mice injected intraperitoneally with β-mercaptoethylamine (*i.e.*, cysteinamine) prior to a lethal exposure of 700 r whole body x-irradiation survived the 30-day observation period. Some 97% of these are still alive 60 days after irradiation. The CF_1 mice, exposed to the higher dose of 800 r after injection, showed 58% survival while the controls, receiving only 700 r, all died within 20 days. Thus prior injection of this compound definitely affords radiation protection to mice. Injection after x-irradiation, whether single or multiple, had an adverse effect on survival.

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A Filterable Agent, Recovered from Ak Leukemic Extracts, Causing Salivary Gland Carcinomas in C3H Mice.* (20376)

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In previous experiments reported from this laboratory, Seitz filters were used for the filtration of Ak leukemic extracts; the results, however, did not appear to be entirely satisfactory, since only a comparatively small number of C3H mice developed leukemia following inoculation with such filtered extracts (1). The possibility had to be considered that much of the leukemic agent might have been absorbed, and thus retained, by the asbestos pads used in Seitz filters. For that reason a series of experiments has been carried out in which, instead of Seitz, the Selas† microporous porcelain filter candles have been used for filtration of leukemic extracts. Since the leukemic extracts became more active following centrifugation at 7000 x g(2), such centrifugation was carried out, in this study, prior to filtration of the extracts. Newborn (less than 16 hours old) C3H or C3H(f) mice were inoculated with such filtered extracts, and were then observed for development of leukemia. The results were surprising. Some of the inoculated mice developed typical generalized leukemia. Others, however, developed bilateral carcinomas in the cervical region, arising probably in the salivary glands.

Materials and methods. Leukemic extracts. Mice of the Ak line that developed leukemia either spontaneously, or as a result of transplantation of Ak leukemic cells, were sacrificed by ether inhalation; the livers, spleens, mesenteric tumors and peripheral lymph glands were removed aseptically, and ground for 2 to 3 minutes in a porcelain mortar, chilled (+4°C) physiological solution of sodium chloride added in sufficient quantity to obtain cell suspensions of 20% concentration. These leukemic cell suspensions were

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† Selas Corp. of America, Philadelphia, Pa.

TABLE I. Results of Inoculations of Filtered (Selas) Ak Leukemic Extracts into Newborn C3H or C3H(f) Mice.

No. of individual exp.	Prelim. centrif.,* 7000 × g, min.	Filter candle (Selas) porosity	Filter pressure†	Strain of mice inoc.	Avg age at inoc., hr	Sex	Fresh‡ fil. extr. No. of mice inoc.	Fresh‡ fil. extr. No. of mice dev. neck tum.	Fresh‡ fil. extr. No. of mice dev. leuk.	Fresh‡ fil. extr. Avg age when neck tum. (leuk.) appeared, mo	Heated (68°C ½ hr) fil. extr. No. of mice inoc.	Heated (68°C ½ hr) fil. extr. No. of mice dev. neck tum. or leuk.
5	5 to 10	03	pos	C3H	<12	♀	7	2	2	3.6	6	0
						♂	5	1	0		5	0
5	"	"	"	C3H(f)	< 9	♀	7	2	0	3.8	3	0
						♂	7	1	2		5	1§
9	10	"	vac	C3H	<11	♀	10	4	1	4.5	5	0
						♂	9	5	0		9	0
11	5 to 10	"	"	C3H(f)	<12	♀	17	0	2	3.8	7	0
						♂	13	0	0		11	0
1	10	02	"	C3H	<16	♀	2	0	1	3	2	0
						♂	1	0	0			
2	"	"	"	C3H(f)	"	♀	3	0	1	4	1	0
						♂	3	0	0		3	0
				Total			84	15	9		57	1§

* Ak mice which developed "spontaneous" leukemia were used as donors for preparation of leukemic extracts in 16 experiments. In all other (17) experiments Ak mice with transplanted leukemia were used. Leukemic extracts centrifugated in all experiments at 3000 RPM (1400 × g) for 15 min. prior to centrifugation at 7000 × g indicated in this column.

† Positive (10 lb) pressure (95% N mixed with 5% CO_2) used for filtration of leukemic extracts through Selas candles in experiments designated in this column "pos." Experiments designated "vac," vacuum pressure of 20-25 mm of mercury was applied.

