STUDIES ON THE ISOLATION OF AGENTS FROM TUMORS.

By

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A THESIS

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ABSTRACT

Many tumors of both animals and man have been reporte to be caused by viruses, for example, Rous sarcoma (Rous 1910), mouse mammary carcinoma (Bittner 1936), human papilloma (Wile and Kingery 1919), and infectious myxomatosis (Rivers 1926).

In this experiment 17 tumors were obtained from both animals and man. Both tissue culture and serological methods were used in an attempt to isolate the causative agent of these tumors.

Nine tumors grew well in tissue culture. There was n correlation between origin or type of the neoplasm and growth *in vitro*.

Concentrated tumor allantoic fluid, representing the fifth passage of tumor extracts through embryonated chicke eggs, retarded the growth of normal chicken heart tissue i cultures. One exception was encountered in which exceptic growth occurred.

When such concentrated allantoic fluids were tested for hemagglutination with normal chicken cells, no reaction occurred. Concentrated tumor allantoic fluid in many cases produced hemagglutination of chicken erythrocytes previously sensitized with Newcastle disease virus or modified with trypsin. When the concentrated allantoic fluid was tested for hemagglutination with sensitized or modified cells between each passage, hemagglutination was frequently observed. None of the 17 tumors studied contained an agent that was capable of producing hemagglutination through all five consecutive serial passages.

No interference, *in vitro* or *in vivo*, could be detected between concentrated allantoic fluid tumor passage material and Newcastle disease virus.

Walter N. Mack

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I INTRODUCTION

Most of the theories that have been offered regarding the cause and the nature of cancer fall into one of the following categories: (1) embryonic, (2) biochemical, (3) genetic or (4) infectious agents. (Ackerman and delRegato, 1947)

Cohnheim postulated (Ackerman and delRegato, 1947) that neoplasms arise from embryonal cells which have persisted and which retain a special proliferative potency. Ribbert's modification of the theory was that differentiated but embryologically displaced cells serve as foci for the genesis of neoplasia (Ackerman and delRegato, 1947).

The biochemical theories assume that certain specific biochemical or biophysical alterations in the environment of the cells cause them to acquire neoplastic properties, for example, those caused by chronic irritation, carcinogenic hydrocarbons and other carcinogenic agents.

According to Ackerman and delRegato, "There is adequate proof that genetic background influences susceptibility to neoplastic reactions. Neoplasms are characters and not genic factors, and susceptibility to neoplasms is expressed in degree. The view that cancer is a single Mendelian factor, either dominant or recessive, is no longer tenable. The continued reproduction of cells in neoplasia and the transmission of characters from one cell

to another for an almost limitless number generations are in agreement with the view that cancer is a manifestation of a genetic difference from normal cells, or a genetic alteration of normal cells."

In this investigation we are interested in the infectious agent theory of cancer. There are several types of neoplasia which are associated with a virus-like agent. The virus, being a self-reproducing entity, is thus transmitted through subsequent generations of the cell. Cancer, according to this theory, is an infectious process and the result of a virus-cell symbiosis.

Rhoads' (1952) opinion was that it may not be enough to invoke virus production if a new neoplasm is caused by the injection of a cell-free extract from an old one. He considered that there is an important distinction to be drawn between cancer and an infectious process of the conventional type. He also suggested that neoplasia may be a process of aggressive cellular growth due to the inheritance of new characteristics, consequent to the modification, by mutation, of components filterable, or non-filterable, which are responsible for old characteristics.

Pinkerton (1952) suggested that a virus must possess certain properties if it is to be successful as a cause of cancer: (1) It must be well adapted to life within its host cells. This means that its reproduction must be geared to that of the cells, but not necessarily as closely as

that of genes, which, in normal mitosis, multiply precisely once for each cellular division. (2) It must be of low virulence and incapable of inducing a high degree of cellular immunity in its host. This suggests that it must be rather closely related to normal cytological constituents, so that it does not behave as a "foreign" protein. (3) It must stimulate its host cells to continuous aggressive proliferation.

Pinkerton finally concluded "Until then, I see nothing unscientific in accepting tentatively the hypothesis that cancer may be caused by self-duplicating virus-like agents."

LITERATURE REVIEWS

II LITERATURE REVIEWS

The Infectious Sarcomata of Chickens

Rous in 1910 described the first of a series of transplantable, malignant sarcomas of the chicken. In 1911 he showed that the tumor could be induced with dried cells, with cells that had been destroyed with glycerol, and with cell-free filtrates. The tumor is now known as the Rous sarcoma. It is a spindle-cell sarcoma which metastatizes freely and usually destroys its host within one month. In 1912 Rous, Murphy, and Tytler described another chicken tumor transmissible with filtrates, an osteochrondrosarcoma, and a third, a spindle-cell angiosarcoma.

