BIOPHYSICAL STUDIES OF EXTRACTS OF TISSUES OF HIGH- AND LOW-BREAST-CANCER-STRAIN MICE

by

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Historically, J. A. MURRAY[1] was the first to observe the importance of ancestry in cancer of the breast in mice and to substantiate the observation by experiment. By selective breeding experiments begun in 1906 he obtained 18.1% cancerous offspring amongst progeny of cancerous ancestry as compared with 8.3% amongst offspring of non-cancerous ancestry. He summarized his results in these words:

"From these observations the conclusion is drawn that female mice in whose ancestry cancer of the mamma has occurred not further back than the grandmothers are distinctly more liable to develop the disease spontaneously in this organ than those in whose ancestry cancer is more remote".

However, a proper analysis of the part played by genetic constitution in the development of breast cancer in mice was possible only after the establishment of genetically homozygous strains by selecting for and against breast tumour and by brother-to-sister matings over a number of years[2, 3].

After the establishment of these inbred strains, the predominance of the maternal influence in the hereditary transmission of the tendency to develop breast cancer was observed in reciprocal crosses between high- and low-breast-cancer strains[4, 5, 6, 7]. It was shown in these experiments that this influence was not transmitted through the chromosomes and had to be attributed to an unspecified extra-chromosomal factor.

BITTNER[8–11] was first to point out that this extra-chromosomal factor is conveyed by the milk of high-cancer-strain female mice. He described this factor as the 'milk factor' or the 'milk influence'. Similar results were obtained by many workers in various laboratories, and the general validity of the role played by the milk factor in the origin of spontaneous breast cancer in inbred strains of mice was established[12–17].

BITTNER postulated[9, 10] that in the origin of breast cancer in certain strains of mice three factors are involved: the genetic factor or inherited susceptibility, transmitted in a dominant manner; the hormonal factor or hormonal stimulation; and the milk factor or influence which has also been described as the mammary tumour inciter[26, 29, 30] or as a virus[24].

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The milk factor has been shown to possess the following properties. It is present during the entire period of lactation and there is a quantitative correlation between the amount of milk ingested and the time of appearance and incidence of breast cancer[11, 18, 19]. It is widely distributed throughout the normal tissues and organs of high-breast-cancer strains of mice, and it can be transmitted to susceptible mice by extracts of such tissues[11, 18, 20, 21]. It is present also in extracts of breast tumours of high-cancer-strain mice[22–25] and in the blood of these mice[26, 27], and it has been shown to be present in the organs of low-breast-cancer-strain mice fostered by high-cancer-strain females in a concentration sufficient to induce tumours in susceptible mice[28].

The tumour-inducing property of the factor or agent is preserved for six months in lyophilized breast-tumour tissue[21, 31], for short periods in glycerolated extracts of tumour tissue[22, 32], and for as long as two years in breast-tumour tissue desiccated over phosphorus pentoxide and kept in sealed and evacuated containers[33]. The milk factor remains active following filtration of lactating-breast tissue extracts through Seitz filters[22] and through Berkefeld candles[29]. It is destroyed in breast-tumour tissue at 60° C. after an hour, remains stable within the range of p_H 5–10.2, is not inactivated by petroleum ether or acetone, and is not appreciably soluble in them[34]. A temperature of 66° C. for 30 minutes destroys the tumour-inducing property of tumour extracts[32].

Ultracentrifugation experiments with extracts of lactating-breast tissue of high-cancer-strain mice[24], tumour tissue and milk[23, 35] indicated that the milk factor or agent may be a protein of high molecular weight, although complete sedimentation of the active principle was not obtained even at speeds corresponding to 110,000 times gravity[36]. However, these experiments, as well as the desiccation and filtration experiments, demonstrate that breast cancer in mice can be transmitted by cell-free filtrates.

Finally, it was shown that the milk factor has antigenic properties and stimulates the formation of antibodies in other species of animals[32, 37].

Taking advantage of these established properties of the so-called milk factor, electron microscope and ultracentrifugation studies of normal and malignant tissues from high- and low-breast-cancer strains were undertaken in an attempt to elucidate its nature.

MATERIALS AND METHODS

The following inbred strains of mice were employed as source of material: C3H, Strong A, and RIII high-breast-cancer; and for the purposes of control, C57 black, CBA, and IF low-breast-cancer.

Normal tissues and tissues of spontaneous and induced tumours were used in the experiments. Extracts of the following tissues were made: normal lactating breasts and breast tumours of C3H and RIII strains and breast tumours of Strong A strain; normal lactating breasts of C57 black and CBA strains; lactating breast tissues of mixed stock mice; breast tumours induced in C3H males, either by oestrone alone or by oestrone together with methylcholanthrene[38]; breast tumours similarly induced in C57 black males and females[38], and in IF mice kindly supplied by Dr J. W. Orr[39]; sarcoma induced in C57 black mice by oestrone and methylcholanthrene[38]; transplantable 37 S sarcoma of the *Imperial Cancer Research Fund;* normal organs of C3H males; and C57 transplantable breast tumour, originally induced by fostering C57 mice by RIII females.

The tissues were usually obtained from several mice of each strain, and were minced together, weighed and desiccated as previously described[25, 33, 40, 41]. In some cases tissues were obtained from a single mouse.

In several experiments milk from C3H and C57 black strains was obtained either by a specially constructed milking device[42] lent to us by Dr S. K. Kon of the *National Institute for Research in Dairying, Shinfield, Reading,* or by simple gentle squeezing of the breasts. The quantities obtained were small and amounted to only a few cubic centimetres from as many as 15–20 mice. Milk was also obtained from stomachs of 5-day-old C3H and C57 black mice, by killing the mice under ether

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anaesthesia half-an-hour after suckling and then removing their stomach contents. The milk obtained by various methods was desiccated in the same manner as for other tissues and stored in sealed tubes in an ice-chest.

In the preparation of tissue extracts several methods were tried before one was finally selected. Usually in each experiment tissue extracts from high- and low-breast-cancer strains were examined at the same time, and the procedure was kept strictly the same for both types of tissue.

First method. Dried tissue was ground with sand in a mortar, distilled water being added gradually to give a final proportion of 1 ml to 10 mg of dried tissue. The suspensions were then centrifuged for 30 minutes at 3,700 rpm (corresponding to 2,600 times gravity) in a Baskerville and Lindsay standard type (Manchester) centrifuge so as to remove sand and larger tissue particles. The supernatant obtained from centrifugation was filtered through Whatman No. 542 filter paper. The filtrates, after further dilution, were examined under the electron microscope immediately, and again after 4–14 days standing in the ice chest.

Second method. Dried tissue was ground for 20 minutes in a mortar with sand and petroleum ether in a proportion of 1 ml to 10 mg of tissue. After the petroleum ether had been poured off, the residue was ground with distilled water in a proportion of 1 ml to 10 mg of the original amount of dried tissue, the suspension was centrifuged at 3,700 rpm for 30 minutes, the supernatant filtered through Whatman No. 542 filter paper (and sometimes also through a Berkefeld N candle), and the filtrate, after further dilution, examined under the electron microscope immediately, and again after 4–14 days standing in the ice-chest.

Third method. Dried tissue was ground with sand, and petroleum ether was added in the proportion of 1 ml to 10 mg of dried tissue, then after 20 minutes the ether was poured off and the residue was ground with distilled water (1 ml to 10 mg of tissue). The distilled water suspension having been centrifuged at 3,700 rpm for 30 minutes, the supernatant was filtered through No. 542 filter paper. Allan and Hanbury trypsin was then added to this filtrate in a proportion of 1 : 1 and incubated, after adjusting p_H to 7.4–7.6, for 30 minutes at 37° C. After incubation with trypsin the extract was filtered through a Berkefeld N candle under a negative pressure of 15–20 cm Hg. The Berkefeld filtrate, after further dilution, was examined under the electron microscope immediately and again after standing for 4–14 days in the ice-chest. This method was finally adopted as routine.

Trypsin used in the experiments was prepared in the following way: 5 g of powdered Allan and Hanbury trypsin was re-suspended in 25 ml distilled water and dialysed against five changes of distilled water at 4° C. during the course of 3 days. The dialysed suspension of trypsin was then centrifuged at 3,700 rpm for 15 minutes and the supernatant filtered through Whatman No. 3 filter paper. The filtrate, after testing against milk to which normal calcium chloride in the usual quantity had previously been added, was used for mixing with tissue extracts in a proportion of 1 volume of filtrate to 1 volume of extract.

In a few experiments, after the addition of trypsin, the p_H was adjusted to 6.0–6.4 or 8.0–8.2. The p_H of high- and low-cancer-strain tissue extracts before addition of trypsin was between 5.9–7.0. The majority of the samples lay within the range of p_H 6.4–6.8. Extracts of dried breast milk gave an average p_H of 7.9; the average p_H of extracts of stomach milk was 6.3.

