Correlation of Physical and Biological Properties of Mouse Mammary Tumor Agent*

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PLATES 34 TO 37

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ABSTRACT

Biophysical procedures have been used to determine the size and structure of the biologically active agent responsible for the transmission, through milk, of mouse mammary adenocarcinoma. Filtration of milk from RIII high-breast-cancer mice through gradocol membranes with decreasing pore sizes indicated that a minimum of activity passed through intermediate pore sizes (100 to 160 mμ). Filtrates through smaller pores were significantly active. Milk treated with small doses of deuteron irradiation produced more tumors than the control, unirradiated milk; larger doses indicated a particle size much less than 100 mμ. Free diffusion experiments indicated that the activity was associated with particles of two different sizes. Altogether the data denoted the presence of a large agent about 100 mμ in diameter and a small agent 20 to 30 mμ in diameter or possibly smaller. Furthermore, the presence in the milk of an inhibitor 40 to 60 mμ is indicated by the results of all three approaches. The complex nature of the milk agent disclosed by the physical measurements agrees with the picture of one of the structures revealed by electron microscopy as well as with seemingly conflicting measurements reported in the literature. The large agent defined by these indirect methods corresponds to the whole particle seen in the electron microscope and the small agent corresponds to its internal core or nucleoid. It is suggested that the nucleoid is essentially a nucleic acid which may, in the absence of the "inhibitor," retain its activity after being stripped of its outer membrane or sac.

The establishment of genetically homozygous strains of mice with high and with low incidence of mammary tumors has permitted extensive biological studies of mammary adenocarcinoma by reciprocal cross-breeding and foster nursing (1–3). Since Bittner (3) demonstrated that an extrachromosomal carcinogenic factor was conveyed through the milk of high-cancer-strain females many attempts have been made to characterize or to isolate the milk agent. Passey *et al.* (4) prepared extracts from malignant and normal tissues from mice of the C3H, Strong A, and RIII high-cancer strains by drying the tissue, treating with petroleum ether, extracting with distilled water, then treating the extract with trypsin for 30 minutes at 37°, and filtering through Berkefeld N candles. Dispersions of these preparations examined in the electron microscope indicated a predominance of particles 15 to 35 mμ in diameter which were active in assay mice, whereas extracts similarly prepared from tissues of low cancer strains or from chemically induced tumors contained neither the characteristic particles nor did they produce tumors in assay mice. By ultracentrifugation of preparations from high-cancer strains they reported that supernatants could be obtained which were free of the 15 to 35 mμ particles and were concomitantly devoid of activity. These experiments indicated a

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high degree of correlation between the presence of characteristic particles revealed by electron microscopy in milk extracts and the latter's ability to induce tumors.

While these experiments were being carried on in Leeds, Porter and Thompson (5) reported the presence of 130 mμ bodies associated with epithelial cells cultured from the high-cancer strain, C3H tumors. These bodies could be seen in great abundance in the whole mounts of thin epithelial cells which were examined in the electron microscope without embedding and sectioning. Close examination of these bodies indicated that they were complex in structure consisting of a dense central portion approximately 75 mμ in diameter, surrounded by a less dense zone. One year later Graff *et al.* (6) reported on the isolation of the mouse mammary carcinoma virus from RIII milk. In this report the purified agent was found to form two sedimenting boundaries in the analytical ultracentrifuge, one sharp with a sediment constant of 900S, and the other diffuse with a sediment constant of 500 to 700S. Dispersions of these preparations contained particles from under 50 to over 100 mμ in diameter, the predominant size being about 100 mμ. The preparations were thought to contain nucleic acid because their absorption curves showed a hump at 2600 A. No similar substance was obtained from milk of C57 agent-free mice except when they were foster-nursed on RIII agent-bearing mothers.

After it became possible to section tissue suitably for electron microscopy, mammary tumors from agent-bearing mice were found to contain a variety of virus-like particles (7–12). One variety, measuring about 65 mμ in diameter, consisted of a dense shell with a less dense homogeneous interior and was found mostly in the Golgi region of the cytoplasm of cells (8, 10). Another variety consisted of a well defined thin-walled spherical sac with an average diameter of about 100 mμ which contained an inner very dense body, centered or excentric, measuring 20 to 35 mμ (7–12). These were found in the intercellular spaces and pictorial evidence suggested that they were formed by a budding process at the cell membrane.

The experiments and observations reported in this paper have involved the use of different procedures for correlating activity with particle size and form, and were made in an effort to determine which, if any, of the several particles observed possess the ability to induce mammary cancer and is indeed the milk agent.

Materials and Methods

Mouse Colonies.—Milk from the Columbia University Colony of RIII mice was used as the source of the milk agent. This colony was started in 1929 from mice kindly provided by Dr. A. Lacassagne, and is locally designated as the Paris strain. Three to five thousand mice are maintained in this colony and the incidence of mammary adenocarcinoma is 97 per cent in the females.

All assays were performed in C57 black mice which were originally provided by E. C. McDowell of Cold Spring Harbor, New York, and which have been maintained at Columbia since 1929. This colony of approximately 4000 animals has been completely free of mammary carcinoma since 1947. The environment of the colonies is meticulously controlled, and pedigrees of the mice in each colony are carefully maintained. The milk preparations were injected intraperitoneally (0.1 ml.) into 12 to 15 day-old females.

Preparation of Milk.—Five ml. samples of RIII milk, expressed by means of a special breast pump into ice-packed centrifuge tubes were centrifuged at 1500 R.P.M. in an International Equipment Company, Boston, trunnion cup head for 1 hour while under refrigeration. The milk was removed with a syringe and long needle, leaving the cream and a small pellet in the centrifuge tube. Care was taken always to keep the milk cold. After filtering, irradiating, or allowing to diffuse as described below, the samples were diluted in Tyrode's solution which contained 2 per cent serum from C57 mice, 1000 units/ml. penicillin G and a trace of phenol red. The serum was added to coat the glassware used and thus prevent the loss of the milk agent by its adherence to the glass at the higher dilutions. The following dilutions were used: 10^{-2}, 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-9}. Tumors were occasionally obtained at all dilutions with many more at the lower dilutions. However, for convenient presentation in the tables, the data have been pooled for all dilutions in each experiment.