‡ Filtered leukemic extracts used for inoculation immediately after preparation in 15 experiments. Extracts kept in ice water at 0°C prior to use for inoculation for less than 7 hr in 4 exp., for less than 24 hr in 10 exp., and for less than 48 hr in 4 exp.

§ This mouse developed a neck tumor at 4 mo of age.

centrifuged at 0°C in an International PR-1 Centrifuge at 3000 RPM (1400 x g) for 15 minutes. The supernatant was then removed, placed in a chilled (0°C) celluloid tube, and centrifugated at 0°C, at 9500 RPM (7000 x g) for periods of time varying from 5 to 10 minutes (Table I) in the International PR-1 Centrifuge. It required 2 to 3 minutes for the head of the high-speed attachment to reach 7000 x g. After centrifugation at this speed for the specified time (Table I), the motor was turned off, the pulley disconnected, and the centrifuge head came gradually to a standstill (requiring additional 7 to 10 minutes). Approximately two-thirds of the upper part of the resulting supernatant was then carefully removed with a syringe, and immediately passed through the filter, as described below.

Filtration. Selas† microporous filter candles with filter elements 2″ long and of a 5/8″ diameter, porosity 03 (FPS-52-03), and in a few instances of porosity 02 (FPS-52-02), were used (Table I). In these candles, the filter elements are glazed directly to the porcelain connectors, forming single units, without any metal or rubber connections. The filter candles were inserted through a rubber stopper into standard, 125 cc (or 250 cc) pyrex vacuum flasks, with the filter element placed downward, inside the flask, and the porcelain connector extending, through the stopper, outside the top of the flask. The centrifugated leukemic extracts, ready for filtration, were then introduced, by means of a sterile syringe, directly inside of the porcelain connector extending outside the flask (Fig. 1). Vacuum pressure of approximately 20 to 25 mm mercury was then applied, and the extract passed through the filter into the flask. The extract did not contact with the rubber tubing or any other part of the filtration system except the filter candle and the pyrex flask. In a few experiments (Table I), positive pressure, instead of vacuum pressure, was applied (mix-

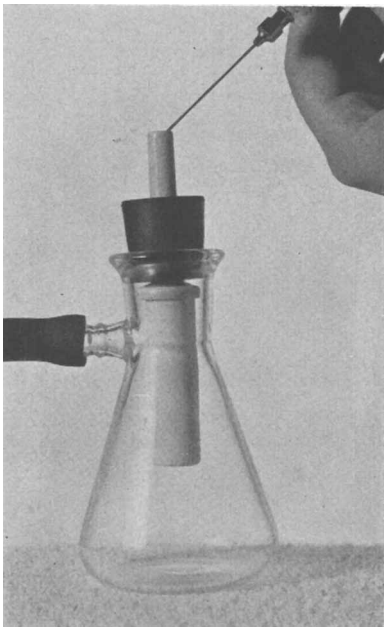

FIG. 1. Selas filter candle mounted for vacuum filtration. The leukemic extract is introduced directly, by means of a syringe, into the porcelain connector of the filter candle extending outside the flask.

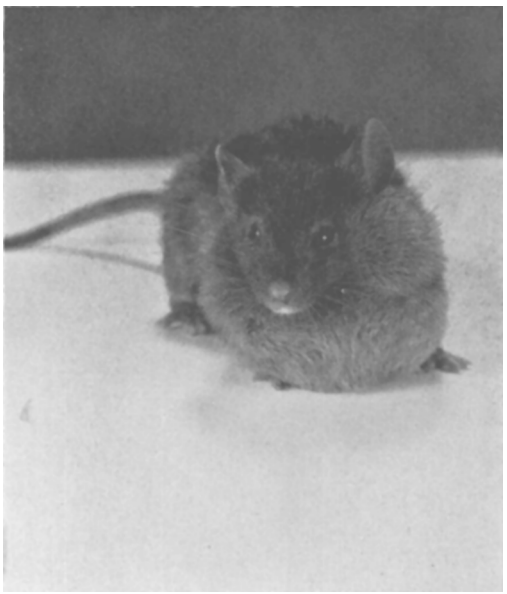

FIG. 2. Salivary gland epithelioma developed in a C3H male at 4 mo of age. This mouse had been inoculated with Ak leukemic filtered extract (Selas candle porosity 03) within 12 hr after birth. Photograph taken after tumor had been allowed to grow for 1 mo.