The centrifuge used by Dr PALEY JOHNSON in the experiments at the *Royal Institution* in London was a high-speed vacuum centrifuge of the Beams and Pickels type. C3H breast-tumour tissue extracts, prepared in the usual way, were transported to London in ice containers and examined the following day. The proportion of dried tissue to the re-suspending fluids varied from the usual 10 mg to 1 ml to as much as 150 mg to 1 ml. In each experiment the chloride content of the extracts was estimated by Dr L. H. STICKLAND, and sodium chloride was then added to give a final concentration of 0.85%.

In the few centrifugation experiments so far carried out the centrifuge was run at various speeds corresponding to 40,000–120,000 times gravity for lengths of time varying from 1 to 2 hours. In each case samples of the supernatant and sediment were taken for examination under the electron microscope.

Biological tests. The extracts of various tissues examined with the electron microscope and in the ultracentrifuge were tested at the same time for tumour-inducing activity in 5–6 weeks old susceptible C57 × RIII hybrid mice. The extracts in each experiment were injected into the test mice subcutaneously in quantities of ½ ml.

Electron microscopy. In the preparation of tissue extracts for electron microscope examination it is most essential to maintain a high standard of cleanliness during the various stages of e.m. preparation and examination. Small test tubes were used for dilution purposes; these were new tubes which had stood in chromic acid for 12 hours, after which they were washed for 48 hours in running tap water, and finally washed several times with distilled water which had been filtered through three thicknesses of Whatman No. 42 filter paper. After draining the clean tubes, a known amount (usually 1 ml) of filtered distilled water was run into each tube, which was then stoppered with a cellophane-covered cork. Dilutions of tissue extracts were made by adding the necessary number of micro-drops from clean micro-pipettes. In most cases a satisfactory dilution ratio proved to be 1 part

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of tissue extract to 500 parts of filtered distilled water. (In some cases when the tissue extracts contained very large numbers of particles, it was necessary to alter the dilution ratio in order to obtain particle separation on the e.m. mounting film). Thorough mixing of the diluted extract was carried out by shaking the tube.

The quantitative determination of particle-size distribution by the electron microscope is by no means a certain matter, since it is difficult to control the regular deposition of particulate material over the small area of an e.m. mounting film. In this work, however, an attempt was made to keep conditions on as firm a quantitative basis as possible by (a) keeping the dilution strictly constant, and (b) using a standardized method of preparing the specimen mounts. Mounting consisted of placing a small drop of diluted extract in the middle of a filmed specimen grid (collodion mounting films were used throughout the work). To achieve even distribution and to prevent particle aggregation during drying, the drops were immediately drained with small pieces of filter paper; in this way very thin layers of liquid were formed on the filmed grids. Such layers dry almost immediately and the chance of particle aggregation is minimized. After drying, the mounted grids were metal-shadowed by sending a beam of gold or chromium atoms at a small angle to the plane of the mounting film, following the vacuum technique of WILLIAMS AND WYCKOFF[43].

The specimens thus prepared were examined in an RCA Type B instrument, using a beam of 45 KV electrons. All photographic recordings were made on fast plates (Kodak B.10 Photoscript)[44].

Some mention is necessary of the fields "sampled" and actually recorded in the electron microscope. The small particles are not easily seen; they can be observed more easily by increasing the contrast by depositing more metal during the shadowing stage of the preparation, but if this is done, there may occur granulation of the background due to movement and aggregation of metal during exposure to the electron beam. Accordingly, the method used was to deposit very thin coatings of metal and then to take many photographs from widely different parts of the preparation. No plan of selection was followed, the fields being photographed as they came into view. This routine procedure was made easier by (a) focussing on an edge of the supporting grid and then moving quickly away to the more central region of the film; and (b) rapid photography, since the plates used (Kodak B.10 Photoscript) are fast plates. The usual way was to repeat the procedure for two or more preparations of the same tissue extract made at the same time. Thus the conclusions regarding any particular tissue extract were arrived at from consideration of 15–20 photographs of each specimen, those illustrated here being typical.

RESULTS

In the earliest experiments distilled water extracts (1st method) both of RIII breast-tumour tissue and of normal lactating-breast tissue of mixed-stock mice were found to contain roughly spherical particles of diameters from about 200 Å to 1200 Å (Table I). No qualitative difference could be seen between the extracts of tumour and normal tissue. Re-examination of these extracts after 4–14 days standing in the ice-chest revealed, however, similar numbers of particles in the tumour extracts but much fewer in those of the normal lactating-breast tissue of mixed stock mice (Table I). A similar experiment with distilled water extracts of lactating-breast tissue of C3H and C57 black mice gave also this kind of result due to standing in the ice-chest.

Extracts of C3H lactating-breast tissue, after treatment with petroleum ether, showed the presence of particles, but corresponding extracts of C57 showed them only occasionally (Table I). Also, extracts of C3H breast-tumour tissue treated with petroleum ether, when compared with corresponding extracts of C57 lactating-breast tissue, showed a similar contrast (Table I). Examination of extracts of 37S transplantable sarcoma treated with petroleum ether revealed the presence of only few particles.

Particles similar to those seen in C3H breast-tumour tissue treated with petroleum ether were found also in comparable extracts of Strong A and RIII breast-tumour tissue (Plates 1 and 2).

Extracts of C3H normal lactating breasts and breast-tumour tissue (3rd method) showed considerable improvement; there was much less tissue debris and the particles were much more easily visible. There were very few particles present in extracts of similarly treated C57 lactating-breast tissue (Table I).

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TABLE I

Tissues	Treatment					
	Distilled Water Extraction		Petroleum Ether followed by Distilled Water Extraction		Petroleum Ether followed by Distilled Water Extraction followed by Trypsinization followed by Filtration through Berkefeld Candles	
	Examined Immediately	After 4–14 Days in Ice-Chest	Examined Immediately	After 4–14 Days in Ice-Chest	Examined Immediately	After 4–14 Days in Ice-Chest
Lactating Breast Tissue of High-Breast-Cancer Strains (C3H, RIII)	+	+	+	+	+	+
Lactating Breast Tissue of Low-Breast-Cancer Strains (C57, CBA)	+	±	±	± —	± or —	± or —
Spontaneous Breast Tumours of High-Breast-Cancer Strains (C3H, RIII, Strong A)	+	+	+	+	+	+

\+ Indicates Particles present
± Indicates Particles present, but few in numbers
± — Indicates Particles present, but very few in numbers
— Indicates Particles absent

In extracts (3rd method) of IF-strain breast tumours, C57 breast tumours and a C57 sarcoma, all induced by oestrone and methylcholanthrene (Table II), either no particles or only occasional particles could be seen. Similar results were obtained with extracts of CBA lactating-breast tissue.

In extracts (3rd method) of a C57 breast tumour, originally induced by foster-nursing C57 black mice by RIII high-breast-cancer-strain females and transplanted for 42 generations in C57 black mice, particles were found to be present although in smaller numbers than in high-breast-cancer-strain lactating tissue or in breast tumours of high-breast-cancer strains.

In extracts of normal organs of C3H-strain males particles were also found but in smaller numbers than in extracts of breast tumours induced in C3H male mice by oestrone or by oestrone plus methylcholanthrene (Table III).

In breast milk of C3H mice particles were found in varying numbers, while only

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TABLE II

	Tissues	Particles
Low-breast-cancer strains males and females	Breast tumours induced by oestrone and methylcholanthrene (C57, IF)	± – or – (very few) (absent)
	Sarcoma induced by oestrone and methylcholanthrene (C57)	– (absent)
	37 S transplantable sarcoma	± – (very few)

TABLE III

	Tissues	Particles
High-breast-cancer strain males (C3H)	Normal organs (lung, spleen, thymus, kidneys, heart)	± (few)
	Breast tumours induced by oestrone	+
	Breast tumours induced by oestrone and methylcholanthrene	+

occasional particles were present in similarly obtained C57 milk. In C3H milk obtained from the stomachs of young mice, a particularly rich crop of particles was obtained in one case (Plates 3 and 4), but it has not yet been possible to repeat this result at will. In the preparation of C57 stomach milk only a few particles were present (Table IV).