Electron microscopy on sections of cultured tumors is included in this report. The procedure is described in the succeeding article.

RESULTS

Filtration of Milk through Gradocol Membranes.—Gradocol membranes, made according to Elford's (13, 14) specifications and calibrated by the manufacturer were obtained from Gallenkamp, Ltd., London. The filtration process was hastened either by evacuating the receiving chamber or by applying 15 pounds of pressure from a nitrogen tank to the reservoir. The results recorded in Table I indicate that pores of intermediate size prevent the passage of activity more effectively than did smaller pores. Elford found that in this size range,

10 to 200 mμ, the pores were about twice as large as the particles passing them.

Ionizing Irradiation.—The determination of size of active areas by this method involves probability considerations, the theory and application of which have already been amply documented (15). In our experiments 4 mev. deuterons in the cyclotron were employed to bombard samples of frozen-dried RIII milk. 0.05 ml. of milk from which the cream had been removed was placed on each of 12 glass coverslips which were affixed to a 6 inch diameter brass disc later to go into the cyclotron. The disc, precooled with dry ice, was then evacuated for 2 to 3 hours while remaining in an ice chest or sometimes for ½ hour at room temperature before transferring to the ice chest. The disc was quickly transferred from the vacuum desiccator to the high vacuum of the cyclotron where the temperature was from 30 to 35°C. Nine of the glass coverslips holding the dried milk were then irradiated, one at a time. Three served as controls. Three different doses of irradiation were used, groups of 3 coverslips being given the same dose. The discs were then carried from New Haven to New York where they were kept cold until the following day,

TABLE I

Filtration through Gradocol Membranes

	No. mice inoculated	Tumor incidence
		per cent
Whole milk—unfiltered	42	53
Passing membranes with 780 mμ pores	78	58
" " " 160 " "	46	4
" " " 100 " "	44	0
" " " 38 " "	45	11
" " " 7 " "	36	5
Residue held on membrane	12	50

TABLE II

Deuteron Irradiation

Irradiation	Minimum size destroyed	Assay	
		No. mice inoculated	Tumors
*beam-sec.**	*mμ*		*per cent*
180	30	67	1.5
14	100	65	18.5
4	200	46	15.2
0	Infinite	47	0

* 1 beam-sec. = 10^{10} deuterons/cm.^{2}.

TABLE III

Deuteron Irradiation of Versene-Treated Milk

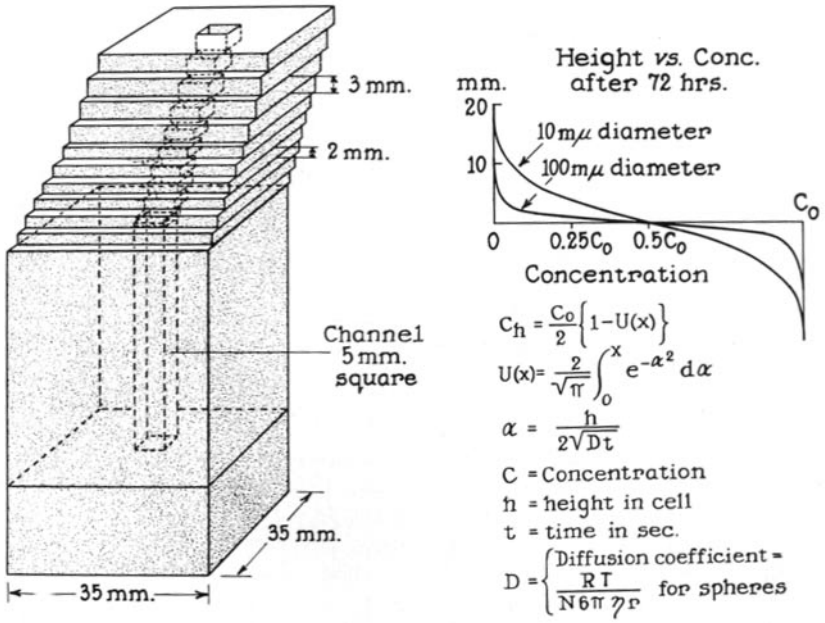
Irradiation	Minimum size destroyed	Assay	
		No. mice inoculated	Tumors
beam-sec.	*mμ*		*per cent*
180	30	48	0
14	100	38	21.0
4	200	42	21.4
0	Infinite	40	22.5
Wet* c versene		43	11.6
Wet* s "		34	14.7

* Control milk, not frozen-dried.

when they were dissolved in the diluting phosphate buffered saline (pH 7.4) and inoculated into the 12 to 15 day-old female C57 mice. The results of these assays are recorded in Table II along with the calculated doses of irradiation sufficient to destroy 99.9 per cent of the particles of different cross-sectional areas. The number of tumors obtained in our C57 test mice is not very large, but these assays are to be evaluated against the knowledge that in 11 years with a colony of more than 4000 mice there has not been a single spontaneous tumor. There is no significant difference in the assay data for the irradiation calculated to inactivate the 100 and the 200 mμ particles and there was one tumor even for the high radiation dose calculated to inactivate 30 mμ particles. In these experiments the control samples that went into the cyclotron but received no irradiation produced no tumors. The reason for this is not understood.

Chelation.—In working with RIII milk a few years ago, Dr. Otto Plescia found that defatted milk could be clarified by adding a trace of the chelating agent, versene.[1] Dispersions of untreated milk (cream removed) yield the type of electron micrograph illustrated in Fig. 1. There are numerous spherical particles having a wide range of sizes. After adding 5 per cent of a 0.15 M versene solution to the milk, no particles larger than 5 to 10 mμ can be found in the usual dispersion, as is illustrated in Fig. 2. However, the versene-treated milk remains active in producing tumors, and particles varying in size from 20 to 100 mμ can be seen in dispersions after the milk is concentrated by ultracentrifugation (Fig. 3). Chelated milk was subjected to the cyclotron experiments with re-

[1] Trisodium *N*-hydroxyethylethylenediaminetriacetic acid.



