

All tissues used for morphologic studies were fixed in glutaraldehyde, processed as before,[9] and examined with an RCA-EMU-3G electron microscope.

*Results.—Presence of virus particles in chicken tissues free of infectious virus:* In a previous publication, the presence of virus particles in embryos free of infectious ALV was reported.[3] This finding has been confirmed in embryos from 5 of the 6 flocks used in this study. Eggs from each flock were incubated and, after 18–19 days, a sample of liver and pancreas was removed aseptically from each embryo. The liver was frozen at −60°C for later virus assay, and pieces of liver and pancreas were fixed immediately for examination with the electron microscope. Each liver was tested for virus by the COFAL test. In addition, the remaining carcasses of some embryos were trypsinized and tissue cultures were prepared. These cultures were passaged 6–8 times at 3- or 4-day intervals and the final passage cells were tested for infectious virus by the COFAL test.

The results of tests on embryos from all 6 flocks are summarized in Table 1. Out of 186 embryos examined, none contained infectious ALV when liver extracts

TABLE 1

PRESENCE OF VIRUS PARTICLES IN CHICK EMBRYOS FREE OF INFECTIOUS VIRUS

| | Number Positive over Number Tested | | |
|---|---|---|---|
| | COFAL Test | | |
| Flock | Tissue culture infected with liver extract | Tissue culture from tested embryo | Virus particles in pancreas (EM) |
| UC | 0/83 | 0/22 | 31/33 |
| SPAFAS | 0/18 | — | 6/6 |
| JH | 0/14 | 0/8 | 4/5 |
| RPL | 0/34 | 0/12 | 2/6 |
| Shamrock | 0/17 | 0/7 | 4/4 |
| Sachs | 0/20 | — | 0/9 |
| Total | 0/186 | 0/49 | 47/63 |