In the centrifugation experiments with extracts of C3H breast-tumour tissue (3rd method) the same kinds of particles were found in the supernatants and sediments

TABLE IV

	Tissues	Particles
Milk from	High-breast-cancer-strain breasts (C3H)	+
	Low-breast-cancer-strain breasts (C57)	± or ± – (few) (very few)
	High-breast-cancer-strain stomachs (C3H)	+ or ± (few)
	Low-breast-cancer-strain stomachs (C57)	± or – (few) (none)

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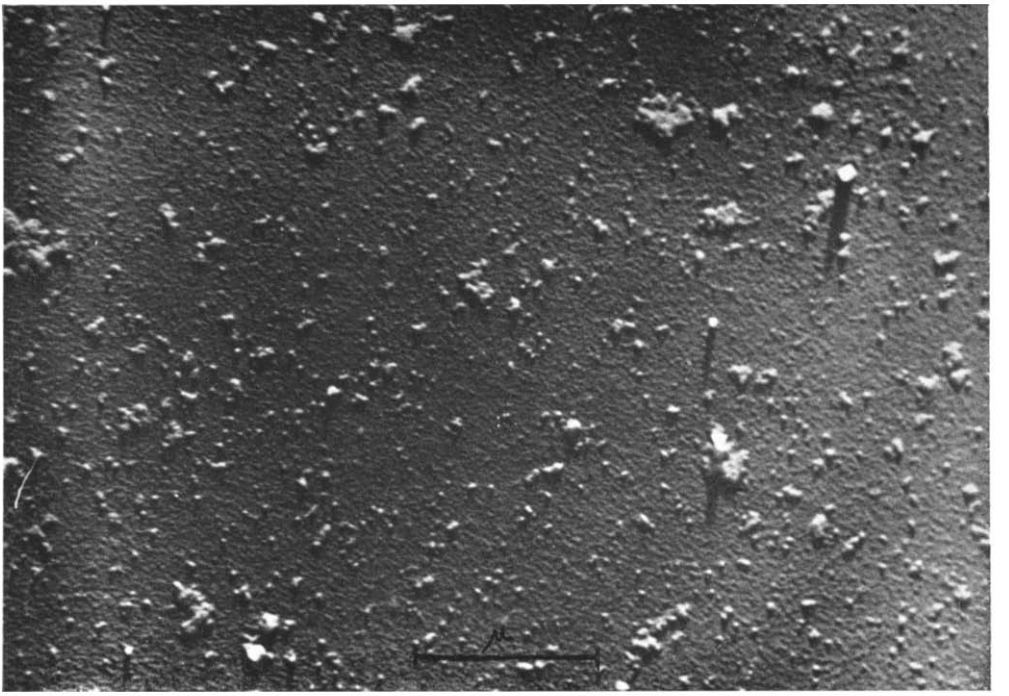

Plate 1. Strong A (high-breast-cancer strain) spontaneous breast tumour tissue: desiccated, treated with petroleum ether, extracted with distilled water, filtered through Berkefeld N candle. Chromium shadowed.

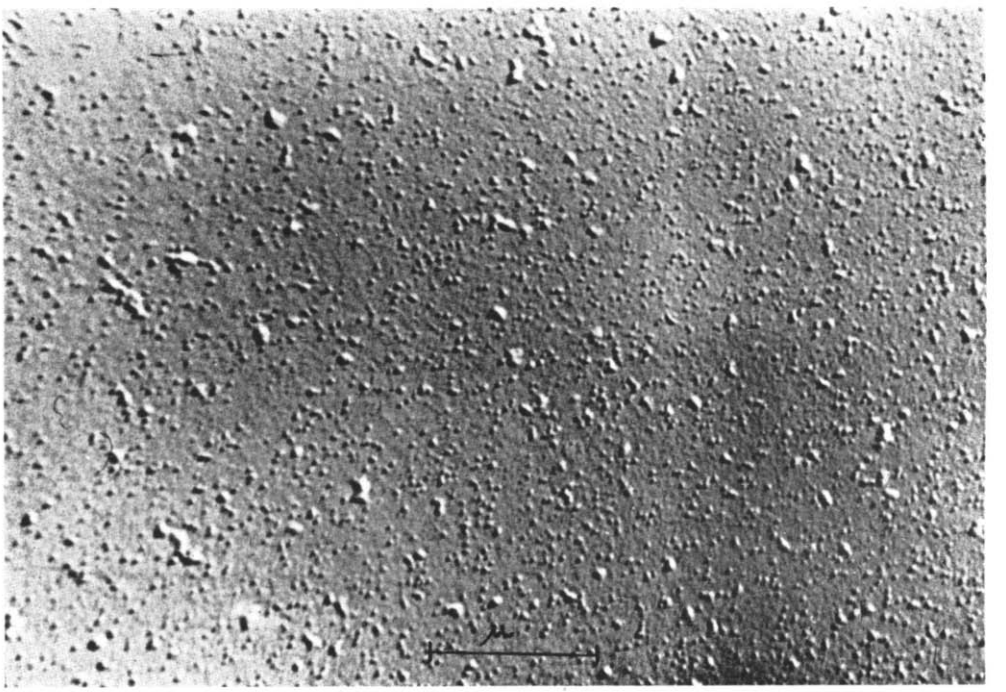

Plate 2. RIII (high-breast-cancer strain) spontaneous breast tumour tissue: desiccated, treated with petroleum ether, extracted with distilled water, filtered through Berkefeld N candle. Chromium shadowed.

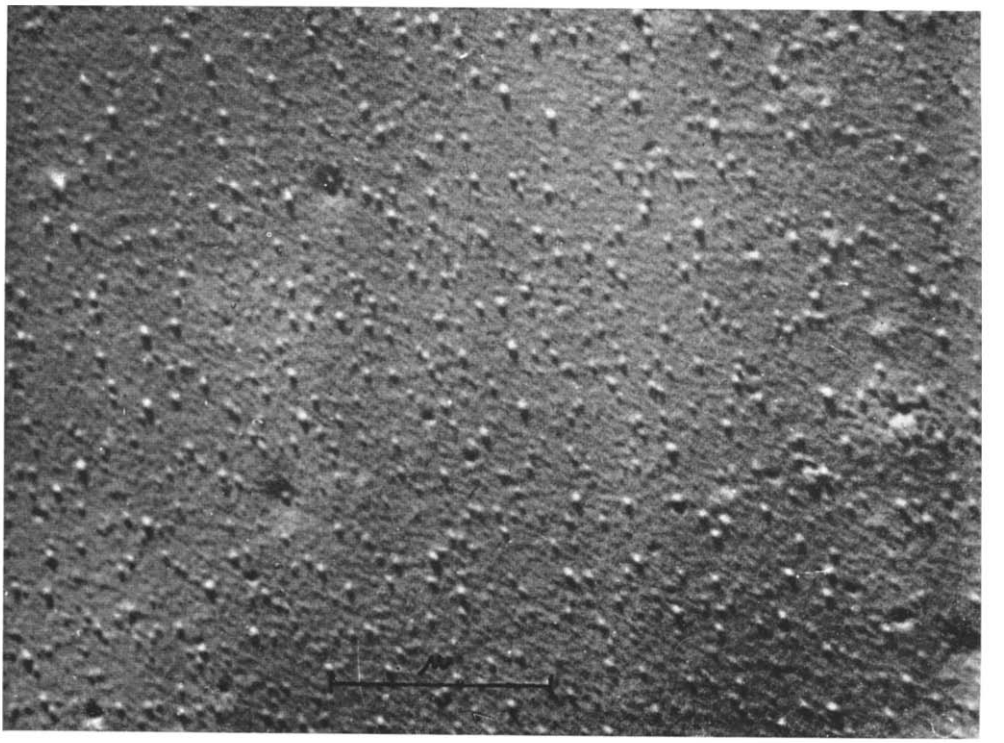

Plate 3. C3H (high-breast-cancer strain) stomach milk: desiccated, treated with petroleum ether extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Gold shadowed.

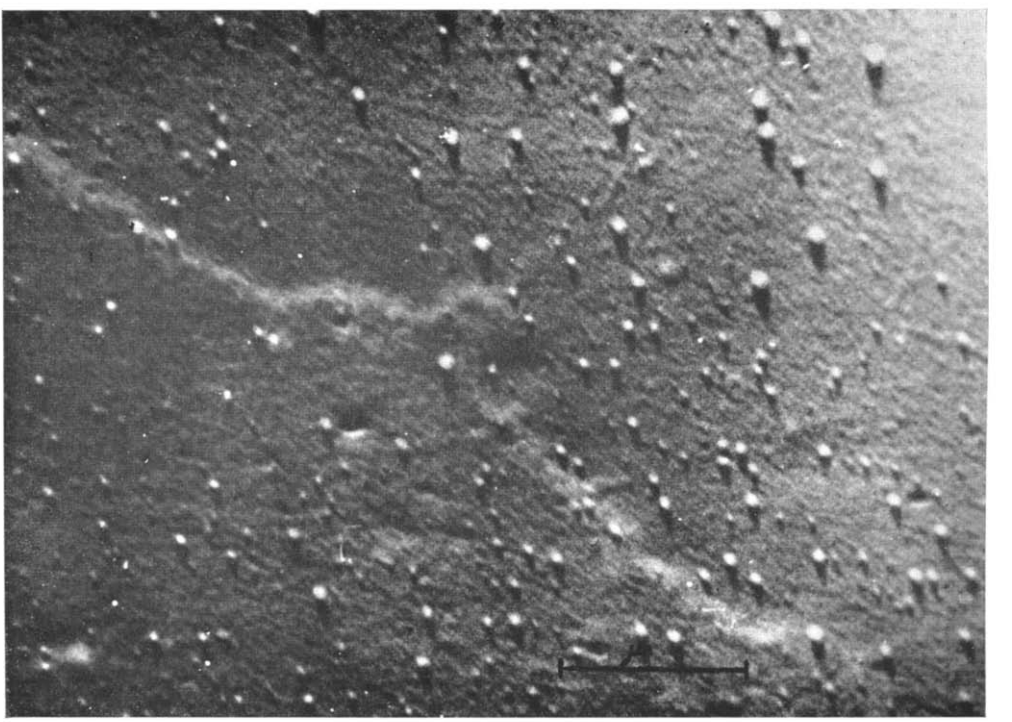

Plate 4. Same as Plate 3. Variation in size of particles can be seen.

