The purpose of this note is to emphasize and augment published descriptions of cytoplasmic inclusion bodies in mouse mammary tumors. First noted by Borrel in 1903, eosinophilic bodies were studied in some detail by Guérin (1955). Guérin found inclusions most often in mammary tumors developing after whole-body irradiation of 3- to 6-month-old female mice of low-tumor strain IC, and in some cases in spontaneous tumors. The present report deals with similar bodies observed in mammary tumors of C3H/Crgl mice following treatment with large amounts of cortisol acetate. Bernhard, Bauer, Guérin, and Oberling (1955) indicated that the inclusion bodies were microscopically visible aggregates of the virus-like particles seen with the electron microscope. Porter and Thompson (1948) first demonstrated such particles associated with mouse mammary tumors; since then they have been seen by a number of investigators (cf. Bernhard, 1958; Dmochowski, 1960). These particles are spherical double membranes averaging 700 Å in diameter (A particles), clustered within the apical cytoplasm. B particles, about 1100 Å in diameter and of a more complex structure, occur in lumina and occasionally intracellularly (Pitelka, Bern, DeOme, Schooley, and Wellings, 1958). They are found not only in mammary tumors, but also in large numbers in hyperplastic alveolar nodules, and infrequently in normal tissues. An abundance of these particles appears to be diagnostic of hyperplasias with preneoplastic significance (Pitelka, DeOme, and Bern, 1960).

METHODS

Samples of mammary tumors from multiparous female mice of the high-tumor strain C3H/Crgl were obtained both before and after daily injections of 0.5 mg. cortisol (Hydrocortone acetate, Merck, Sharp, and Dohme) for 14 days. Tissues for electron microscopy were fixed in Palade's buffered osmium tetroxide, methacrylate-embedded, and examined in an RCA-EMU3E microscope. Cytochemical studies were made on tissues preserved in Carnoy's or Bouin's fluids; in addition to routine histologic staining with hematoxylin and eosin, methods used were the Feulgen reaction, methyl-green pyronin, mercuric bromphenol blue, and the periodic acid-Schiff reaction (PAS), with appropriate enzyme digestion controls.

RESULTS AND DISCUSSION

Cortisol is known to cause atrophy of mouse mammary adenocarcinomas (Sparks, Daane, Hayashida, Cole, Lyons, and Li, 1955), and in our experience cortisol inhibits tumor growth and even delays the appearance of palpable tumors in our C3H mice. In the present study, atrophy of smaller tumors often followed treatment, and central necrosis was frequent. However, in non-necrotic regions of the cortisol-treated tumors, virus-like particles are frequently present in such large masses that inclusion bodies are visible in the light microscope. Fig. 1 is a high-power photomicrograph of a thick methacrylate section showing an acinus in which several cells have eosin-stained inclusion bodies in the apical cytoplasm. Fig. 2 is a low-power electron micrograph of a similar acinus in a thin section cut from the same methacrylate block. Aggregations of virus-like particles clearly correspond to the eosinophilic inclusion bodies. The inhomogeneous character of the inclusion bodies in Fig. 1 reflects the clustering of elementary particles about vesicles, as seen in Fig. 2. The distribution of inclusion-bearing cells varies considerably within areas showing a histology typical of C3H mammary tumors. The bodies seem to be more common in non-secreting acinar areas; however, they also are found in papillary and secreting regions.

In agreement with Guérin (1955), we found that the inclusion bodies display somewhat greater ribonuclease-sensitive pyronin staining than the surrounding cytoplasm, whereas DNA is not demonstrable by the Feulgen reaction. It is tempting to postulate that the RNA staining is due to the particles themselves, since reference to Fig. 6 shows that small granules resembling Palade's ribonucleoprotein particles abound in the cytoplasm but are absent from the immediate vicinity of the virus-like particles. The masses...
FIGURE 1
Photomicrograph of acinus from cortisol-treated tumor, fixed and embedded for electron microscopy, but sectioned at 2 μ, and stained with hematoxylin and eosin. Several dark inclusion bodies (arrows) are present, 3 of them indenting nuclei. The inclusion bodies have a vacuolated appearance. × 2,400.

FIGURE 2
Low power electron micrograph of a section cut from the same block as that in Fig. 1. The vesiculated masses of A particles (arrows) correspond to the inclusion bodies in Fig. 1. A few B particles appear in the lumen (the large open space in the center of the picture). × 8,200.

clearly contain protein, as shown by their intense staining with mercuric bromphenol blue (Fig. 4). The PAS reaction is faintly but variably positive (i.e., the inclusion bodies are sometimes slightly pinker than the cytoplasmic background). This reaction is not prevented by prior salivary amylase treatment, indicating that it is probably due to some mucopolysaccharide. Guérin, on the other hand, found the inclusion bodies to be PAS-negative.