TEXT-FIG. 1. Diagram of diffusion cell with many displaceable sections for isolating samples. Concentration curves for spherical electrically neutral particles of two different sizes after diffusing for 72 hours at 1°C. are shown at right along with the formulae for calculating them.

R, the gas constant, 8.3×10^7 ergs per degree per mole

T, absolute temperature

N, Aragadro's constant, 6.02×10^{23}

η, coefficient of viscosity in poises

r, the radius of the particle in centimeters

sults which indicate no change in size, but the total frequency of tumors is greater, as is indicated by the data of Table III. The tumor incidence was lower, however, for the control milk that was not dried.

Diffusion.—The other procedure that we have used in determining particles size is that of free diffusion. If the agent-bearing milk is placed in the bottom part of a cell and overlaid with a buffer of suitable pH and ionic strength, thermal activity will cause the molecules and particles of the underlying solution to diffuse up into the overlying solution. The rate of mixing is a function of the size and shape of particles. For these experiments a special cell, illustrated in Text-fig. 1, with many sliding ground glass plates, 2 and 3 mm. thick, was constructed.[2] The channel of the bottom section plus that of a single plate was filled with defatted milk diluted with 2 parts of buffer pH 7.4. The plate, sealed with silicone grease, was then displaced thus sealing the milk in the bottom. The excess milk was removed from the channel of the covering plate and the channel was thorougly washed and rinsed. Six more plates were then added and filled with buffer. The assembly was placed in a thermostatic bath at 1°C. and after being allowed to reach thermal equilibrium the plates were aligned with the bottom part of the cell. The initial very sharp boundary was allowed to diffuse for 72 hours, whereupon the channel was segmented into many small sections by displacing consecutive plates in opposite directions. It is possible to calculate theoretically the concentration of particles in the various sections of the cell for spheres of different diameters (16). Curves for particles of two different sizes are plotted in the graph of Text-fig. 1. At 6 mm. above the boundary the concentration of 100 mμ spheres in 72 hours should be 4×10^{-4} the original concentration, C_o, whereas at 11 mm. the concentration should be $3 \times 10^{-10} C_o$. For spheres of 10 mμ a second curve is obtained. At 11 mm. the concentration should be 0.024 C_o. The results of the assays in mice are shown in Table IV. The

[2] We are grateful to Mr. Emil Maier of the Pyrocell Manufacturing Company, New York, for the construction of this special diffusion cell.

highest tumor incidence is found in the first section, but there is some activity in sections 6 to 8 mm. above the boundary. This same experiment was repeated with milk clarified with versene, the results being recorded in Table V. Since all samples taken from the diffusion cell were injected into the mice at dilutions of 10^{-3} or greater, the concentrations become extremely low for 100 mμ particles. Clearly there is no correlation between the theoretical concentrations and the number of carcinomas obtained; but since with the versene-treated milk tumors are produced by the contents of sections above the location expected for 100 mμ particles, activity must be associated with smaller particles, as was also indicated by filtration and irradiation. The highest carcinoma-producing dilution obtained for RIII milk has been 10^{-10}. Therefore, the milk agent cannot consist only of particles as large as 100 mμ.

TABLE IV

Diffusion of Activity in RIII Milk after 72 Hours at 1.0°C.

Distance above boundary	Calculated concentration* for assumed sizes		Assays	
	100 mμ (Diff. coefficient 6×10^{-8})	10 mμ (Diff. coefficient 6×10^{-7}	No. mice inoculated	Tumors
mμ				*per cent*
11–14	3×10^{-10} C_o	0.024 C_o	21	0
6–8	4×10^{-4} "	0.18 "	22	9
4–6	1×10^{-2} "	0.25 "	28	0
2–4	0.13 "	0.36 "	32	3.1
0–2	0.5 "	0.5 "	36	16.6
Below boundary	1.0 "	1.0 "	28	10.7

* The small samples removed from the cell were diluted 10^{-3} before assay, making the total dilutions greater than the values given.

TABLE V

Diffusion of Activity in Versene-Treated RIII Milk after 72 Hours at 1.0°C.

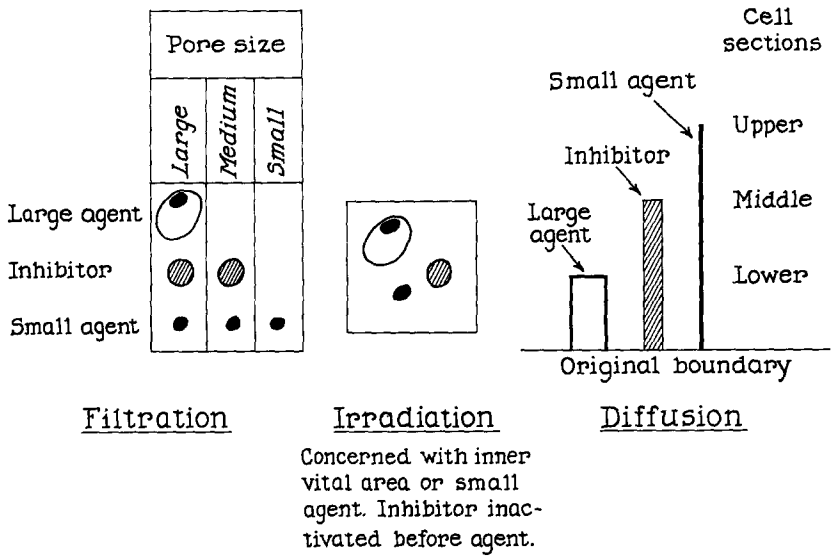
Distance above boundary	Calculated concentration* for assumed sizes		Assays	
	100 mμ (Diff. coefficient 6×10^{-8})	10 mμ (Diff. coefficient 6×10^{-7})	No. mice inoculated	Tumors
mm.				*per cent*
12–16	5×10^{-11} C_o	0.017 C_o	17	5.9
9–12	3×10^{-7} "	0.056 "	26	3.8
6–9	4×10^{-4} "	0.18 "	31	6.5
3–6	4×10^{-2} "	0.29 "	31	0
0–3	0.5 "	0.5 "	20	10.0
Below boundary	1.0 "	1.0 "	24	29.2

* The small samples removed from the cell were diluted 10^{-3} before assay, making the total dilutions greater than the values given.