ture of 95% nitrogen, and 5% carbon dioxide, under 10 lb pressure, blown into the filter candle through a rubber tubing pulled over the porcelain connector). Each filter had a record number, and was first tested with, and found to be impervious to, *E. coli*. The fresh suspension of *E. coli* culture in tryptose phosphate broth, diluted 1:10 with physiological saline solution, was passed through the filter candle, and the resulting filtered extract proved to be sterile, as evidenced by inoculation of tryptose phosphate broth media. After this preliminary test, the filter candle was washed with distilled water, autoclaved (one hour at 115°C at 15 lb) and used for the filtration of the leukemic extracts. Immediately after filtration, the candle was again tested with, and found to be impervious to, *E. coli*. The candle was now baked in a Selas filter candle furnace, in order to burn out organic matter remaining after filtration, then washed with distilled water, and autoclaved. For the second time the candle was now used for filtration of another leukemic extract; immediately afterwards, the candle was again

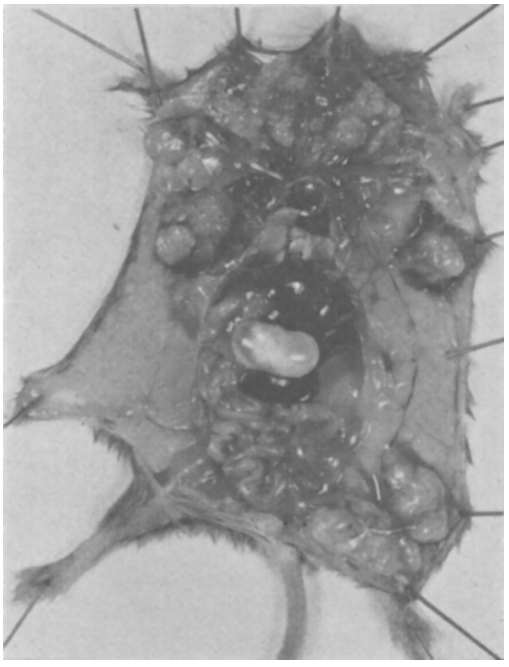

FIG. 3. This C3H(f) female was inoculated (within 16 hr after birth) with a centrifugated (7000 × g) extract prepared from normal Ak embryos, and developed bilateral salivary gland carcinomas at 3 mo of age. Two weeks later, additional tumors developed in both axillary pits, and in the left groin.