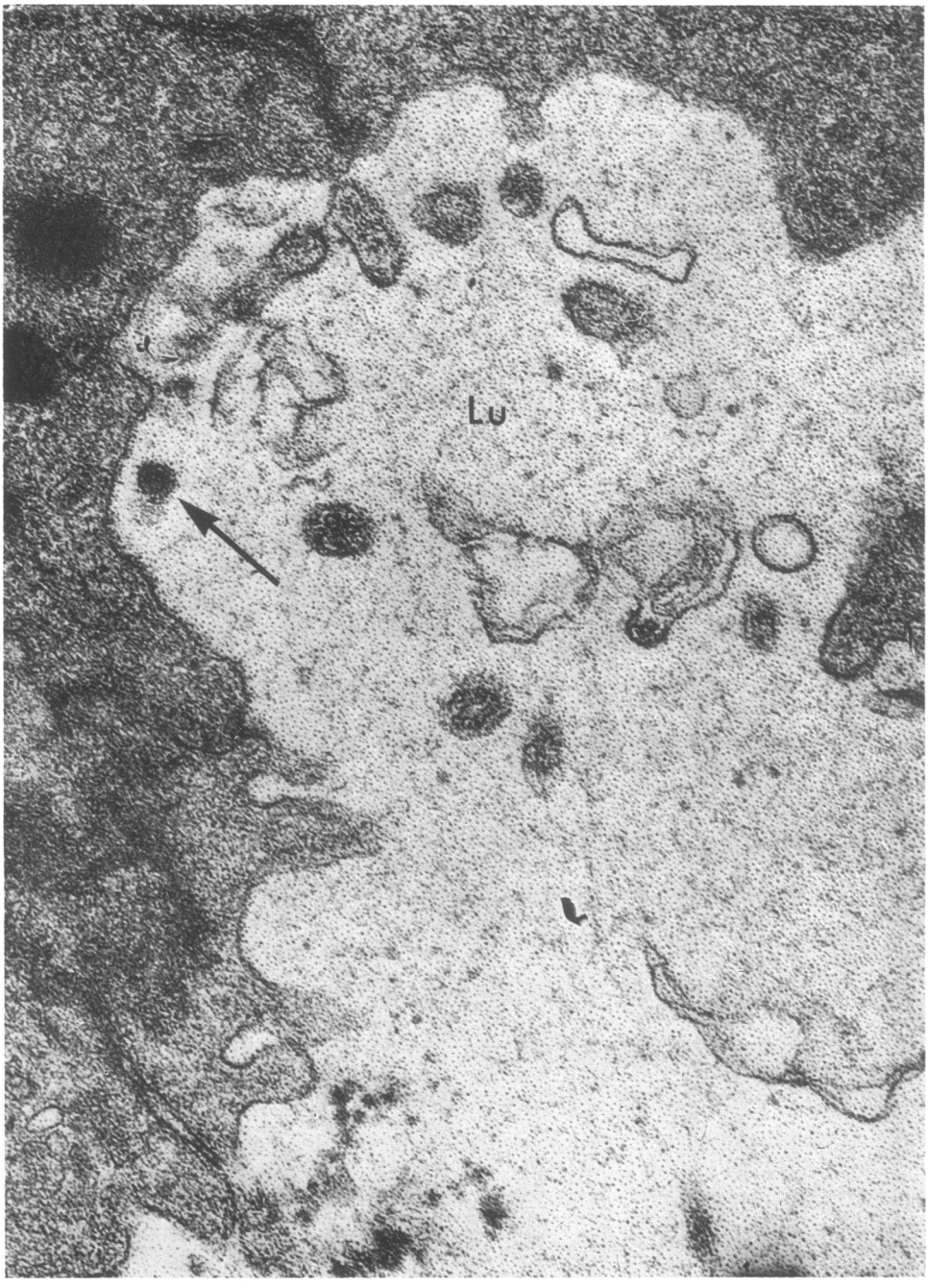


FIG. 1.—Arrow indicates either an immature virus particle, or a late budding stage in which the point of attachment is in a different section, in the lumen (*Lu*) of a pancreatic acinus from an 18-day chick embryo free of infectious viruses. ×51,000.

were tested on C/O cells. Further, tissue cultures prepared directly from 49 of these embryos likewise failed to yield evidence of infectious virus. However, virus particles were found in pancreas and/or liver of most embryos from 5 of the 6 flocks representing a total of 47 of the 63 embryos examined by electron microscopy. Figure 1 represents an electron micrograph showing a virus particle (either an immature free particle or a late bud whose point of attachment is not in this section) in a pancreatic acinar lumen of one of these "virus-free" embryos. It should be emphasized that particles were difficult to find in these embryos. At best, 1 or 2 particles might be found in an entire section that included profiles of several pancreatic acini; particles were even scarcer in the liver. This is in contrast to the picture seen routinely in the pancreas of chick embryos from a flock of chickens, congenitally infected with ALV, that is maintained in this laboratory[10] (Fig. 2). This representative electron micrograph demonstrates the large numbers of free virus particles and budding forms always seen in the lumina of pancreatic acini of ALV-infected embryos.

The presence of virus particles in chick embryos can best be explained on the basis of congenital transmission by an infected hen, and it has been shown that the pathway for congenital transmission of ALV is probably through the female reproductive system.[10] It was therefore of interest to determine if a similar mech-