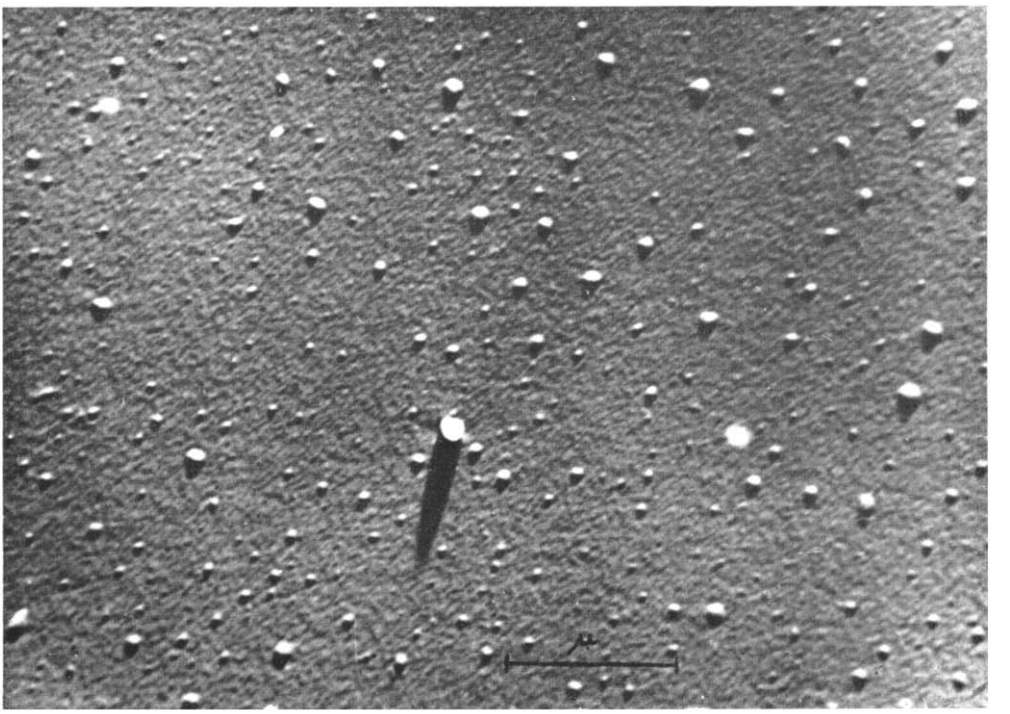

Plate 5. C3H (high-breast-cancer strain) spontaneous breast tumour tissue: desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Supernatant fluid after centrifugation for one hour at 60,000 times gravity. Chromium shadowed.

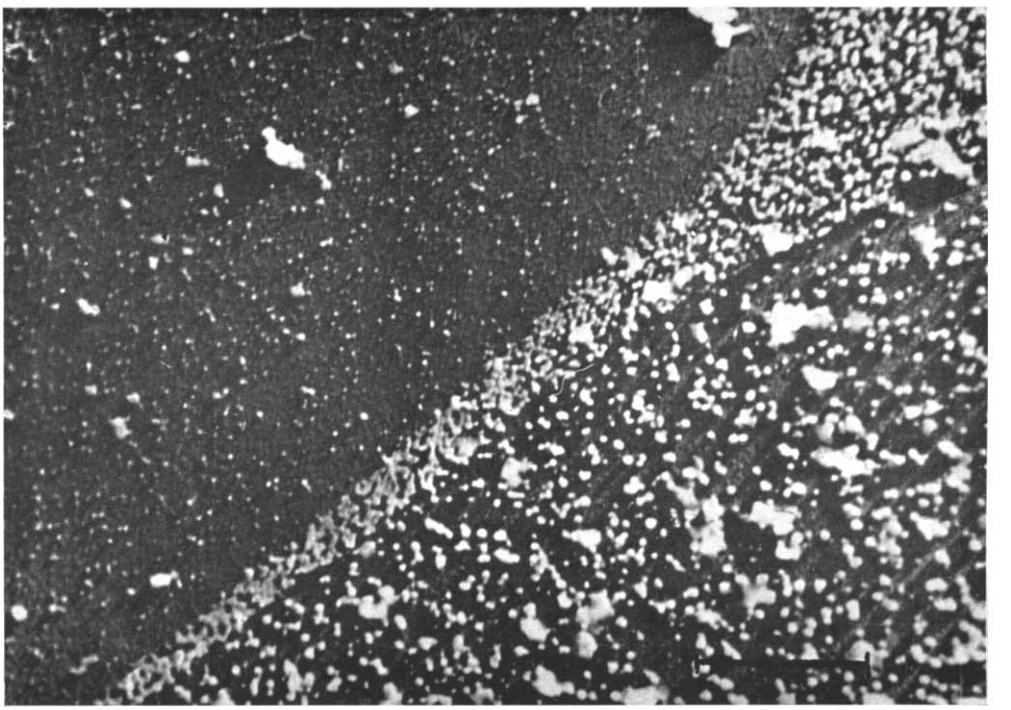

Plate 6. Same tissue as for Plate 5. Sediment after centrifugation for one hour at 60,000 times gravity. Chromium shadowed.

obtained at speeds corresponding to 40,000–60,000 times gravity (Plate 5). In some preparations obtained by centrifuging extracts of C3H breast-tumour tissue at 60,000 times gravity, a tendency to regular crystallographic arrangement was observed (Plate 6). After centrifugation for 2 hours at a speed equivalent to 120,000 times gravity, the supernatant was found to be entirely free from particles as observed under the electron microscope. The deposit consisted of particles of various sizes corresponding to those encountered in all high-breast-cancer-strain tissue extracts.

Variations in particle size in all preparations were constantly observed, but the general impression has been that diameters greater than about 300 Å have been few. There were many particles below 300 Å in diameter.

The biological tests are still far from complete, and the situation to date is summarized in Table V.

TABLE V

Number of mice injected	Source of material	Number of mice alive at the earliest tumour appearance	Number of mice with tumours	Number of mice still alive
274	High-cancer-strain tissues (normal and malignant tissues)	221	56	73
190	Low-cancer-strain tissues (normal and malignant tissues)	185	6*	51

* Workers in other laboratories both here and in America have reported the spontaneous appearance of tumours in untreated hybrid mice. For literature see DMOCHOWSKI[86].

The results of nitrogen, phosphorus, dry weight, viscosity and density estimations on tissue extracts have been so variable that no significant difference between high- and low-breast-cancer strains could be detected**.

DISCUSSION

The results of the present investigations have demonstrated that extracts of normal and malignant tissues of three high-breast-cancer strains contained approximately spherical particles of various sizes up to about 300 Å, with a few considerably larger. Extracts of normal and malignant tissues obtained from low-breast-cancer strains either did not contain similar particles or contained only a few.

The number of particles found in extracts of normal lactating-breast tissues of C3H high-cancer strain, as seen in the electron micrographs, was smaller than that encountered in C3H breast-tumour extracts. This was found repeatedly, though variations in the numbers of particles in extracts of normal tissues from the same strain occurred frequently, yet not to such an extent as between extracts of normal and malignant tissues of the same high-breast-cancer strain.

** These estimations have been carried out by Dr H. L. STICKLAND.

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Usually, though not always, electron micrographs of RIII breast-tumour extracts contained more particles than micrographs of C3H and Strong A breast-tumour extracts. It is not known at present whether this finding bears any relation to previous[41] higher tumour-inducing indications given by RIII breast-tumour extracts as compared with C3H and Strong A breast-tumour extracts. The presence of particles in extracts of a C57 black transplantable breast tumour in its 42nd transplant may be connected with the observed tumour-inducing property of distilled water extracts of dried tumour tissue of the same generation of transplants[49].

The extracts of normal organs of C3H-strain males showed the presence of a smaller number of the characteristic particles than was found in lactating-breast tissue of C3H females. The number of particles seen in the extracts of breast tumours induced in C3H males by oestrone alone, or by combined treatment with oestrone and methylcholanthrene[38], was larger than that in the extracts of normal organs of these males.