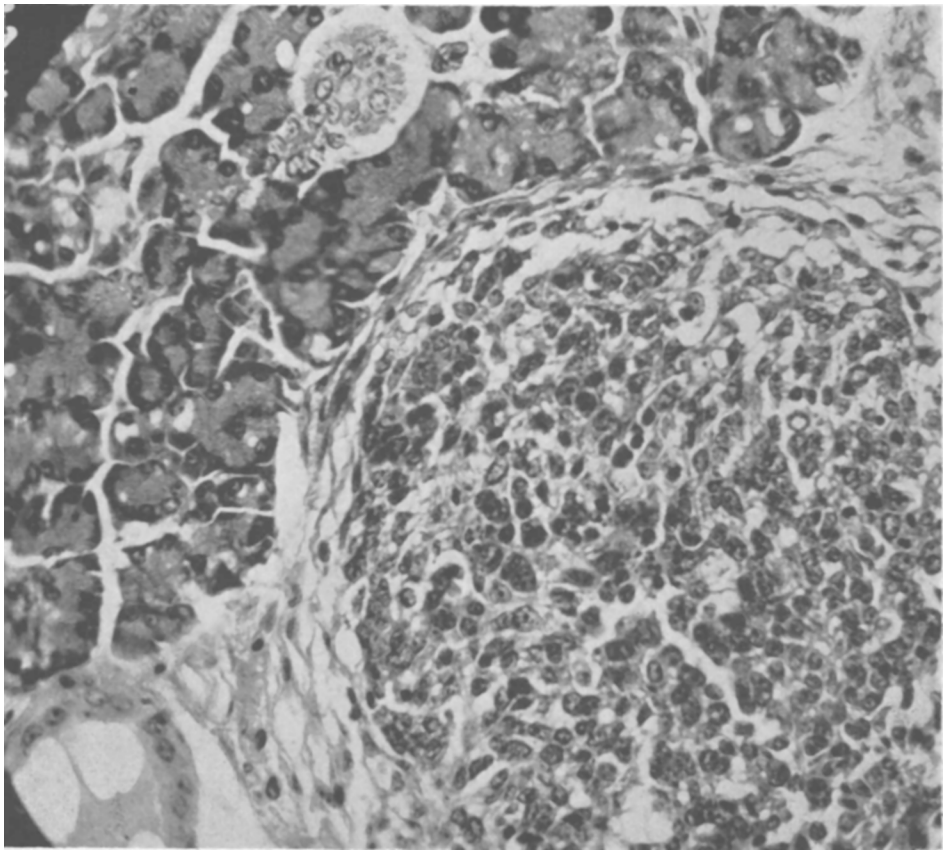

FIG. 4. Photomicrograph of a salivary gland epithelioma, most probably arising in the parotid gland. This tumor developed in a C3H female, at 3½ mo of age, following inoculation (within 12 hr after birth) of a filtered (Selas, porosity 03) Ak leukemic extract. (H. & E. × 475.)

tested with, and found to be impervious to, *E. coli*. The procedure of baking in an oven, washing, and autoclaving, was then repeated, and again the candle was used for the filtration of a third leukemic extract, followed immediately by testing with *E. coli*. After 4 or 5 of these consecutive filtrations, the candle was discarded.

The filtration procedure was carried out as follows: The autoclaved Selas filter candle was first briefly washed by passing 10 to 15 cc of chilled (0°C) sterile physiological saline solution through the candle, under the usual vacuum pressure of 20 to 25 mm of mercury. The leukemic supernate resulting from the 7000 x g centrifugation was then passed through the filter candle using either positive or vacuum pressure. Immediately after filtration, the filtered leukemic extract was removed from the flask aseptically, placed in a sterile pyrex glass tube immersed in ice-water, and designated "fresh filtered leukemic extract." Part of the filtered extract was heated in an electric waterbath to a temperature of 68°C for 30 minutes (Table I), then placed in a sterile pyrex glass tube immersed in ice-water, and designated "heated filtered leukemic extract." The extracts were inoculated in most instances immediately or within 24 hours, occasionally, however, within 48 hours, after their preparation (Table I), having been kept in tubes immersed in a container filled with ice tubes, and placed in an electric refrigerator.

Inoculation of mice. Newborn (less than 16 hours old) mice of our colonies(1,2) of either the C3H line or of the foster nursed C3H(f) subline, bred in our laboratory by brother-to-sister mating, and having an incidence of spontaneous leukemia far below 0.5%, were then inoculated with the extracts.

Each newborn litter was divided, one part inoculated with the fresh, and the other part with heated filtered extract. Inoculations were subcutaneous (0.1 cc each). The skin punctures were dried off gently with a sterile sponge, and then sealed off with a drop of collodion. For the purpose of identification, the mice of each litter injected with either the fresh or the heated supernate had their tails tied off at half length and cut immediately after inoculation. The mice were weaned at 21 days of age; they were then identified by individual numbers, and separated by sex.

Results. The results were surprising: while only 9 of the 84 mice that had been inoculated with the fresh filtered extracts developed generalized leukemia, 15 other mice developed, at an average age of 3.3 months, bilateral tumors in the cervical region (Fig. 2). These tumors slowly enlarged, and eventually formed enormous confluent masses around their necks. On dissection (Fig. 3) these tumors presented grape-like clusters of individual small tumors, or, in more advanced cases, solid masses of hard (difficult to cut and grind) white tumor tissue, infiltrating the cervical region, on both sides of the neck. Microscopic examination of the tumors (Fig. 4) revealed undifferentiated tumor cells with large, vesicular nuclei, and numerous atypical mitotic figures, frequently growing either in bands of cells, or forming glandular pattern; occasionally, however, no pattern was discernible, the tumor cells growing chaotically (Fig. 5). In spite of their individual differences, these tumors, apparently arising in the salivary glands, could be classified as carcinomas.‡ Additional tumors could sometimes be found in the axillary pits, and occasionally also in the inguinal region (Fig. 3). Animals with these tumors showed no other signs of disease: microscopic sections of liver showed no changes; the peripheral blood picture as well as the bone marrow were normal. The cervical tumors reported in this study were essentially similar to those recently observed in C3H mice following inoculation of centrifugated (144000 x g) Ak leukemic extracts(3). The neck tumors could be transplanted, by cell inoculation, into mice of the C3H, but not those of the Ak, line(3,4). They were found to grow in some of the inoculated C3H mice, after an incubation period of 4 to 8 weeks, at the site of implantation (subcutaneous or intraperitoneal), infiltrating the surrounding tissues and eventually forming enormous tumors.