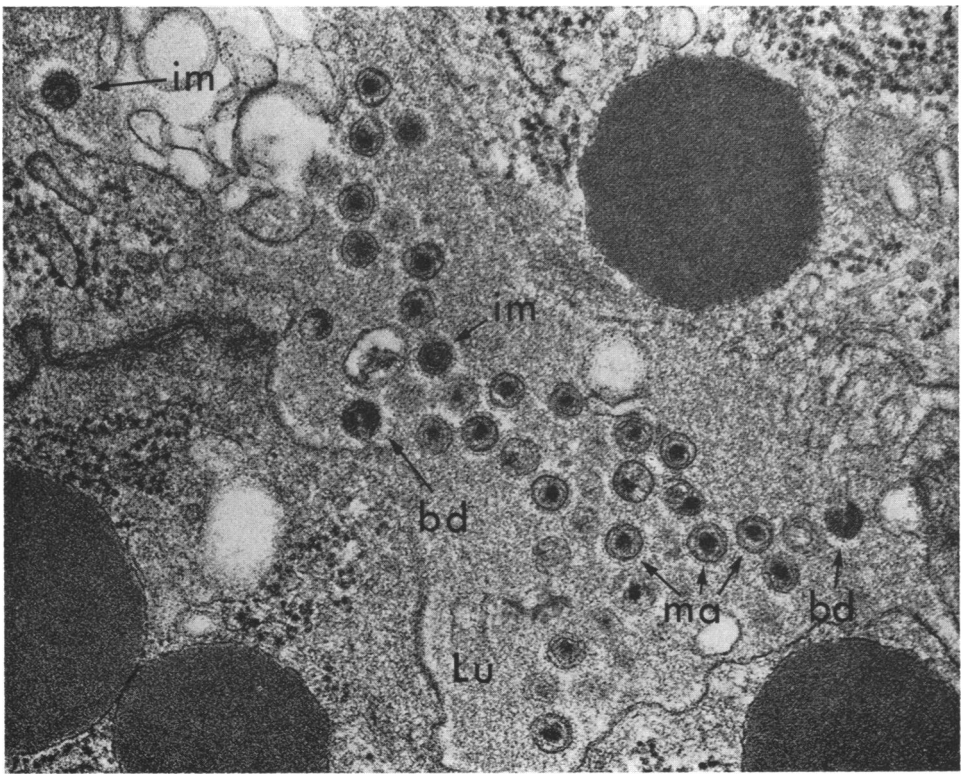


FIG. 2.—Lumen (*Lu*) of a pancreatic acinus from an 18-day chick embryo with congenital ALV infection showing many free virus particles and buds at the surface of the epithelial cells (buds, *bd;* immature particles, *im;* mature particles, *ma*). ×48,500.

TABLE 2

PRESENCE OF VIRUS PARTICLES IN MATURE HENS FREE OF INFECTIOUS VIRUS

| Tissue | Number Positive over Number Tested | |
|---|---|---|
| | Virus particles (EM) | COFAL test |
| Pancreas | 5/6 | 0/6 |
| Liver | — | 0/6 |
| Kidney | — | 0/6 |
| Ovary | 4/5 | 0/6 |
| Oviduct | 0/1 | 0/6 |

anism might explain the presence of the noninfectious particles in embryos. Trap-nested fertile eggs, marked to designate the hen that laid each egg, were obtained from the University of Connecticut flock. Most of the embryos that developed proved to have noninfectious virus particles. Figure 3 shows a virus bud in the pancreas of one of these embryos. Six of the hens that had produced particle-containing embryos were subsequently obtained through the generosity of Dr. R. Luginbuhl, and samples of tissues were taken for virologic and morphologic examination. The results are shown in Table 2. No infectious virus was found in any of the tissues. However, in tissues examined with the electron microscope, virus particles were found in the pancreas of five of the six hens, and of these five, virus particles were found in the ovary of four. Figures 4 and 5 show virus particles budding from the surface of a pancreatic acinar cell and an ovarian follicular epithelial cell in the parent hen of the embryo whose pancreas is illustrated in Figure 3. Figure 6 shows a mature particle in the ovarian stroma of this same hen. Again the number of particles was small. For comparison, a representative field of ovarian stroma from a mature hen congenitally infected with ALV is shown in Figure 7.