The active principle of these tumors, particularly of Rous sarcoma, has been extensively studies by Murphy and co-workers (1931, 1932) and by Sittenfield, Johnson, and Jobling (1931). The active agent has not been isolated in a pure state, but relatively pure extracts have been made by chemical precipitation. They possess the power of stimulating neutralizing antibodies when injected into rabbits, and in other respects do not differ in behavior from viruses that cause infectious diseases.

Mouse Mammary Carcinoma

Since Bittner's discovery (1936) of the "nursing

influence," it has been possible to define mouse mammary carcinoma as a disease of the adult female, acquired in infancy through an agent transmitted in the mother's milk. The definition is neither complete nor exclusive, but it is a consequence of Bittner's demonstration of the "nursing influence." With this in mind, many laboratories attempted to isolate the agent from milk. Graff et al. and Passy et al. (1947), with an electron microscope, found small spherical particles having a diameter of from 20 to 200 mu. in samples of milk obtained from nursing mice known to carry the mammary carcinoma. Graff et al. (1947) and Dossing et al. (1949) reported that similar spherical particles have also been found in tumor extracts. Porter and Thompson (1948) also reported that they found these particles in cells cultured from mouse mammary carcinomas.

It is difficult, at the present time, to form an opinion concerning the nature of these particles. One has to take into consideration that particles similar in shape and size have been found in extracts prepared from presumably normal mouse tissue, and in milk samples collected from mice apparently free from the mammary carcinoma agent. The differentiation of the various particles with the electron microscope is, at the present time, quite difficult. Many particles which may be of an entirely different origin may appear alike to the critical observer. This is particularly true when particles are observed after they have

been shadowed with heavy metals.

It is true that the exact nature of the particles detected in mouse milk have not yet been determined. Their consistent presence, however, in mouse milk known to contain the mammary carcinoma agent, and their only occasional presence in samples of milk collected from mice known to be free from the tumor agent, suggests that they may represent the mouse mammary carcinoma agent. Such an assumption was strengthened by the results of Graff et al. (1949) and Passey et al. (1950). Milk samples containing the particles were fractionated by centrifugation. Upon injection of this material into susceptible mice, mammary carcinomas were produced. Graff et al. (1952) found that these preparations from milk obtained both ultracentrifugally and electrophoretically, contain two components and all components produced tumors in high yield.

Immunological specificity is an important consideration in cancer and considerable work has been reported by some laboratories on the immunological behavior of one of the milk agent preparation. Andervont and Bryan (1944) and Green and Bittner (1946) showed that the agent was able to stimulate the production of neutralizing antibodies, and that the agent is antigenically different from mouse tissues.

These particles transmitted the disease in high dilution in characteristic manner in mice, and elicited anti-

bodies in the rabbit. They were shown by immunochemical techniques to be antigenically distinct from normal protein of the mouse or mouse milk. They concluded that these particles constitute the virus responsible for mouse mammary carcinoma.

Kidney Carcinoma in the Leopard Frog

Lucke (1934) found that in the leopard frog (Rana pipiens), the kidney is frequently the site of a malignant tumor, an adenocarcinoma. In 1938 he made a survey of over 10,000 frogs and found the incidence of this tumor to be 2.7 per cent.

The frog tumors are ivory-white in color, contrasting well with the brownish renal tissue. Most of them are somewhat firmer than the surrounding normal kidney. They range in size from tiny, early tumors to large masses that have destroyed all but small portions of the kidney, displacing the neighboring organs and nearly filling the coelomic cavity.

Lucke (1952) showed that the outstanding characteristic of this frog tumor is the frequent presence of acidophilic intranuclear inclusions which, in general appearance, are like those found in herpes and certain other diseases known to be caused by viruses. In their typical, fully developed form, the inclusions are readily recognizable and they were observed in over one-half of the series. It is

obvious that there must be developmental stages of the inclusions, but the appearance of the early stages is still a matter of uncertainty. The inclusions are invariably confined to the neoplastic cells. They have never been observed in normal renal epithelium of tumor-bearing kidneys, or in the normal cells of other organs. The number of inclusions in the neoplastic tissue varies greatly; sometimes, relatively few are present; sometimes, nearly every cell in some portion of the tumor is affected.

Lucke performed transmission experiments on tumor material containing no living cells. He prepared the tumor desiccates from ten frog tumors by freezing the minced tumors at approximately -80°C. in a mixture of cellusolve and solid CO_2, and then drying them by high vacuum distillation from a frozen state. The containers were sealed under vacuum and stored at refrigerator temperature for at least two or three weeks. The dried material was then ground to a fine powder under aseptic conditions and suspended in sterile water. This material was used as inoculum in frogs.