DISCUSSION

The results of the several experiments described above do not lead to an easy description of the milk agent. As has always been the case in the many attempts to characterize this agent, the results are sometimes erratic and inconsistent. It has been well established, however, that the milk agent is not the only influence entering into the development of the mammary carcinomas, but that other factors such as heredity and hormones are also determinants (17). Still other entities, as yet obscure, may enter into the process as well. In making the assays we are controlling only one variable; *i.e.*, the amount of milk inoculated. We are able to control the amount of ionizing irradiation used or the size of pores in the filter membranes, but we do not know all the effects that irradiation or filtration have on the milk. Nor do we have knowledge of the hormonal status of the animal or the intricacies of its genetics. At this point not all of the inconsistencies can be unequivocally clarified. We believe, however, that some of them can be explained by assuming that the agent is complex in nature, existing in two different sizes, and that there is an inhibitor capable of destroying one form of the agent.

Inhibitor in Milk.—In both the diffusion experiments, whether versene was or was not used, there was a location in the column 3 to 6 mm. above the original boundary where the activity fell to zero and then reappeared at a higher level. Again, in the filtration experiments the material passing through 100 mμ pores showed no activity whereas that passing smaller pores did. Furthermore, in the deuteron irradiation experiments unirradiated milk produced fewer tumors than did samples receiving intermediate doses of irradiation. Such results might be explained by assuming the presence of an inhibitor which diffuses, filters, and responds to irradiation at a different rate from the tumor-producing agents. Indeed, if it is assumed that the activity is asso-



TEXT-FIG. 2. Schematic diagrams illustrating the effects of filtration, ionizing irradiation, and diffusion on the three assumed active particles in the milk of high-breast-cancer, RIII strain mice. The first diagram indicates the particles passing through membranes with different average pore diameters, and the third indicates roughly the maximum relative height to which the different sized particles rise after diffusing for a period of time from the originally sharp boundary.

TABLE VI

Activity of RIII Milk as Influenced by Treatment

Treatment	No. mice inoculated	Tumors
		per cent
Untreated	17	23.5
Chymotrypsin (10 min.)	75	49.5
Chymotrypsin (2 hrs.)	28	46.4
From reference 6		
Untreated	382	6.3
Versene-treated	289	12.8

From all deuteron irradiation experiments including Tables II and III

ciated with particles of two distinct sizes and that there is an "inhibitor" having an intermediate size which reacts with *only* the smaller of the milk-agent particles, the data can, in general, be explained (see Text-fig. 2).

In filtration, for instance, membranes with large pores allow all three particle sizes to pass. Membranes with intermediate sized pores (100 mμ) retain only the larger milk agent, passing the "inhibitor" and the smaller agent, which is then unable to produce tumors. Small pores, however, retain the "inhibitor" leaving the small agent undestroyed.

Similarly, in free diffusion the larger agent is confined to the lower sections of the diffusion cell; whereas a little higher there is only the small agent and "inhibitor" which give zero activity; slightly higher still, the small agent alone is found with a return of activity.

In the deuteron irradiation experiments the active area as determined by the irradiation would be the same for both the large and the small agent, because the activity may be associated with only the core of the larger particle. Here we find the "inhibitor" being destroyed by small doses of irradiation with the accompanying increase in tumor incidence, whereas a large dose of irradiation destroys also the agent. The sizes of these assumed particles would seem to be as follows: large agent, 90 to 120 mμ; small agent, 15 to 35 mμ; and the inhibitor, 40 to 60 mμ. Treatment with chymotrypsin and with versene seems to stimulate the tumor-inducing activities of milk, as is demonstrated in Table VI. Drying in the presence of versene seems also to increase the activity of the agent as is demonstrated in Table III. The samples that went into the cyclotron produced more carcinomas than the controls that were not dried.

The nature of the "inhibitor" is not known, but its existence was suggested as early as 1944 because extracting with petroleum ether seemed to increase the activity of the milk agent (27). It is possible that the "inhibitor" is either an enzyme or an antibody. In order to fit the filtration, irradiation, and diffusion data the size would have to be unusually large, but both of these functions are known to be sometimes associated with large molecular complexes.

Structure of Milk Agent.—The hypothesis that the mammary tumor agent exists in two different sizes is supported by previous data, as observed above, and the actual appearance of the milk agent is believed to be represented in the electron micrograph of Figs. 4 to 7. The characteristic structure seen here consists of a dense body, a nucleoid (18), 20 to 35 mμ in diameter contained within a membranous sac 90 to 120 mμ (occasionally larger) in diameter. The membrane forming the sac appears to be of the same structure and density as the cell membrane. It is 8 to 10 mμ thick. Aside from the nucleoid the sac does not contain any other structure; usually the nucleoid is near or in contact with the sac. Occasionally a sac contains more than one nucleoid (Fig. 5). The membrane is designated as a sac because there is considerable variation in its size and shape and it does not seem to be rigid enough to be designated as a shell or capsule. In looking at cultured epithelial sheets of C3H tumors placed directly in the electron microscope without embedding and sectioning, Porter and Thompson (5) observed the presence of the nucleoid and reported that "in gold-shadowed preparations it is possible in some cases to note that the central dense portion protrudes above a flattened border," indicating that the dehydrated sac had collapsed over the more solid nucleoid. The size range of the nucleoid reported by Porter and Thompson was necessarily larger than that seen in the profiles obtained from sections (Figs. 4 to 7) because their measurements included also the folded sac membranes. Their measurements ranged from 50 to 95 mμ for the internal body and from 90 to 200 mμ for the over-all particle, the latter also being larger due to flattening, because their specimens were not embedded and the sacs collapsed and spread over a greater area upon drying.

Passey and his collaborators (4) were probably dealing with only the nucleoid of this particle. They used petroleum ether to extract frozen-dried tissues before extracting the milk agent with distilled water. Furthermore, they usually incubated the water extract with trypsin. They state (reference 2 page 402) that "the preponderance of particles no larger than about 30 mμ in the extracts of high-breast-cancer-strain tissues and their comparative absence from extracts of low-breast-cancer-strain tissues indicates that such particles are a characteristic feature of high-breast-cancer-tissues." It is possible that the ether and trypsin treatment destroyed the sac and that the centrifugation and filtration yielded a fairly pure preparation of nucleoids.