An interesting aspect revealed in these studies was the unusual ratio of free virus

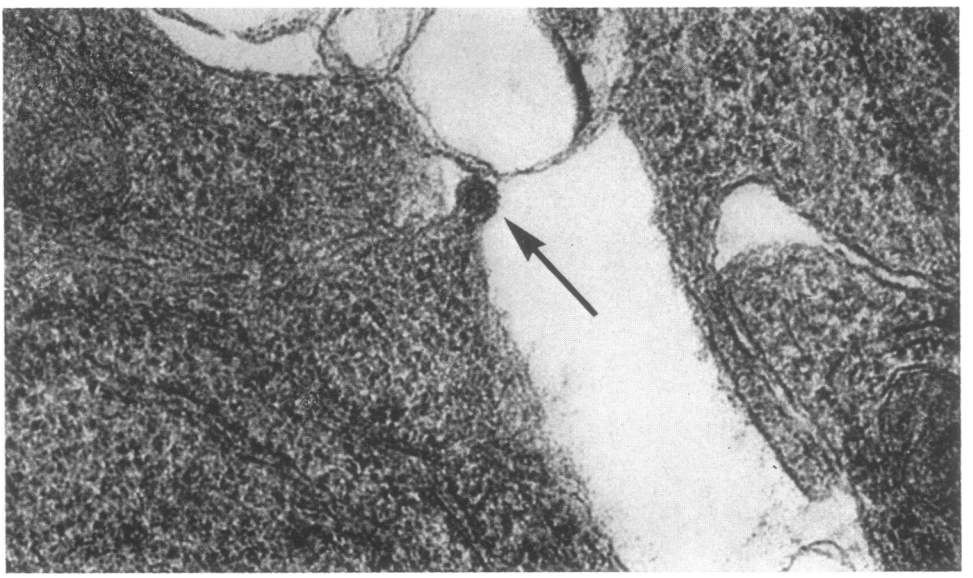

FIG. 3.—A virus particle (*arrow*) budding from the lateral surface of a pancreatic acinar cell in an 18-day chick embryo free of infectious viruses. ×72,500.