It has not been possible to prove that the virus-like particles are identical with the viral milk factor associated with the development of mouse mammary cancer (Pitelka, DeOme, and Bern, 1960); however, the cytochemical picture fits well with what little is known about milk agent chemistry. Present indications are that the milk agent contains protein and RNA, but little if any DNA (Dmochowski, Grey, Pearson, Ward, Hurlbert, Griffin, and Bresson, 1959).

The same tumors biopsied prior to cortisol treatment showed some inclusion bodies, as would be expected in view of the relatively large aggregations of virus-like particles sporadically encountered in C3H mammary tumors generally. However, sections from 13 of 15 treated tumors could be distinguished easily from pre-treatment sections on the basis of the abundance of inclusion bodies (compare Figs. 3 and 4). The large inclusions found after cortisol treatment represent virus-like particles in far greater numbers than have been seen in untreated spontaneous mammary tumors, with the exception of tumors in DBA mice (Goldfeder, Gelber, and Moore, 1960).
FIGURE 3
Untreated tumor stained with mercuric bromphenol blue for proteins. Nuclei are not well shown by this method. × 800.

FIGURE 4
After 2 weeks of cortisol treatment, the same tumor as in Fig. 3 contains numerous inclusion bodies, which stain intensely with bromphenol blue. × 800.

The increase involves both A and B particles, as large acinar lumina have been found completely filled with B particles in some of the treated tumors (Fig. 5).

The virus-like particles in cortisol-treated tumors are morphologically identical to those seen previously in our laboratory in hyperplastic nodules and tumors of several Crgl strains: C3H, C3Hf, RIII, DBA, and A (Pitelka, Bern, DeOme, Schooley, and Wellings, 1958; Pitelka, DeOme, and Bern, 1960; unpublished data). A particles have the usual spherical shape (appearing flattened because of lateral compression during microtomy) (Fig. 6). The double nature of the A particle membrane is shown more clearly at higher magnification (Fig. 7). An irregular, diffuse background density is characteristic within the aggregates of particles. The significance of this matrix is not understood, although Goldfeder, Gelber, and Moore (1960) have suggested that it may be the source of A particles.

The normal appearance of other cell constituents (Fig. 6) leaves the impression that the only effect of cortisol on the structure of individual tumor cells is to increase particle concentration.

The mechanism of this apparently specific effect is unknown, but cortisol treatment is the first known method for experimental manipulation of particle concentration in established tumors. (Irradiation, as used by Guérin, 1955, induced the formation of tumors.) It is hoped that use can be made of this finding to elucidate the “life history” of the particles, their chemical nature, and their relationship to tumorigenesis.

SUMMARY
Following cortisol treatment of mammary-tumor-bearing C3H mice, cytoplasmic inclusion bodies frequently are visible in the tumor cells. Histochmical studies indicate that these inclusion bodies contain protein, RNA, and probably mucopolysaccharide, but no demonstrable DNA. Electron micrographs reveal that the inclusions are composed of large aggregations of the virus-like particles usually found in preneoplastic and neoplastic mouse mammary tissues.

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Figure 5
Electron micrograph of part of an alveolar area of a tumor from a cortisol-treated animal. A branching lumen (L) is entirely filled with a granular mass composed of B particles. An inclusion body is seen in the adjacent cytoplasm (arrow). X 11,000.

Figure 6
Parts of 2 acinar cells from cortisol-treated tumor, one containing an aggregate of A particles. Particles are seen mostly lined up around various-sized vesicles, some of which appear nearly empty. One vacuole near the bottom, however, contains a number of B particles (BP) identical to those seen in the lumen. Note normal-appearing mitochondria (M), endoplasmic reticulum (ER), Golgi (G), RNP particles. X 31,600.

Figure 7
High magnification electron micrograph of portion of particle mass. A particles clearly have a double membrane. Their diameter averages 700 A; the thickness of the double wall 140 A. Note the diffuse background density associated with the groups of particles, and the lack of RNP granules within the particle aggregation. Edge of nucleus (N) at bottom right; mitochondrion (M) at upper left. X 89,000.
particles in hyperplastic alveolar nodules of the mammary gland of the C3H/He CRGL mouse, 