Graff *et al.* (6), using milk as the source of the agent, extracting not with ether but by treating with chymotrypsin, and purifying by means of differential centrifugation, obtained a preparation which was predominantly the size of whole particles. Similar preparations of milk from the C57 noncancerous mice did not provide any particles at all when examined in the analytical ultracentrifuge or the electron microscope.

In spite of the fact that not much reliance can be placed on these early experiments, as was pointed out by Howatson (19), they at least do not conflict with the present hypothesis nor in light of it, with each other. Much more experimental work needs to be done, however, to clarify results obtained by different preparative procedures.

The Nature of the Nucleoid.—The recent experiments of Schramm (20), Fraenkel-Conrat (21), Colter (22), Alexander (23), and others have shown that nucleic acids from viruses are able to carry the activity of the virus. Latarjet (24) has presented evidence that nucleic acid from leukemic tissues of Ak mice inoculated into newborn isologous animals accelerated the appearance of leukemia and in some cases caused a variety of malignant tumors which never existed in the donors of the extract. Epstein (25) has demonstrated that under certain conditions the central bodies of Rous sarcoma virus particles can be destroyed by incubating with ribonuclease. Pollard (15) has shown that the active elements of a number of viruses are contained within a much larger shell. By analogy the nucleoids described are believed to represent the active nucleic acid or nucleoprotein of the milk agent.

All of our experiments indicate that most of the activity is associated with large particles, but in almost every experiment activity is found to be associated also with small particles. In working

with influenza virus Bourdillon (26) reported that its infectivity was associated with two distinct diffusion rates. One represented a particle quite small, probably about 6 mμ; the other a large particle over 100 mμ in diameter. The small particles were found to be labile, whereas the large ones were more stabile. Similarly, the naked nucleic acids of TMV (20, 21), neurotropic viruses (22), and polio (23) virus has been found to be more labile than the whole particle. In our experiments the small milk agent seems to be vulnerable to certain components of the milk (the "inhibitor"), whereas the large agent with outer membrane is more stabile.

Bibliography

1. Staff of the Roscoe B. Jackson Memorial Laboratory, *Science*, 1933, **78,** 465.
2. Murray, W. S., and Little, C. C., *Am. J. Cancer*, 1936, **27,** 516.
3. Bittner, J. J., *Science*, 1936, **84,** 162.
4. Passey, R. D., Dmochowski, L., Reed, R., and Astbury, W. T., *Biochim. et Biophysica Acta*, 1950, **4,** 391.
5. Porter, Keith R., and Thompson, H. P., *J. Exp. Med.*, 1948, **88,** 15.
6. Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., and Haagensen, C. D., *Cancer*, 1949, **2,** 755.
7. Kinosita, R., Erickson, J. O., Armen, D. M., Dolch, M. E., and Ward, J. P., *Exp. Cell Research*, 1953, **4,** 353.
8. Dmochowski, L., Haagensen, C. D., and Moore, D. H., *Acta Internat. Contra Cancrum*, 1955, **11,** 640.
9. Dmochowski, L., and Moore, D. H., *J. Nat. Cancer Inst.*, 1954, **15,** 785.
10. Bernhard, W., Bauer, A., Guerin, M., and Oberling, C., *Bull. Assoc. franç. étude cancer*, 1955, **42,** 163.
11. Bang, F. B., Vellisto, I., and Libert, R., *Bull. Johns Hopkins Hosp.*, 1956, **98,** 255. Bang, F. B., Andervont, H. B., and Vellisto, I., *Bull. Johns Hopkins Hosp.*, 1956, **98,** 287.
12. Pitelka, D. R., Bern, H. A., DeOme, K. B., Schooley, C. N., and Wellings, S. R., *J. Nat. Cancer Inst.*, 1958, **20,** 541.
13. Barnard, E. J., and Elford, W. J., *Proc. Roy. Soc. London, Series B*, 1932, **109,** 360.
14. Elford, W. J., *Proc. Roy. Soc. London, Series B*, 1933, **112,** 384.
15. Pollard, E. C., The Physics of Viruses, New York, Academic Press, Inc., 1953.
16. Tables of the Error Function, and Its Derivative, 2nd edition, National Bureau of Standards, Applied Mathematics Series 41, Washington, D. C., 1954.
17. Bittner, J. J., *Ann. New York Acad. Sc.*, 1958, **71,** 943.
18. Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H., *J. Exp. Med.*, 1954, **100,** 301.
19. Howatson, A. F., *Brit. J. Cancer*, 1953, **7,** 393.
20. Gierer, A., and Schramm, G., *Nature*, 1956, **177,** 702.
21. Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, 1956, **78,** 882.
22. Colter, John S., Bird, H. H., and Brown, R. A., *Nature*, 1957, **179,** 859. Colter, John S., Bird, H. H., Moyer, A. W., and Brown, R. A., *Virology*, 1957, **4,** 522.
23. Alexander, H. E., Koch, G., Mountain, I. M., Sprunt, K., and Van Damme, O., *Virology*, 1958, **5,** 172. Alexander, H. E., Koch, G., Mountain, I. M., and Van Damme, O., *J. Exp. Med.*, 1958, **108,** 493.
24. Latarjet, R., 7th International Cancer Congress, London, July, 1958, Abstracts of Papers, 138.
25. Epstein, M. A., 7th International Cancer Congress, London, July, 1958, Abstracts of Papers, 109. Epstein, M. A., *Nature*, 1958, **181,** 1808.
26. Bourdillon, J., *J. Gen. Physiol.*, 1941, **25,** 263.
27. Barnum, C. P., Ball, Z. B., Bittner, J. J., and Visscher, M. B., *Science*, 1944, **100,** 575.