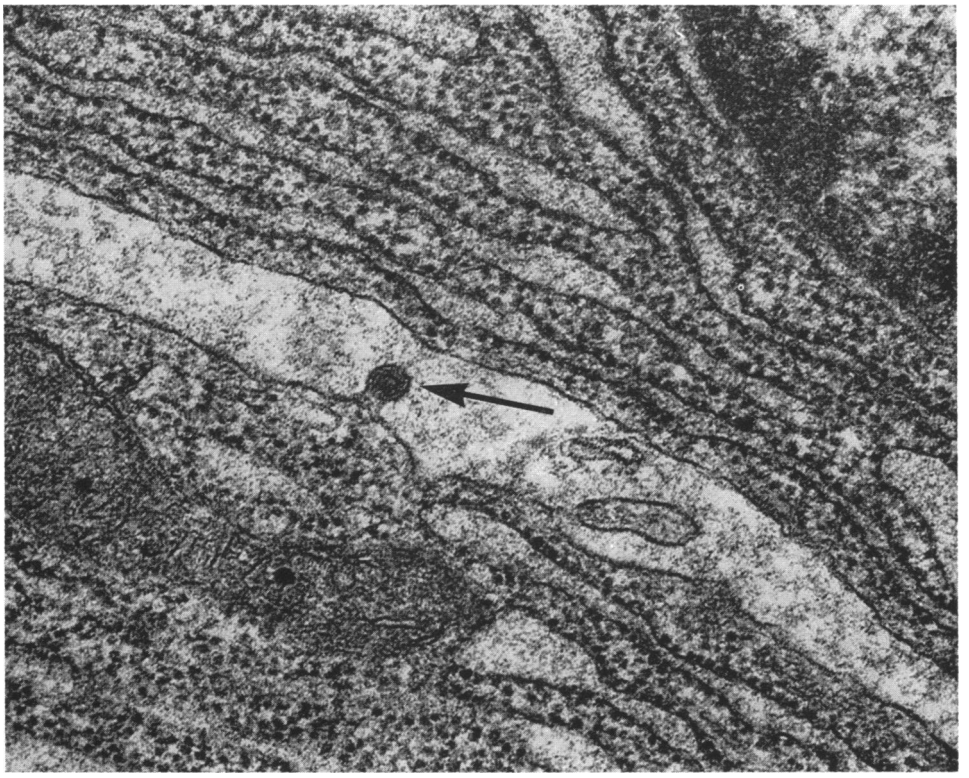

FIG. 4.—A virus particle (*arrow*) budding from the lateral surface of a pancreatic acinar cell in the hen that gave rise to the embryo seen in Fig. 3. This hen was free of infectious viruses. ×72,500.

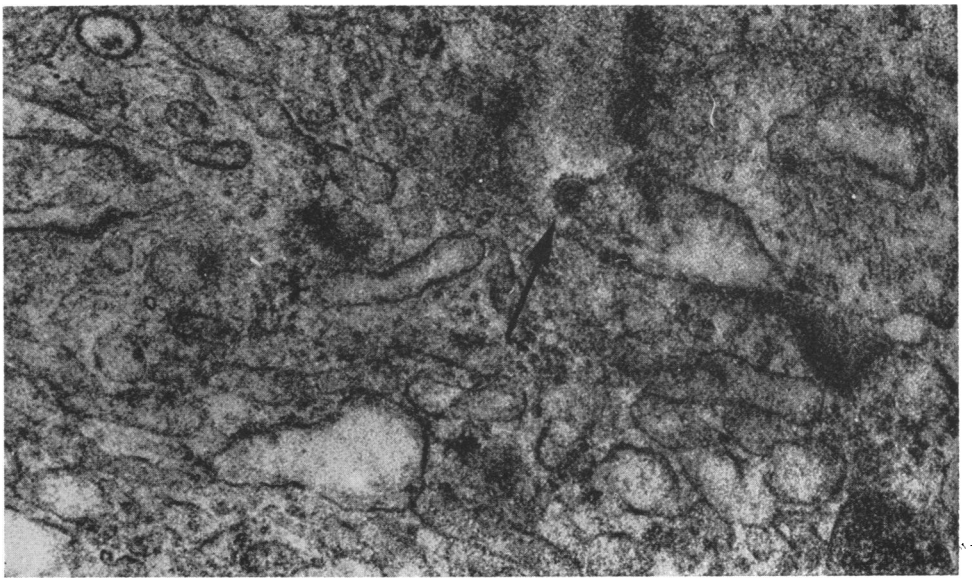

FIG. 5.—A virus bud (*arrow*) at the surface of a follicular epithelial cell in the ovary of the same hen used in Fig. 4. ×70,000.