That the neck tumor agent could be inactivated by heating to 68°C for 30 minutes, was evident from a series of experiments (Table I), in which 54 control litter-mates were inoculated with the heated leukemic ex-

‡ Dr. Maurice N. Richter, Department of Pathology, New York University-Bellevue Medical Center, kindly reviewed the tumor slides, and commented as follows: The sections of neck tumors suggest that the tumor is in the salivary glands, often forming cell accumulations around small glands or ducts, and eventually displacing most of the glandular tissue. In the less involved areas, small groups of tumor cells suggest the possibility of multiple foci of origin. The cells are rounded or elongated. I am inclined to believe that all of the cells are epithelial. It is possible that these tumors arise from myoepithelial cells of the salivary glands, as suggested by Lippincott and associates(6). However, I have been unable to see longitudinal fibrils, and consider the origin uncertain at the present time.

Dr. Jacob Furth, Biology Division, Oak Ridge National Laboratory, dissected in our laboratory two C3H mice that had developed bilateral neck tumors as a result of inoculation with Ak leukemic filtered (Selas) extracts. Dr. Furth also reviewed the microscopic slides of these tumors. His comments follow: "The 'neck' tumors in the two animals which I have dissected involve the parotid gland and extend in the region of the submaxillary glands, but the latter were free from tumor. Likewise the lymph nodes of these animals examined showed neither leukemic involvement nor metastatic tumor. The site of the primary tumors is the parotid gland. The sections which you have shown me indicate multicentric origin of the tumors in the parotid glands. The appearance of the tumors in sections, some resembling carcinomas, others sarcomas support the belief that they originate in a salivary gland. Further dissections are indicated to find out if salivary glands other than the parotid can give rise to primary tumors. Whether the subcutaneous tumors in the axilla and groins are primary or secondary remains to be determined; if primary, the possibility exists that your agent can also cause cancer in the mammary gland. I recommend designating the 'neck' tumor as 'salivary gland tumor'."