Explanation of Plates

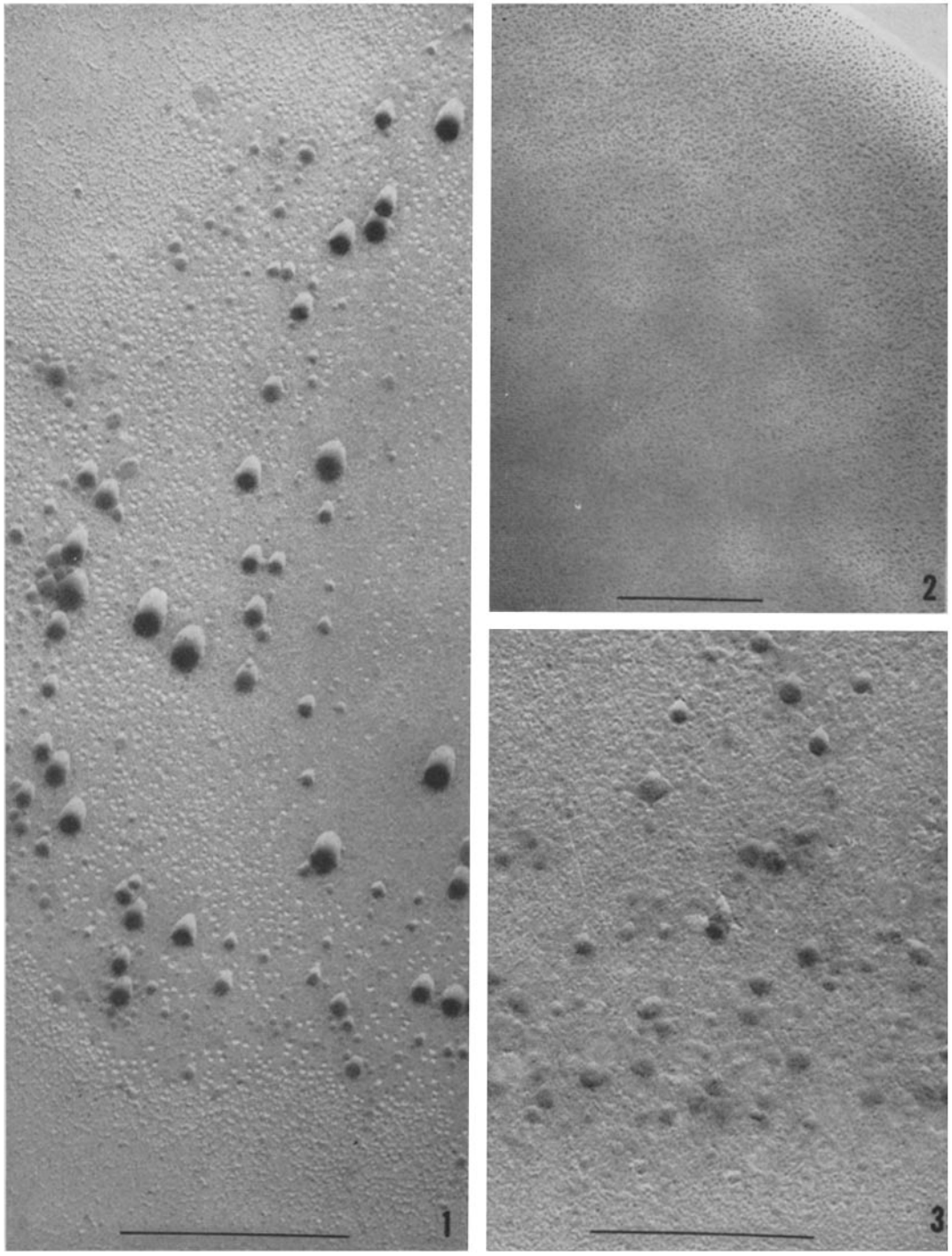
Plate 34

Microdroplets of milk were dispersed with a nebulizer before shadowing with chromium.

Fig. 1. Mouse milk (RIII). The cream was removed by low speed centrifugation. × 34,000.

Fig. 2. Same as Fig. 1 except that 0.05 ml. of a 0.15 M versene solution was added to 1 ml. of the milk. × 21,000.

Fig. 3. Sample from bottom of centrifuge tube after centrifuging the versene-treated milk for 1 hour at 40,000 R.P.M. (approximately 100,000 *g*). × 34,000.