Electron micrographs of C3H-strain milk obtained from the breasts of C3H female mice yielded results similar to those from normal lactating-breast tissue of C3H females. Extracts of C57-strain milk, similarly obtained, showed either none or only few particles. Considerable variation in the numbers of particles was observed in the extracts of milk obtained from the stomachs of C3H young mice, an observation which cannot for the moment be explained. It may be due to the variation of material obtained from young mice belonging to the first, third or fourth litters. BITTNER[29, 50, 51] observed a difference in the tumour incidence of successive litters born to the same mother: the mice of the third and fourth litters showed a higher incidence of breast cancer than those of the first and second litters. This indicates that the milk factor may vary in concentration during the reproductive period of the mouse. On the other hand such variations have not been observed in litters of C3H strain maintained by ANDERVONT[52]. It is not clear at present whether the variation in the numbers of particles was due to variation in the tumour incidence of successive litters or to other factors, and the point is under investigation.

The use of trypsin improved the electron micrographs of extracts although it must be stressed that the particles were present before trypsinization. The addition of trypsin did not affect their appearance, but the preparations were much clearer and the number of particles was undiminished. The stimulating action of trypsin in acid medium on the formation of Rous tumour was observed long ago[53], and trypsin has been used extensively in experiments with Rous chicken tumour. Trypsin has been used for purification of plant viruses and no differences in activity have been noticed between virus purified with trypsin and that purified by other methods[54]; it has also been employed for obtaining a better yield of virus from infected plant material[55]. The milk factor can produce tumours when fed to young mice, which suggests that it may be either resistant to the action of the digestive enzymes or dependent on these enzymes for degradation into active material. The question whether trypsin influences the biological activity of the extracts is under investigation.

The desiccation of tissue does not affect the presence of particles, for they were seen in electron micrographs of similar extracts of RIII breast-tumour tissue, whether fresh or dried.

The clearing-up effect in distilled water extracts of high-breast-cancer-strain tissues and to a similar extent in petroleum ether-treated tissue extracts after standing frozen in the ice-chest for at least 4 days seems to be of interest. The particles were more

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clearly visible in the electron micrographs, which were much cleaner and more free from tissue debris than those of extracts examined immediately. A similar phenomenon has been observed with ether-clarified aqueous extracts, frozen and stored for at least 5 days, of tissues infected with the Lansing strain of poliomyelitis virus[56, 57]. In this case the extracts subjected to ultracentrifugation yielded material much more active than the extracts before freezing, and the electron micrographs showed much improvement. Extracts from normal tissue, frozen and stored for several weeks, yielded negligible amounts of sedimentable protein, while extracts from infectious material gave small amounts of highly infectious macromolecular protein[58]. It would appear that normal proteins rather than virus were denatured by such treatment, as the nitrogen content of the material stored and cleared by ultracentrifugation was 50–75% of that of material before freezing. Whether the same interpretation can be applied to the extracts of high-breast-cancer-strain tissues is not known at present; it may be considered only after clear evidence of a connection between the characteristic particles and breast tumour-inducing activity.

It has been shown[24, 35] that material of high tumour-inducing activity could be at least partially sedimented from saline extracts of high-breast-cancer-strain tissue and milk, at a speed corresponding to 90,000 times gravity. The activity seemed to be associated with particles having a sedimentation constant between 25 s and 200 s. In the present experiments only quantitative centrifugation was carried out. The presence of particles, similar to those in tissue extracts, in supernatants after centrifugation at 60,000 times gravity for two hours, and their complete absence in supernatants after spinning at 120,000 times gravity for two hours, indicates that a centrifugal force lying somewhere between these extremes is required to bring down the particles. The results of tests for tumour-inducing activity at various stages of centrifugation are awaited. Recently the isolation of "a characteristic material of large particle size" from high-breast-cancer-strain milk has been reported[59]. These heavy particles (their exact size is not stated) were seen not at all or only in low concentrations in C57-black-strain milk. It is difficult at this stage to assess the significance of the difference in size between such particles and those encountered in the present experiments.

From our centrifugation experiments it may be presumed that the spherical particles are not in the large particle class of the filterable chicken tumour[60]; and also, from the high centrifugal force required to give complete sedimentation in extracts of high-cancer-strain tissues, it seems probable that there is very little, if any, similarity between them and the macromolecular components isolated from certain mammalian tumours as well as from chick embryo[61, 62, 63] and other tissues[64, 65, 66, 67]. The particles isolated in CLAUDE's experiment by differential centrifugation ranged from 500 Å to 3000 Å in diameter[68]. Some of the particles encountered in the present experiments fall within this range, but it is not possible at the moment to draw any further comparison. The preponderance of particles no larger than about 300 Å 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-strain tissues, though their origin, whether extracellular or from other cell constituents, must remain for the present only a matter of speculation. The particulate components, of diameter 500 Å to 1500 Å, that have been isolated by CLAUDE[61] from normal and malignant cells of chicken and mice embryo and sarcoma were strikingly similar in physical and chemical properties whether they came from normal

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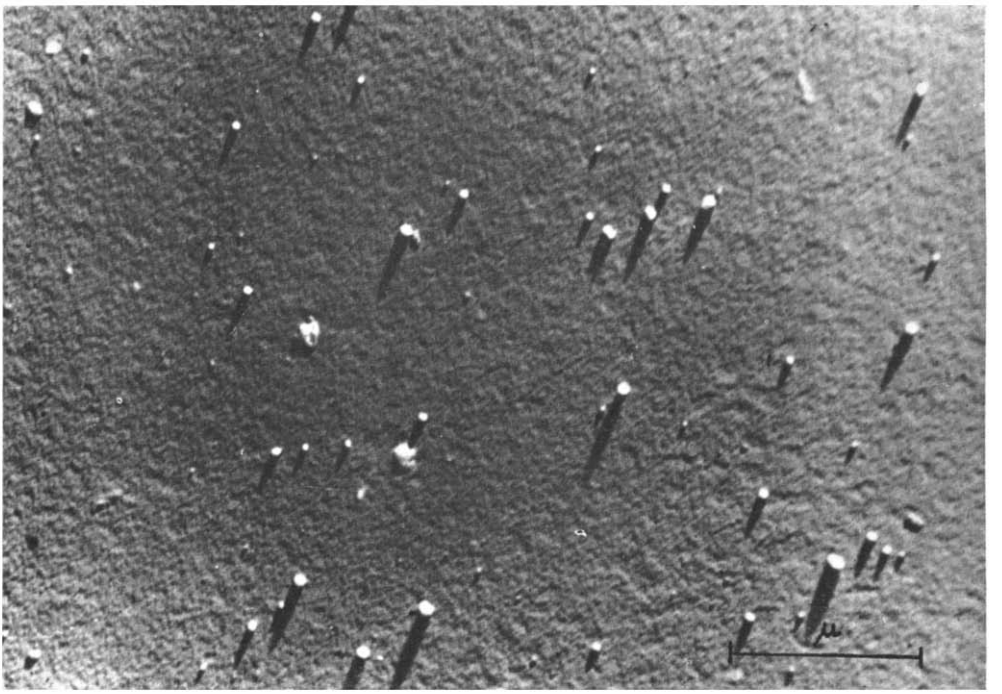

Plate 7. C3H (high-breast-cancer strain) male breast tumour induced with oestrone and methylcholanthrene: desiccated tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Chromium shadowed.

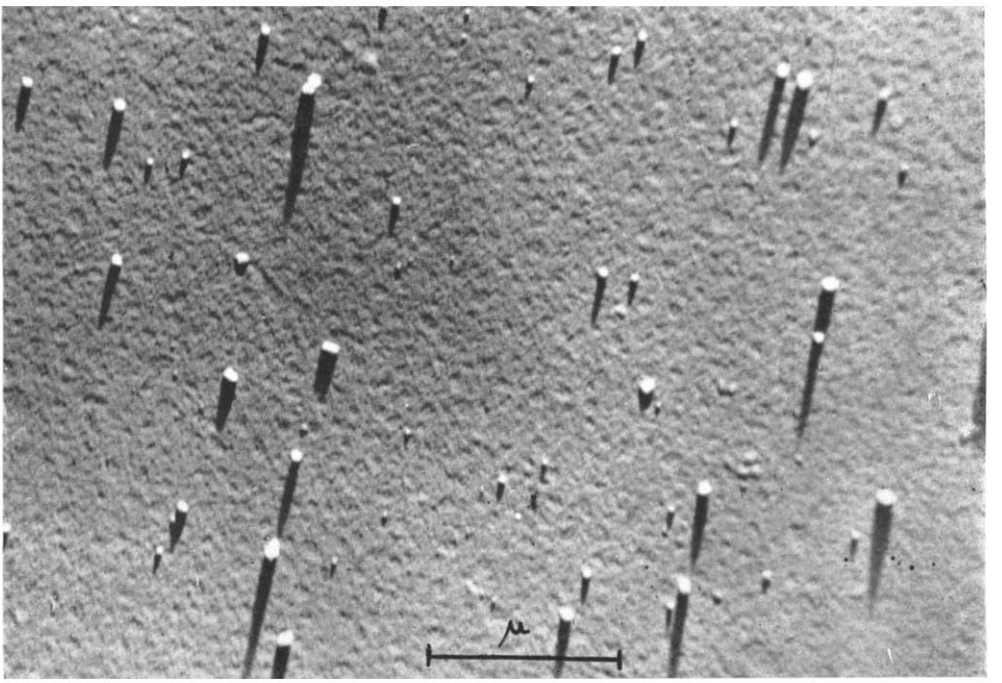

Plate 8. Same tissue as for Plate 7.