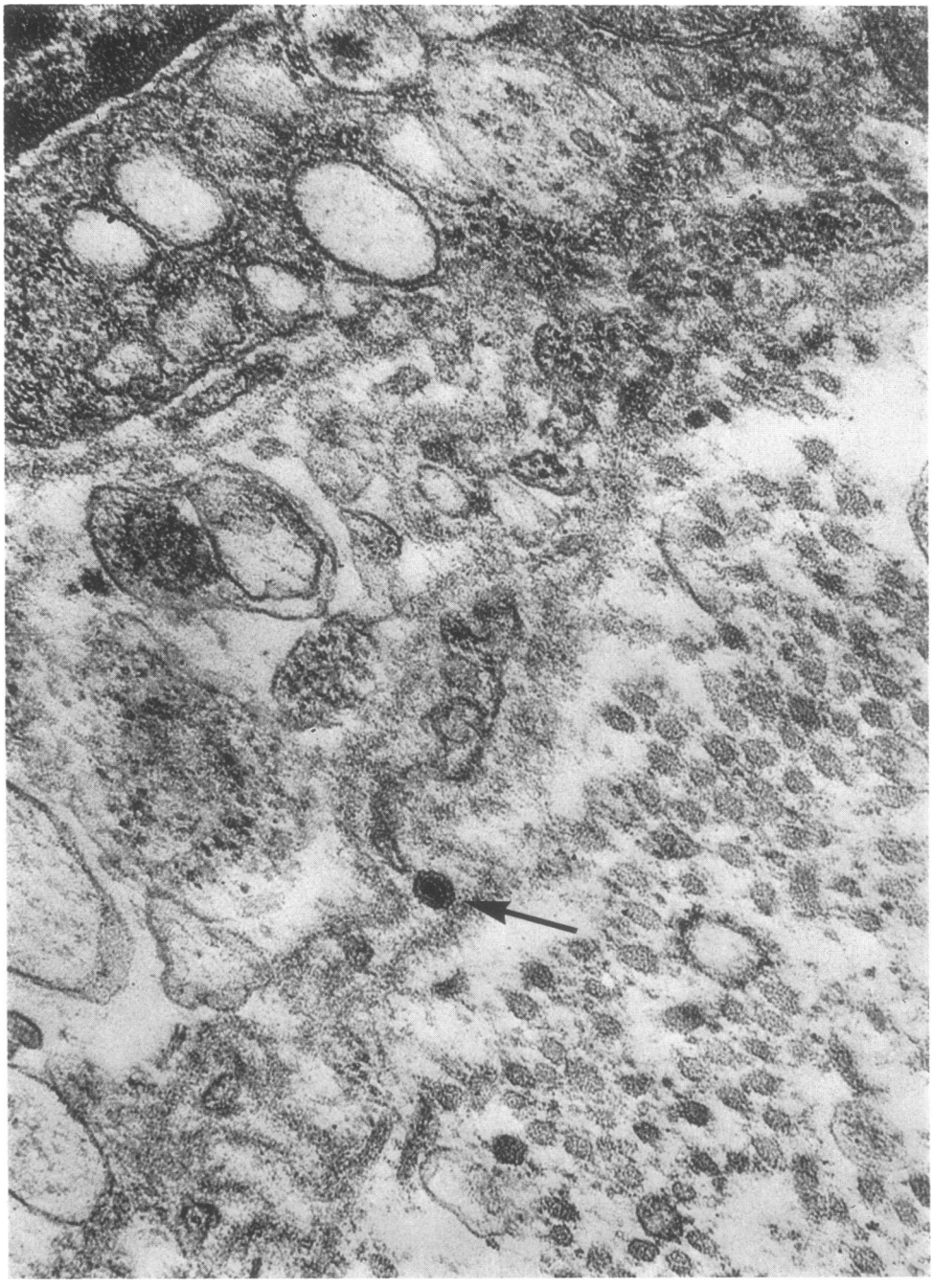

FIG. 6.—A mature virus particle (*arrow*) in interstitial space of ovarian stroma of the same hen used in Fig. 4. ×68,000.