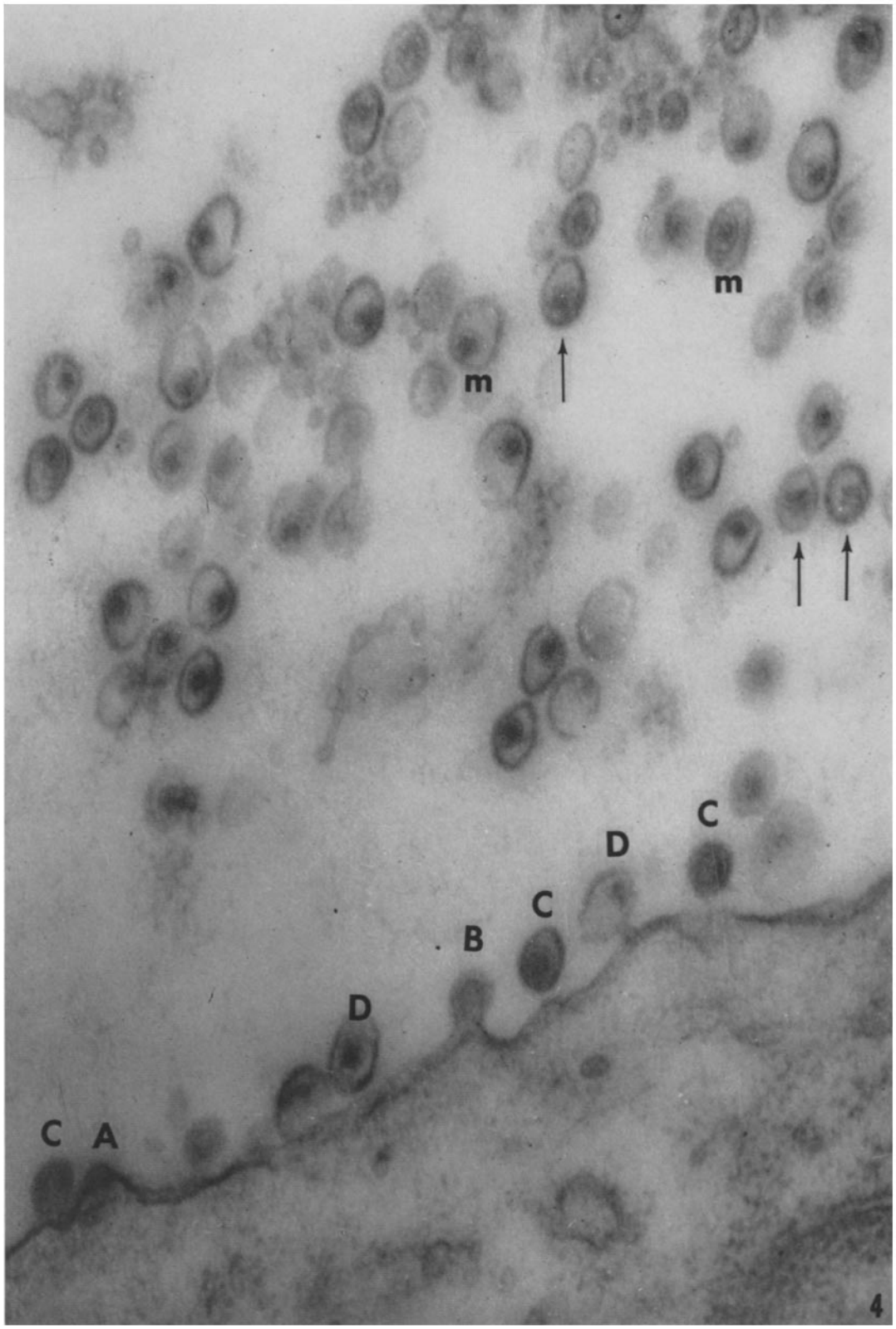


(Moore *et al.*: Mouse mammary tumor agent)

PLATE 35

For details of electron microscopy see the following article.

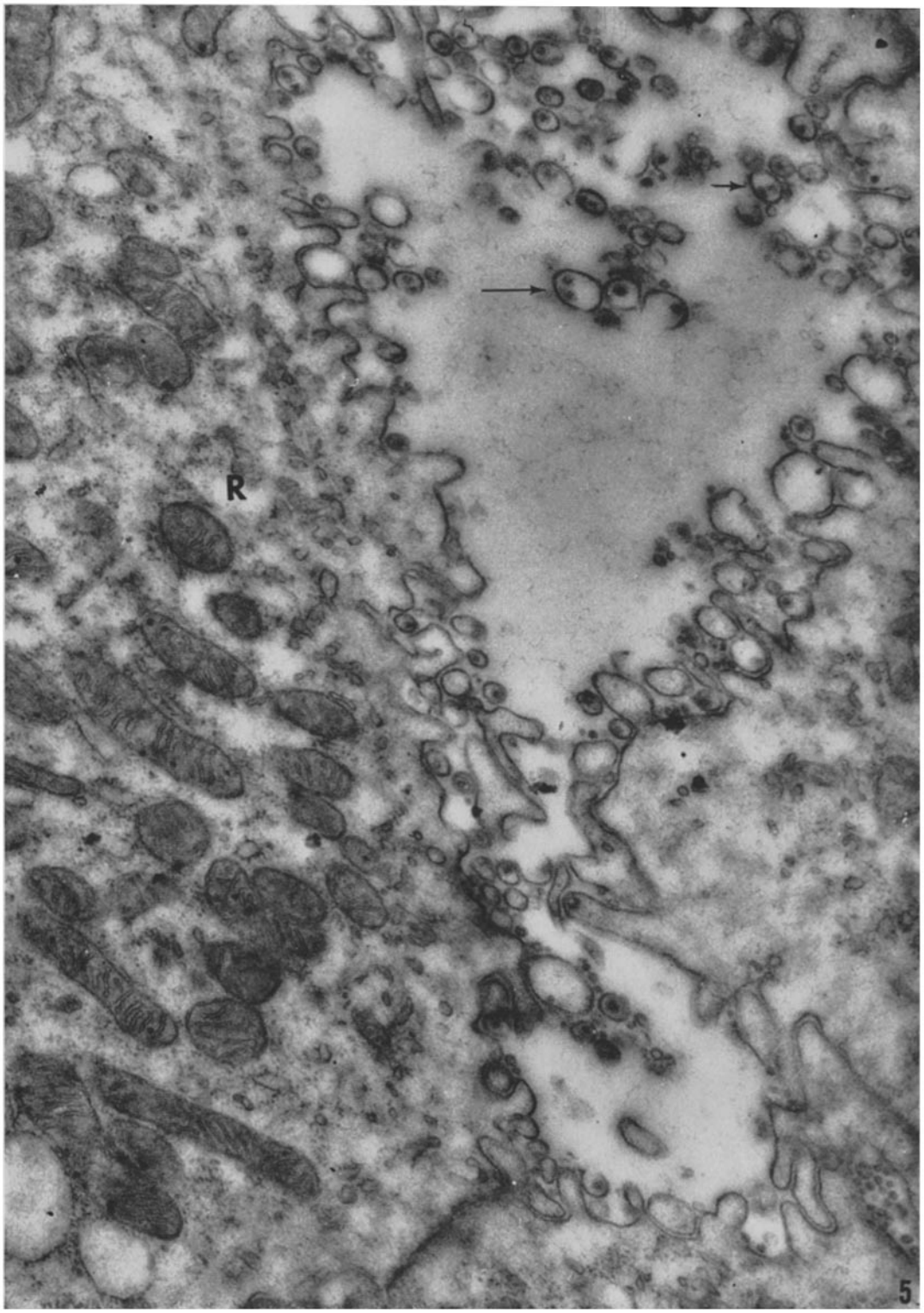
FIG. 4. RIII tumor grown in tissue culture for 14 days. Many particles consisting of sac and nucleoid are seen near the cell membrane and in the wide intercellular space. They appear to be irregular elipsoids with their major axes all pointing the same direction. This indicates that they were spherical but have been deformed by the microtome knife. Their outer diameters average 95 to 100 mμ, the membranes being about 10 mμ thick. The nucleoids are difficult to measure. They are usually most dense at their centers and fade away at the periphery, although a few reveal a granular nature (arrows). They also show variation in size, and some, *M*, seem to have exceedingly delicate membranes. Their mean diameters would seem to be about 25 to 30 mμ. Interdispersed with viral particles are also many smaller bodies which have a lesser density than the nucleoids. The viral bodies seem to be formed at the cell membrane by a budding process. At the lower left the cell membrane is particularly thick and dense. The presumed initial stage of particle formation is represented at *A*, and a later stage is seen at *B*. Particles *C* and *D* appear to have just been released. (See text of following article.) × 90,000.