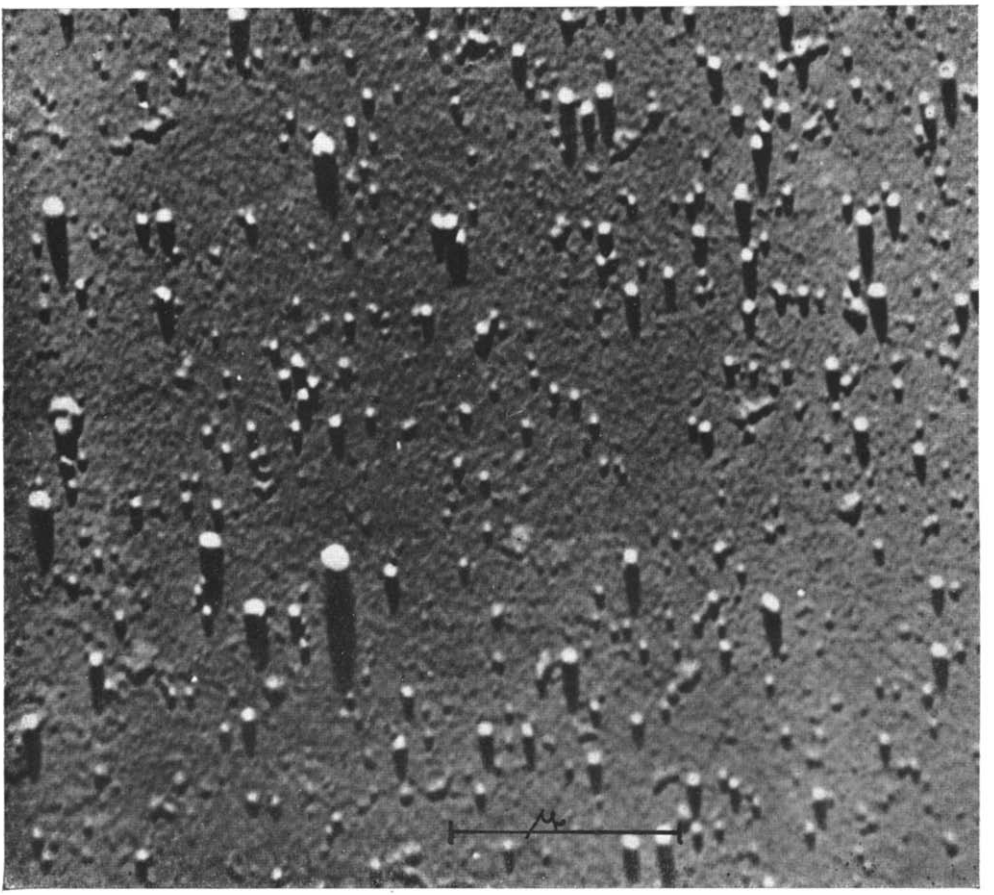

Plate 9. C3H (high-breast-cancer strain) spontaneous breast tumour tissue: desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Chromium shadowed.

or from malignant tissues. In the experiments described here, extracts from C57 low-breast-cancer-strain sarcoma showed few only of the characteristic spherical particles of diameters up to about 300 Å. This may be due to the technique of these experiments differing from that of CLAUDE. Ultracentrifugation studies of normal and infected tissues with influenza virus[69, 70] showed the presence of particles of 1000 Å in the electron micrographs of both types of tissue, whereas in infected chorio-allantoic fluid there were found particles of 100 Å to 300 Å diameter. Similar particles were absent from normal and embryonic fluid. However, LAUFFER AND STANLEY[71] and STANLEY[72] showed that infectivity is associated not with the smaller particles but only with particles of 1000 Å diameter, and KNIGHT[73] isolated particles of 400 Å diameter from normal chorio-allantoic fluid which were antigenically similar to influenza virus particles but were not infective. The isolation of animal viruses is therefore complicated by the presence of normal particles which are often indistinguishable from infectious material. GESSLER AND GREY[74] recently demonstrated the presence of spherical bodies of 500 Å to 1500 Å diameter in sections of human breast tumours, but it is difficult to draw any comparison

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between this finding and ours because of the difference in particle size and in origin of tissues in which they were found.

PORTER AND THOMPSON[75] have recently published the interesting results of their electron microscope investigation of epithelial cells, grown *in vitro*, from spontaneous breast tumours of high-breast-cancer-strain mice. They have observed in these cells spherical particles, most of them with a double structure consisting of a central dense portion of approximately 750 Å average diameter (varying from 500 to 900 Å) and a less dense outer portion of 1350 Å average diameter (varying from 900 to 1700 Å). They have advanced the view that these particles represent the milk factor and have drawn attention to the great difference in size between their particles (1350 Å) and those observed by us[45]. At the time of writing their paper it would appear that they had not seen our second communication[46]. In that communication attention was drawn to the variation in size of the particles encountered (see also Plates 7, 8 and 9), and actually our range overlaps that reported by PORTER AND THOMPSON, although there is a difference in average size. It is possible, indeed probable, that not all of the particles observed in the experiments outlined here are of the same biological nature, but at the moment there is no evidence on this point. Porter and Thompson rightly mention that the difference in size cannot be entirely a result of different techniques, but they are of the opinion that this difference may have been brought about by the methods employed, and suggest that the study of tumour cells grown *in vitro* may be a technique less subject to criticism than the present experiments. It should be pointed out, however, that the particles described here were found in fresh as well as dried, and in trypsinized and non-trypsinized tissues.

It must be stressed that no claim has been put forward that the particles observed in the tissue extracts are indeed the milk factor; on the contrary, it has been emphasized that at the present stage of these investigations it is not known what connection, if any, they have with milk factor. At the same time, electron microscope investigations of particles contained in the cells of tissue culture may be open to criticism in that they may not necessarily show the true size of the particles. The basis of this criticism is as follows. Combinations between viruses and host cells appear very frequently, and they can take place as virus-cell surface, virus-intracellular, virus-extracellular or tissue component union (HORSFALL, HARDY, DAVENPORT[76]. It has been shown that digestion with trypsin releases the aggregates of virus from infected tissue[77], and that in addition to trypsin a cellulase from the snail (*Helix aspersa*) can liberate some of the viruses from infected plant tissue[78]. It has also been shown that some virus-host cell combinations remain firm even after purification attempts[79]. In view of this and the frequency of virus-host cell combinations, HORSFALL *et al.*[76] point out that such combinations, if unrecognized, may lead to observations difficult of interpretation.

Observations on the virus which causes pneumonia in mice (HORSFALL AND HAHN[80]) are of particular interest in connection with the present problem. This virus infects the lungs and combines with lung tissue particles but not with other tissue particles. The union is normally stable, but it can be broken down by heat and alkali which, however, destroy the infectivity (MILLS AND DOCHEZ[81]; CURNEN AND HORSFALL JR.[82, 83]); or by relatively small amounts of crystalline trypsin (VOLKERT AND HORSFALL JR.[84]); or by an appropriate concentration of electrolytes which does not destroy the infectivity of the agent (HORSFALL *et al.*[76]). It has been found that the free virus is several times smaller than the combined virus, its size being 400 Å (CURNEN, PICKELS, HORSFALL JR.[85])

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as compared with about 1400 Å for the combined virus. In view of the combination with tissue particles which occurs with other viruses, HORSFALL *et al.*[76] are of the opinion that the mice pneumonia data indicate that the physical and other properties of viruses may require revaluation.

In the light of these findings, it is possible that the results of PORTER AND THOMPSON[75] may be explained as arising from a combination between smaller particles and cell components. The double structure of their particles may also favour this explanation. It is not claimed that the particles observed in our experiments are the actual milk factor, because the biological tests are not yet complete. So far 56 out 221 hybrid mice injected with the earliest extracts containing the particles have developed tumours, but it is necessary to await further results of the centrifugation experiments before connection between the particles and tumour-inducing activity can be definitely established. It is possible to state now that the tumour-inducing activity is associated with suspensions containing particles of a range of diameters, but the experiments are not sufficiently advanced to demonstrate conclusively that these particles are indeed the tumour-inducing factor. Because of the methods of preparation the suspensions containing the particles may be less potent than the original tumour extracts. Such an effect is well known in preparations of virus particles and may be due to a variety of causes. At the moment all we can say is that suspensions containing particles obtained from high-breast-cancer-strain tissues induce tumours in susceptible hybrid mice.