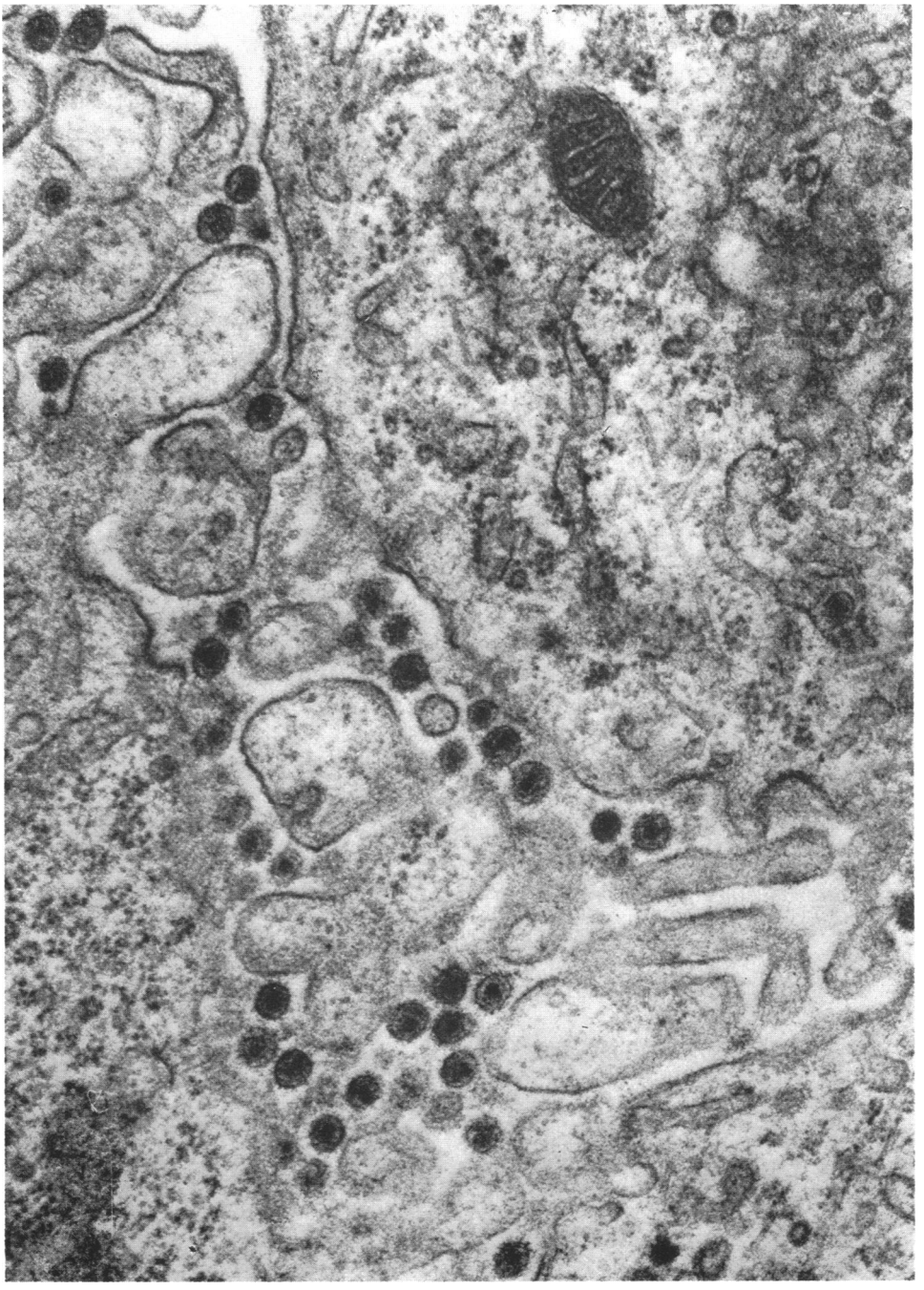

FIG. 7.—Virus particles in ovarian stroma of a mature hen congenitally infected with ALV. ×53,500.

particles to buds. In ALV-infected embryos or hens, free particles predominated (Figs. 2 and 7), while in embryos and hens containing noninfectious particles the budding forms were seen more frequently than free particles.

*Presence of avian leukosis group-specific antigen in embryos free of infectious virus:* The presence of an antigen similar to avian leukosis *gs* antigen in "virus-free" chick embryos has been reported.[3] Antiserum from hamsters with tumors induced by the Schmidt-Ruppin strain of RSV-fixed complement with extracts of various tissues from embryos that did not contain infectious ALV. Further work has been done to identify this antigen. Immunodiffusion tests were carried out with potent hamster Schmidt-Ruppin antiserum and antigens from tissue cultures infected with ALV or RSV, a sodium lauryl sulfate (SLS) extract of avian myeloblastosis virus (AMV), leukemic AMV plasma, and extracts of liver from "virus-free" chick embryos. One such test is illustrated in Figure 8. A single diffuse precipitin line, without crosses or spurs, is seen between the antibody and all antigens, indicating at least one antigen common to all. No precipitin lines developed with antigen from uninfected chick fibroblast tissue cultures, and adsorption of antiserum with uninfected chick fibroblast tissue culture cells did not alter the reaction. Therefore, the precipitin lines between the antiserum and the virus-infected tissue cultures represent a virus-associated antigen. Since all the precipitin lines are connected, and it has been shown that an SLS extract of AMV contains the internal *gs* avian leukosis antigen,[11, 12] we conclude that the "virus-free" embryo liver contains *gs* antigen.