(Moore *et al.*: Mouse mammary tumor agent)

PLATE 36

FIG. 5. Another section from the same tissue as in Fig. 4 showing a small intercellular lumen with many microvilli and characteristic virus particles. Here there is a greater variation in particle size with some of the particles containing double nucleoids (arrows). Many RNP granules can be seen in cytoplasm. These granules are 10 to 15 mμ in size and many, as at *R*, are not associated with the endoplasmic reticulum.

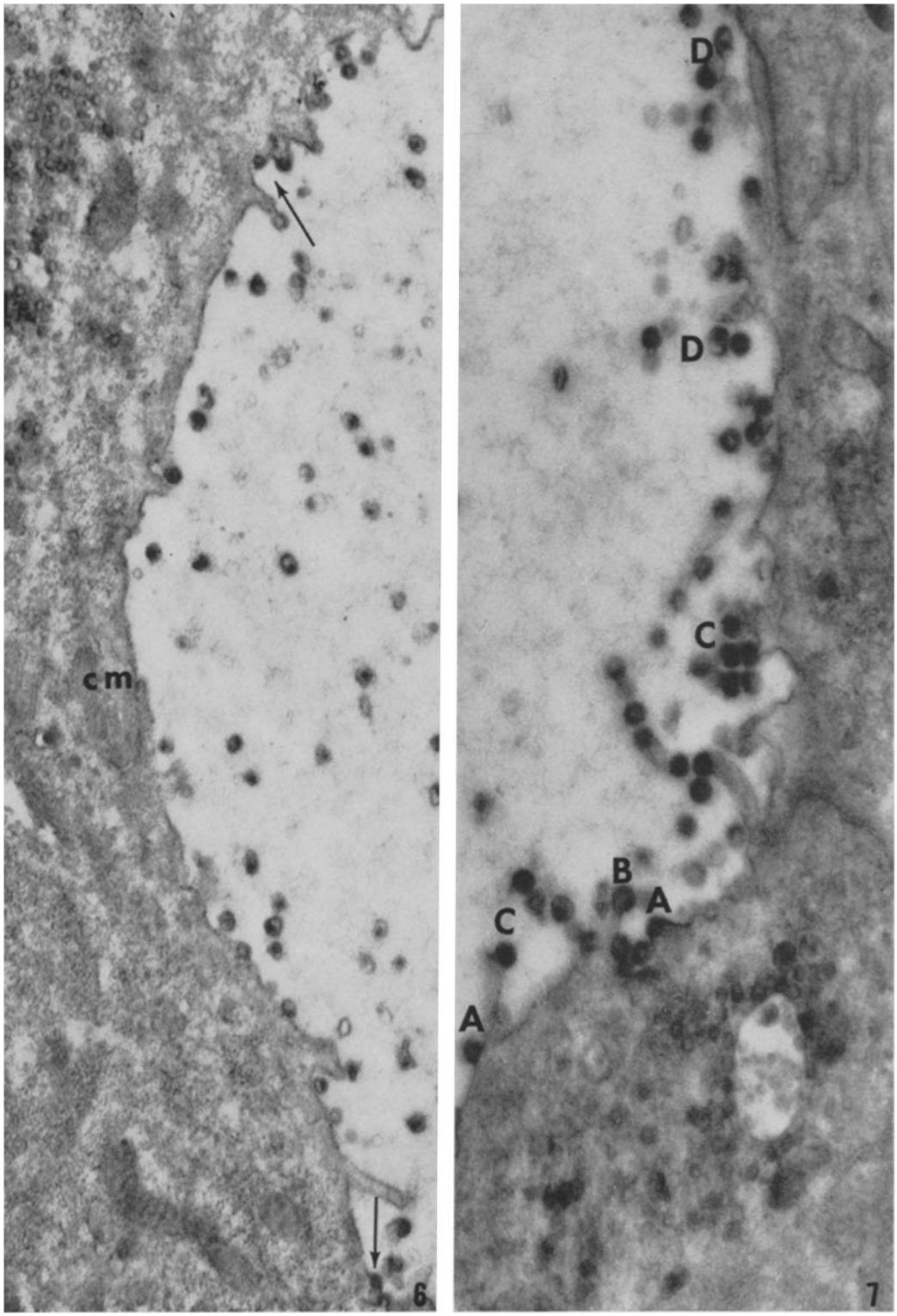


(Moore *et al.*: Mouse mammary tumor agent)

PLATE 37

FIG. 6. Ten-day culture showing portion of a large intercellular space completely surrounded by degenerating cells whose cytoplasms are granular. Neither mitochondria nor nuclei are well defined. Interdigitating cell membranes can only faintly be seen at *cm*. A few partially formed viral particles are evident (arrows). Numerous small vesicles, 65 to 70 mμ, are apparent at upper left. × 30,000.

FIG. 7. Micrograph of section from the same preparation as Fig. 6, including parts of three cells. These cells are not so necrotic as those of Fig. 6, and the formation of many particles is evident. The bodies which appear to be in the cytoplasm are actually on the surface of the cell because the surface is almost parallel with the plane of section. Similarly many of those that appear to be in the intercellular space are on irregularities of the cell surface just caught in the section. This, therefore, depicts an unusual display of viral synthesis. Assumed stages in development are marked alphabetically, *A* to *D*. × 43,000.



(Moore *et al.*: Mouse mammary tumor agent)