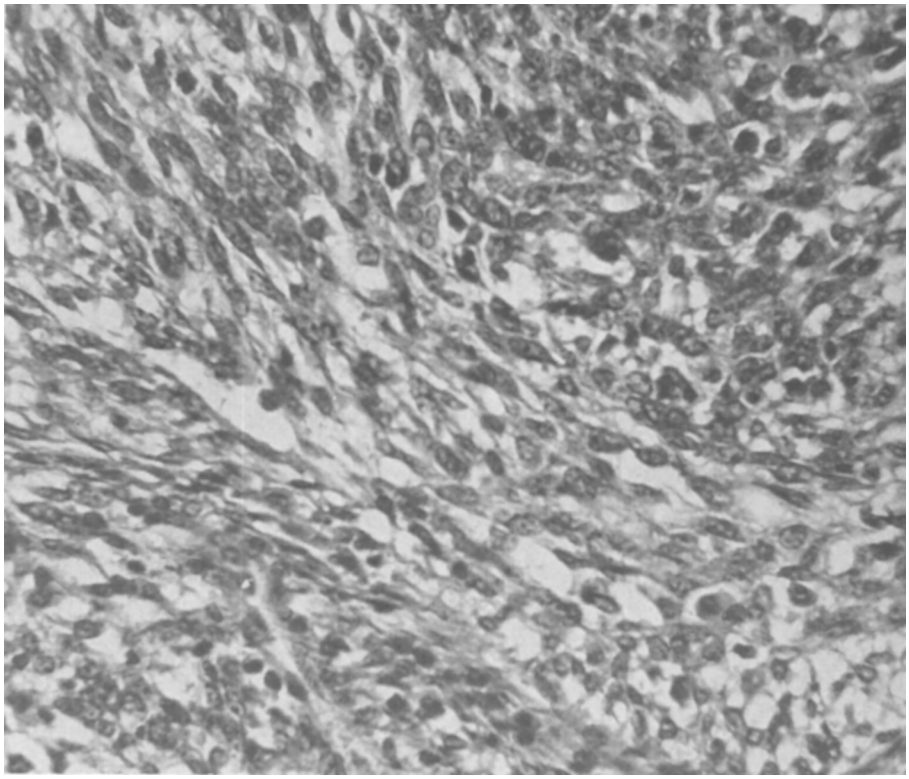

FIG. 5. Photomicrograph of a salivary gland tumor showing a sarcomatous appearance. Elsewhere same tumor had structure of anaplastic adenocarcinoma, particularly on opposite side of neck in same mouse. This tumor developed bilaterally on the neck of a C3H female, at 3 mo of age, following inoculation (within 16 hr after birth) of a filtered (Selas, porosity 03) Ak leukemic extract.

tracts: only one of them developed a salivary gland tumor.

The presence of the tumor agent in normal Ak embryos. In order to determine whether the salivary gland tumor agent is present in normal Ak embryos, 2 young (3-month-old) healthy pregnant Ak females were sacrificed, their embryos removed aseptically, separated from placenta, and ground with physiological saline solution. The resulting embryo cell suspension was centrifugated at 3000 RPM (1400 x g) for 15 minutes; the supernate was again centrifugated at 9500 RPM (7000 x g) for 5 minutes. The final supernate was removed carefully with a syringe, and immediately inoculated into 4 newborn (less than 16 hours old) litters of C3H(f) mice. The litters were divided as follows: 12 infant mice were inoculated with the Ak embryo centrifugated extract, and 11 control litter-mates were inoculated with a similar extract prepared simultaneously under identical experimental conditions from C3H embryos. As a result, 4 of the 12 C3H(f) mice that had been inoculated with the Ak embryo extracts developed typical, bilateral neck carcinomas at 3 months of age; the 11 control litter-mates that had been inoculated with C3H embryo extracts remained in good health. In previous experiments(5), the inoculation of Ak embryos resulted in the development of a generalized leukemia in the injected C3H(f) mice, and not that of neck tumors; however, in previous studies, Ak embryo cell suspensions, and not centrifugated extracts, had been used for inoculation(5).

Discussion. It is apparent from experiments reported in this and previous(1-3) studies, that the inoculation of centrifugated, or filtered Ak leukemic extracts into newborn

(less than 16 hours old) C3H or C3H(f) mice may result in the development of either a generalized leukemia, or bilateral cervical epitheliomas, possibly arising in the salivary glands.‡ One may determine, by selecting proper experimental conditions, which of the two neoplastic conditions, *i.e.* leukemia or cervical epitheliomas will develop in the inoculated animals. When Ak leukemic extracts centrifugated at 3000 RPM (1400 x g), or 9500 RPM (7000 x g), were inoculated into newborn C3H or C3H(f) mice, leukemia resulted and not salivary gland tumors(1,2). When Ak leukemic extracts were filtered through Berkefeld (N) candles, then inoculated into newborn C3H mice, leukemias developed with only very few mice developing cervical epitheliomas(3). On the other hand, when the Ak leukemic extracts were centrifugated at 144000 x g and the resulting supernatant was used for inoculation of newborn C3H mice, cervical epitheliomas developed much more frequently than generalized leukemia(3). In experiments reported in this study the Ak leukemic extracts were filtered through Selas micro-porcelain filter candles, and the extracts were inoculated; as a result, 15 out of 84 inoculated animals developed cervical epitheliomas, and only 9 generalized leukemia. Curiously, only extracts that had been filtered through candles having a very fine porosity of 03, produced neck carcinomas (but occasionally also leukemia); of 9 mice inoculated with extracts filtered through 02 porosity candles, 2 developed leukemia, but none salivary gland tumors (Table I). The number of mice in this series is too small to draw any definite conclusions, and additional experiments have since been instituted(4) to determine whether the finer porosity filter candles (03) must be used for the preparation of extracts capable of producing salivary gland epitheliomas.