Finally, it should be mentioned that particles of the size described by PORTER AND THOMPSON were found by them in only 50% of the cells examined, whereas in our experiments particles of the sizes quoted were constantly present in all malignant tissue extracts from high-breast-cancer strains, whether prepared from fresh or desiccated tissues, and whether trypsinized or not.

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SUMMARY

Electron micrographs prepared from extracts of normal and malignant tissues of 3 high-breast-cancer strains (C3H, RIII, Strong A) of mice show the presence of approximately spherical particles of diameters ranging from about 200 Å to 1200 Å, though mostly not larger than about 300 Å. Similar micrographs from extracts of normal tissues and induced tumours of low-breast-cancer strains (C57 black, CBA, IF) either do not show such particles or show them only occasionally.

Extracts of RIII high-cancer-strain breast-tumour tissue show greater numbers of these particles than extracts of tumours from other high-breast-cancer strains. Electron micrographs of the extracts of breast tumours induced in C3H high-breast-cancer strain males show smaller numbers of characteristic particles than those of spontaneous breast tumours arising in females of this strain.

Electron micrographs of milk of C3H high and C57 black low-breast-cancer strains show similar numerical differences in the presence of the characteristic particles.

Electron micrographs after ultracentrifugation of extracts from high-breast-cancer-strain tumours indicate that the particles are completely sedimented in two hours at a speed corresponding to 120,000 times gravity, but the critical speed has not yet been established.

All extracts tested by the electron microscope and in the ultracentrifuge are being tested for the presence of the tumour-inducing agent by injecting them into C57 × RIII susceptible hybrid mice; 56 out of 221 mice injected with extracts which contained the particles in large numbers have so far developed tumours.

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The relationship between these particles and the tumour-inducing factor is not yet clear. It is necessary to await the final results of the biological tests in hybrid mice before a definite correlation may be regarded as established.

RÉSUMÉ

Des microphotographies électroniques d'extraits de tissus normaux et de tissus tumoraux de 3 races de souris à haut pourcentage de cancer de la mammelle (C3H, RIII, Strong A) montrent la présence de particules à peu près sphériques, de diamètre compris entre 200 Å et 1200 Å, mais le plus souvent pas supérieur à 300 Å. Des microphotographies analogues d'extraits de tissus normaux et de tumeurs induites provenant de races à faible pourcentages de cancer de la mammelle (C57 black, CBA, IF) ne montrent pas ou montrent seulement rarement de telles particules.

Les extraits de tissus tumoraux provenant de la souche à haut pourcentage de cancer de la mammelle RIII montrent un plus grand nombre de ces particules que les extraits de tumeurs des autres races à haut pourcentage de cancer de la mammelle. Les micrographies électroniques des extraits de tumeurs de la mammelle induites chez les mâles de la race à haut pourcentage de cancer de la mammelle C3H montrent un nombre plus faible des particules caractéristiques que les microphotographies des extraits de tumeurs spontanées de la mammelle se développant chez les femelles de cette race.

Des micrographies électroniques du lait de la race C3H à haut pourcentage de cancer de la mammelle, et de la race C57 black à faible pourcentage de cancer de la mammelle, montrent des différences numériques analogues en ce qui concerne les particules caractéristiques.

Des micrographies électroniques faites après ultracentrifugation des extraits de tumeurs provenant d'une race à haut pourcentage de cancer de la mammelle, montrent que les particules sont complètement sédimentées en 2 heures à une vitesse correspondant à 120.000 g, mais la vitesse critique n'a pas encore été déterminée.

Tous les extraits étudiés au microscope électronique et à l'ultracentrifuge sont actuellement essayés en ce qui concerne la présence d'un facteur inducteur de tumeur, en les injectant chez la souris hybride susceptible C57 × RIII. Jusqu'ici, 56 souris sur 221 ainsi traitées ont développé des tumeurs.

Les relations entre les particules en question et le facteur inducteur des tumeurs ne sont pas encore claires. Il est nécessaire d'attendre les résultats finaux des essais biologiques chez les hybrides des souris avant de pouvoir affirmer l'existence d'une corrélation entre ces deux systèmes.

ZUSAMMENFASSUNG

Mit Hilfe des Elektronmikroskops wurde gezeigt, dass Extrakte aus normalen und Tumorgeweben dreier Mäuserassen mit hohem Brustkrebsvorkommen (C3H, RIII, Strong A) annähernd kugelförmige Teilchen enthalten, deren Durchmesser zwischen 200 Å and 1200 Å, meistens aber nicht mehr als 300 Å beträgt. Ähnliche Mikrophotographien von Extrakten aus normalen Geweben oder induzierten Tumoren von Rassen mit einem geringen Brustkrebsvorkommen (C57 black, CBA, IF) weisen solche Teilchen entweder garnicht oder nur vereinzelt auf.

Brusttumor-Gewebextrakte der Rasse RIII, welche hohes Brustkrebsvorkommen aufweist, zeigen eine grössere Anzahl dieser Teilchen als Tumorextrakte von anderen Rassen mit gleichem Brustkrebsvorkommen. Elektronenmikrogramme von Extrakten aus induzierten Brusttumoren von Männchen der C3H Rasse mit hohem Brustkrebsvorkommen weisen eine geringere Zahl der charakteristischen Teilchen auf, als solche aus spontanen Brusttumoren der Weibchen dieser Rasse.

Ähnliche zahlenmässige Unterschiede in den vorhandenen charakteristischen Teilchen zeigen Elektronenmikrogramme von Milch des Stammes C3H mit hohem Brustkrebsvorkommen und des Stammes C57, der diese Krankheit selten aufweist.

Elektronenmikrogramme von ultrazentrifugierten Tumorextrakten eines Stammes mit hohem Brustkrebsvorkommen zeigen, dass die Teilchen nach zweistündigem Zentrifugieren bei einer Geschwindigkeit von 120,000 g vollständig sedimentiert waren; die kritische Geschwindigkeit wurde aber noch nicht ermittelt.

Alle mit dem Elektronenmikroskop und in der Ultrazentrifuge untersuchten Extrakte werden gegenwärtig durch Injektion in empfindliche Mäuse, Kreuzung C57 × RIII, auf das Vorhandensein von geschwulstbildenden stoffen geprüft; 56 von 221 Mäusen, die mit Extrakten von hoher Konzentration charakteristischer Teilchen injiziert worden waren, haben bisher Tumoren entwickelt.

Das Verhältnis dieser Teilchen zu dem geschwulstbildenden Faktor ist noch nicht deutlich. Die Endergebnisse der biologischen Versuche an den hybridisierten Mäusen müssen erst abgewartet werden, bevor ein endgültiger Zusammenhang zwischen diesen beiden Systemen als erwiesen betrachtet werden kann.

References p. 408/409.