Approximately half of the chick embryo livers listed in Table 1 contained *gs* antigen but there was no evident correlation between the presence of antigen and noninfectious particles in the same embryos.

*Discussion.*—A number of workers have described viruslike particles in otherwise normal chick embryos.[13] This finding was not clearly understood until Rubin[14] devised a method for detecting avian leukosis virus in tissue culture. It was possible, then, to explain that the particles in normal embryos were, in fact, avian leukosis virus that had been transmitted congenitally from infected hens. Congenitally transmitted virus multiplies in many tissues of the infected embryo, and the chickens hatch and mature normally but do not develop leukosis before 20 weeks of age, if at all.[10, 15] The techniques employed in the work reported in this paper were adequate to detect congenitally transmitted ALV, but there was no evidence that

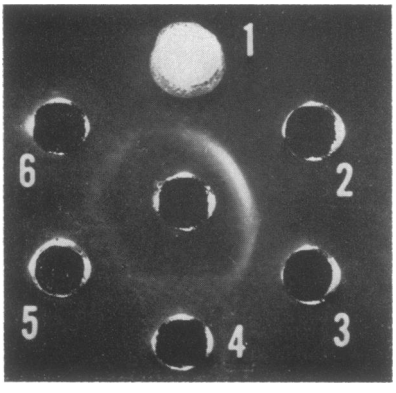


FIG. 8.—Immunodiffusion analysis of avian leukosis *gs* antigens. *Center well:* hamster antibody against Schmidt-Ruppin Rous sarcoma. Well 1, "Virus-free" chick embryo liver, 20% saline extract; 2, sodium lauryl sulfate extract of AMV; 3, chick tissue culture infected with Schmidt-Ruppin RSV; 4, chick tissue culture infected with Prague RSV; 5, chick tissue culture infected with Bryan RSV; 6, leukemic plasma (AMV).

the particles we observed with the electron microscope were infectious virus. Furthermore, the flocks of chickens from which the particle-containing embryos were derived, particularly the SPAFAS and University of Connecticut flocks, are free of lymphoid leukosis. Therefore, it would appear that the particles are not associated with infectious ALV or the disease. They are, nevertheless, morphologically identical with ALV.

Failure to detect infectivity associated with these particles may simply reflect inadequacy of present techniques. This situation may be analogous to that seen with "nonproducer" Rous sarcoma cells, where particles which were apparently noninfectious were found.[1, 4] In view of the work reported at this symposium by Vogt,[6] it is now evident that the particles produced by NP cells are infectious in the appropriate host, and that they are, indeed, RSV with a modified host range.

In ALV-infected tissues, free virus particles are seen more frequently than budding forms because the budding stage is brief in duration and free particles accumulate. Our observation that budding forms of the apparently noninfectious particles predominate over free particles suggests a defect in the mechanism of virus release.

Some embryos that contained noninfectious virus particles also contained an antigen that appeared identical to avian leukosis *gs* antigen, although there was no consistent correlation between the presence of antigen and particles. The sampling techniques of electron microscopy are not sufficiently sensitive to allow us to state that any particular embryo was free of particles. Therefore, it is possible that particles were present in antigen-positive embryos but were not detected with the electron microscope. The converse may also be true, in that it may be possible, by chance, to see a few particles in embryos where the amount of antigen is too low to detect. Therefore, we cannot at this time exclude the possibility that the two are related.

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