Leukemic extracts could be inactivated by heating in a waterbath to 68°C for 30 minutes. Of 54 control litter mates inoculated with such heated extracts, one developed a salivary gland tumor (Table I). There is a possibility, that in this experiment the tube containing the extract had not been completely immersed in the waterbath, and that part of the extract adhering to the top of the test tube, might not have been properly heated and remained active (this technical error has been corrected in subsequent experiments). On the other hand, it is also possible that the neck tumor agent may require heating to a temperature slightly higher than 68°C for 30 minutes for complete neutralization.

While the number of leukemias developing among the mice that had been inoculated with fresh filtered extracts is small (9 out of 84), it must be stated that only 7 months have elapsed since these experiments were initiated; some of the injected mice are only 5 months old at the time of this writing, and may develop leukemia later; moreover, 15 mice developed salivary gland tumors at 3.3 months of age, that is, before they had a chance to develop leukemia.

On rare occasions, salivary gland tumors have been recorded in albino mice of strains C and A(6). However we have not seen salivary gland tumors develop "spontaneously" in untreated mice of either the C3H line or of the C3H(f) subline. We have never seen salivary gland epitheliomas in mice of our colony of Ak mice, which were donors of the leukemic extracts. Moreover, among the C3H or C3H(f) mice that had been inoculated with filtered, or with centrifugated Ak leukemic extracts(1-3), some developed either leukemia, or cervical carcinomas(3), but never both.

The following working hypothesis may explain the experimental results thus far obtained: Mice of the Ak line may carry at least 2 different oncogenic agents, both present in leukemic Ak extracts. One of these agents would cause leukemia ("Ak leukemic agent"); the other salivary gland epitheliomas ("Ak tumor agent"). Since these epitheliomas do not spontaneously develop in mice of the Ak line, whereas spontaneous leukemia is very common in these animals, it would follow that in mice of the Ak line the leukemic agent interferes with the tumor agent, or, that the Ak mice are not susceptible to the pathogenic action of the Ak tumor agent, even though they carry, and transmit it from one generation to another, along with the Ak leukemic agent(4,5). Experiments reported in this, and the preceding(3) article could be explained by the assumption that 2 such dif-

ferent agents are harbored by mice of the Ak line, and can be extracted from organs of leukemic Ak mice. These agents could be separated either by (a) ultracentrifugation (144000 x g), the carcinoma agent remaining mainly in the supernate(3), or (b) by filtering the extracts through fine porosity filters, such as Selas 03. Heating to 63°C or 64°C for 30 minutes may also neutralize the leukemic agent, but not the agent causing salivary gland epitheliomas(4). A higher temperature (at least 68°C for 30 minutes) may be required for complete inactivation.

Should leukemia be caused by a transmissible agent not only in chickens and mice, but also in humans, the possibility would have to be considered that in humans also the leukemic agent may be accompanied by another oncogenic agent transmitted, along with the leukemic agent, from one generation to another. An interference phenomenon could be responsible for the apparent latency of either, or both, in most of the carrier hosts. Occasionally, however, a segregation of such agents may occur, resulting in the development of leukemia in some, and carcinomas in others. This hypothesis may offer an explanation of clinical observations suggesting that in families of patients suffering from leukemia, cancer is more common than in the average population(7,8).

Summary. 1. Extracts prepared from livers, spleens, and lymphatic tumors of leukemic Ak mice, were centrifugated at 1400 x g (15 minutes), then 7000 x g (5 to 10 minutes), and finally passed through Selas porcelain filters. The filtered extracts were then inoculated into newborn (less than 16 hours old) C3H or C3H(f) suckling mice. 2. Of the 84 mice inoculated with fresh, filtered extracts, 9 died from leukemia, and 15 others developed bilateral epitheliomas possibly arising in the salivary glands.‡ Of the 54 control littermates inoculated with heated (68°C for 30 minutes) extracts, only one developed a cervical tumor. 3. Extracts prepared from Ak leukemic cells may therefore contain 2 different agents, pathogenic for newborn C3H or C3H(f) mice: one, causing leukemia, and another salivary gland epitheliomas. These agents may differ in size. 4. It is assumed that the leukemic agent may interfere with the tumor agent preventing it from exerting its pathogenic properties on the cells of its usual Ak, carrier host, or that mice of the Ak line may be naturally resistant to the tumor agent. 5. Salivary gland tumors have not been observed in untreated mice of either the Ak, the C3H, or C3H(f) inbred lines.

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