REFERENCES

1 J. A. MURRAY, *Scient. Rep. Invest. Imperial Cancer Research Fund*, 4 (1911) 114.
2 C. C. LITTLE, *Biology of the Laboratory Mouse*, The Blakiston Company (1941) 248.
3 A. STRONG, *Cancer Res.*, 2 (1942) 531.
4 Staff of the ROSCOE B. JACKSON MEMORIAL LABORATORY, *Science*, 78 (1933) 465.
5 Staff of the ROSCOE B. JACKSON MEMORIAL LABORATORY, *Amer. J. Cancer*, 27 (1936) 551.
6 R. KORTEWEG, *Nederland Tijdschr. v. Geneesk.*, 78 (1934) 240.
7 W. S. MURRAY AND C. C. LITTLE, *Amer. J. Cancer*, 27 (1936) 516.
8 J. J. BITTNER, *Science*, 84 (1936) 162.
9 J. J. BITTNER, *Publ. Health Rep.*, 54 (1939) 1590.
10 J. J. BITTNER, *J. Nat. Cancer Inst.*, 1 (1940–1941) 155.
11 J. J. BITTNER, *Proc. Soc. Exp. Biol. & Med.*, 45 (1940) 805.
12 H. B. ANDERVONT AND W. J. MCELENEY, *Publ. Health Rep.*, 53 (1939) 777.
13 P. J. VAN GULIK AND R. KORTEWEG, *Nederland. Akad. van Wetenschappen Proc.*, 43 (1940) 891.
14 H. B. ANDERVONT, *J. Nat. Cancer Inst.*, 1 (1940–1941) 147.
15 W. S. MURRAY, *Cancer Res.*, 1 (1941) 790.
16 E. FEKETE AND C. C. LITTLE, *Cancer Res.*, 2 (1942) 525.
17 L. DMOCHOWSKI AND W. E. GYE, *Brit. J. Exp. Path.*, 24 (1943) 223.
18 H. B. ANDERVONT AND W. J. MCELENEY, *Publ. Health Rep.*, 54 (1939) 1597.
19 H. B. ANDERVONT, M. B. SHIMKIN AND W. R. BRYAN, *J. Nat. Cancer Inst.*, 3 (1942–1943) 309.
20 J. J. BITTNER, *Publ. Health Rep.*, 54 (1939) 1827.
21 J. J. BITTNER, *Science*, 93 (1941) 527.
22 J. J. BITTNER, *Science*, 95 (1942) 462.
23 W. R. BRYAN, H. KAHLER, M. B. SHIMKIN AND H. B. ANDERVONT, *J. Nat. Cancer Inst.*, 2 (1941–1942) 451.
24 M. B. VISSCHER, R. G. GREEN AND J. J. BITTNER, *Proc. Soc. Exp. Biol. & Med.*, 49 (1942) 94.
25 L. DMOCHOWSKI, *Brit. J. Exp. Path.*, 25 (1944) 138.
26 G. W. WOOLLEY, L. W. LAW AND C. C. LITTLE, *Cancer Res.*, 1 (1941) 955.
27 J. J. BITTNER, *A.A.A.S. Res. Conference*, Washington 25 D.C. (1945) 82.
28 L. DMOCHOWSKI, *Brit. J. Exp. Path.*, 25 (1944) 119.
29 J. J. BITTNER, *Cancer Res.*, 2 (1942) 710.
30 J. J. BITTNER, *Cancer Res.*, 2 (1942) 755.
31 J. J. BITTNER, personal communication quoted by M. B. SHIMKIN AND H. B. ANDERVONT, in *A.A.A.S. Res. Conference*, Washington 25 D.C. (1945) 133.
32 H. B. ANDERVONT AND W. R. BRYAN, *J. Nat. Cancer Inst.*, 5 (1944–1945) 143.
33 L. DMOCHOWSKI, *Brit. J. Exp. Path.*, 27 (1946) 391.
34 C. P. BARNUM, Z. B. BALL, J. J. BITTNER AND M. B. VISSCHER, *Science*, 100 (1944) 575.
35 H. KAHLER AND W. R. BRYAN, *J. Nat. Cancer Inst.*, 4 (1943–1944) 37.
36 J. J. BITTNER, *A.A.A.S. Res. Conference*, Washington 25 D.C. (1945) 81.
37 R. G. GREEN, M. M. MOOSEY AND J. J. BITTNER, *Proc. Soc. Exp. Biol. & Med.*, 61 (1946) 115.
38 L. DMOCHOWSKI AND J. W. ORR (in press).
39 J. W. ORR, *J. Path. & Bact.*, 55 (1943) 483.
40 L. DMOCHOWSKI, *Brit. J. Exp. Path.*, 26 (1945) 192.
41 L. DMOCHOWSKI, *Brit. J. Exp. Path.*, 26 (1945) 267.
42 P. L. TEMPLE AND S. K. KON, *Biochem. J.*, 31 (1937) 2197.
43 R. C. WILLIAMS AND R. W. G. WYCKHOFF, *J. App. Phys.*, 15 (1944) 712.
44 R. REED AND A. MILLARD, *Proc. E.M. Group (Inst. of Physics) Conference*, Leeds, September, 1947.
45 R. D. PASSEY, L. DMOCHOWSKI, W. T. ASTBURY AND R. REED, *Nature*, 160 (1947) 565.
46 R. D. PASSEY, L. DMOCHOWSKI, W. T. ASTBURY, R. REED AND P. JOHNSON, *Nature*, 161 (1948) 759.
47 R. D. PASSEY, L. DMOCHOWSKI, W. T. ASTBURY, AND R. REED, *Report of the 7th Conference on E.M.*, 16–17 September, 1947, p. 13.
48 W. T. ASTBURY, R. REED, R. D. PASSEY AND L. DMOCHOWSKI, *Report of the 8th Conference on E.M.*, 7–8 April, 1948, p. 13.
49 L. DMOCHOWSKI, *Report of the Dept. of Exp. Path. & Cancer Res.*, University of Leeds (1947–'48), Yorkshire Council, British Empire Cancer Campaign.
50 J. J. BITTNER, *Cancer Res.*, 3 (1943) 441.
51 J. J. BITTNER, *Cancer Res.*, 4 (1944) 159.
52 H. B. ANDERVONT, *Symposium on Mammary Tumours in Mice. By Members of the Staff of the National Cancer Inst., A.A.A.S.*, Washington 25 D.C. (1945) 129.
53 S. L. BAKER AND J. MCINTOSH, *Brit. J. Exp. Path.*, 8 (1927) 257.
54 F. C. BAWDEN, *Plant Viruses and Virus Diseases*, 2nd ed., (1943) Chronica Botanica Co., 144.
55 F. C. BAWDEN AND N. W. PIRIE, *Brit. J. Exp. Path.*, 27 (1946) 81.

[56] H. S. LORING AND C. E. SCHWERDT, *Proc. Soc. Exp. Biol. & Med.*, 62 (1946) 289.
[57] H. S. LORING, L. MARTON AND C. E. SCHWERDT, *Proc. Soc. Exp. Biol. & Med.*, 62 (1946) 291.
[58] H. S. LORING AND C. E. SCHWERDT, *J. Exp. Med.*, 75 (1942) 395.
[59] S. GRAFF, D. H. MOORE, W. M. STANLEY, H. T. RANDALL and C. D. HAAGENSEN, *4th Intern. Cancer Research Congress*, Sept. 2–7 (1947) 144.
[60] K. G. STERN AND F. DURAN-REYNALS, *Science*, 89 (1939) 609.
[61] A. CLAUDE, *Proc. Soc. Exp. Biol. & Med.*, 39 (1938) 398.
[62] A. CLAUDE, *Cold Spring Harbor Symposia on Quant. Biol.*, 9 (1941) 263.
[63] A. CLAUDE, *Science*, 97 (1943) 451.
[64] W. HENLE, L. A. CHAMBERS AND V. GROUPE, *J. Exp. Med.*, 74 (1941) 495.
[65] J. FURTH AND E. A. KABAT, *J. Exp. Med.*, 74 (1941) 247.
[66] R. W. GLASER AND R. W. G. WYCKOFF, *Proc. Soc. Exp. Biol. & Med.*, 37 (1937–'38) 503.
[67] R. W. G. WYCKOFF, *Cold Spring Harbor Symp. Quant. Biol.*, 6 (1938) 361.
[68] A. CLAUDE, *J. Exp. Med.*, 84 (1946) 61.
[69] L. CHAMBERS AND W. HENLE, *J. Exp. Med.*, 77 (1943) 251.
[70] L. CHAMBERS, W. HENLE, M. A. LAUFFER AND TH. F. ANDERSON, *J. Exp. Med.*, 77 (1943) 265.
[71] M. A. LAUFFER AND W. M. STANLEY, *J. Exp. Med.*, 80 (1944) 531.
[72] W. M. STANLEY, *J. Exp. Med.*, 79 (1944) 267.
[73] C. A. KNIGHT, *J. Exp. Med.*, 80 (1944) 83.
[74] A. E. GESSLER AND C. E. GREY, *Exp. Med. & Surg.*, 4 (1947) 307.
[75] K. R. PORTER AND H. P. THOMPSON, *J. Exp. Med.*, 88 (1948) 15.
[76] F. L. HORSFALL Jr., P. H. HARDY AND F. M. DAVENPORT, *Bulletin of the N.Y. Academy of Med.*, July (1948) 470.
[77] C. E. WOODRUFF AND E. W. GOODPASTURE, *Am. J. Path.*, 5 (1929) 1.
[78] F. C. BAWDEN AND E. M. CROOK, *Brit. J. Exp. Path.*, 28 (1947) 403.
[79] C. A. KNIGHT, *J. Exp. Med.*, 83 (1946) 281.
[80] F. L. HORSFALL Jr. AND R. G. HAHN, *J. Exp. Med.*, 71 (1940) 391.
[81] K. C. MILLS AND A. R. DOCHEZ, *Proc. Soc. Exp. Biol. & Med.*, 60 (1945) 141.
[82] E. C. CURNEN AND F. L. HORSFALL Jr., *J. Exp. Med.*, 83 (1946) 105.
[83] E. C. CURNEN AND F. L. HORSFALL Jr., *J. Exp. Med.*, 85 (1947) 39.
[84] M. VOLKERT AND F. L. HORSFALL Jr., *J. Exp. Med.*, 86 (1947) 393.
[85] E. C. CURNEN, E. G. PICKELS, AND E. L. HORSFALL Jr., *J. Exp. Med.*, 85 (1947) 23.
[86] L. DMOCHOWSKI, *Brit. J. Cancer*, 2 (1948) 94.

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