Another small group of ten frogs received an emulsien of glycerinated tumor. Slices of the tumor had been stored for 20 days in 50 per cent glycerin at refrigerator temperature. They were washed repeatedly in amphibian Ringer's solution, ground to an emulsion, and 0.5 ml. of the emulsion was injected. Two hundred and thirty-four

frogs received (0.5 ml.) intra-abdominal inoculations of tumor desiccates, and ten frogs received emulsions of glycerinated tumor. The results of both series corresponded closely with those obtained from intra-abdominal injection of living tumor material. The incidence of kidney tumor increased with time. Twenty-one per cent of frogs surviving the injection (of both dried and glycerinated tumor) for more than six months developed renal tumors.

It is unlikely that the tumor desiccates contained living cells. Hence, the conclusion is warranted that the tumor-inducing agent is separable from cells. These experiments, and the frequent presence of intranuclear inclusions within the neoplastic cells, make it very probable that the carcinogenic agent has the attributes of a virus.

Avain Leucosis

The agent of fowl leucosis can be carried from bird to bird indefinitely by intravenous inoculation into susceptible birds. Ellermann and Bang (1908) found that from 20 to 40 per cent of chickens were susceptible to inoculation. When the virus is passed from bird to bird by blood inoculation, the virulence often increases as indicated by a shortening of the incubation period and sometines by an increase in the percentage of birds in which "takes" are obtained. In the beginning of a series of passages the incubation period may be several months in length.

This may be shortened to less than one month after a few serial passages.

Furth and Miller (1932) found that the agent of leucosis passes all siliceous filters. Collodion membrane filtration is uncertain, but they thought that their results indicated that the particulate size of the virus was some what less than 100 mu.

Furth (1932) found that as little as 0.000,001 ml. of plasma was sufficient to produce the disease by intravenous inoculation. He also found that the infectious agent retained its potency for at least 54 days when dried, and for at least 104 days when preserved in glycerol.

Avain Lymphomatosis

Lymphomatosis is an extravascular form of leucosis in which the proliferating tumor cells are primitive, undifferentiated lymphocytes. Several forms of lymphomatosis are recognized: neural, ocular, visceral, and osteopetrotic.

Cell-free transmission experiments have been attempted with great difficulties, not the least of which is that the disease appears spontaneously. Many uninoculated controls frequently develop the disease. Baber (1943) found that when hatches of chickens were separated, some being reared where chickens had not previously been kept, the incidence of lymphomatosis varied according to the age which such chicks were brought back to infected premises.

Hutt and co-workers (1944) found that chicks raised at great distance from infected birds showed a deminished incidence compared with chicks raised in an infected environment.

Lee and Wilcke (1941) found that the incidence of lymphomatosis was much higher among the progeny of birds suffering from the ocular form of the disease, especially when the female was affected. Waters (1947) and Waters and Prickett (1944), working in experimental flocks kept under rigid quarantine, believe they have shown that the agent may be transmitted by infected eggs.

Cottral and co-workers (1949) and Cottral (1950) found that the causative agent of visceral lymphomatosis is contained in the chick embryo and embryonic fluids. The presence or absence of the agent in embryonic material was determined by inoculating this material into groups of 15 to 30 chicks. These chicks were of a line known to be highly susceptible to this disease, although relatively free from infection when hatched, as indicated by the low incidence of the disease when the birds were raised in isolation.

Cottral (1949-50) also performed egg transmission experiments in the production of tumors with material arising from appearently normal individuals. All embryos used were normal and all dams were clinically normal when the eggs were laid, and they remained so for variable

periods. The embryonic material was made into a suspension and then inoculated into susceptible birds. During a period of approximately one year after the use of the embryos, three cases of visceral lymphomatosis developed in the 59 birds, 38, 136 and 144 days respectively following inoculation.

Burmester (1952) mentioned that when passages were made with filtrates prepared from tumors of early developing cases, some of the birds died with suggestive lesions of leukemia in less than 100 days.

The Infectious Papillomas

Papillomas, or common warts, occur in many species of animals. They seem to be most frequent in man, cattle, dogs and rabbits. All these tumors contain filterable agents with which the tumors may be induced in other individuals of the same species. They appear to have a high degree of host specificity, and some of them have specificities for particular kinds of epithelium within a single host. Warts occur in epizootic form in herds of cattle and in kennels of dogs. All varieties are most prevalent in the young of the species. (Hagan and Brunet, 1951)

Bovine Papillomatosis

Bovine papillomatosis frequently occurs in calves and young stock less than 2 years old. Very little is known about the causative agent of bovine warts except that it is filterable. Greech (1929) inoculated eleven calves with unfiltered minced wart material and eleven with filtrates of the same material. The filtrates were bacteriologically sterile. Eight tumors were produced with the unfiltered material and seven with the filtered. The inoculations were made by scarification and intradermal injections. Warts affecting animals usually regress spontaneously after some time. There are indications that animals showing regressing tumors are thereafter resistant to this agent.

Canine Papillomatosis

In dogs, the warts begin around the lips, as a rule, are smooth whitish elevations, which later develop a roughened surface and appear as typical papillomas. McFadyean and Hobday (1898) showed that the warts were infectious by rubbing pieces of tumors on the scarified mucous membranes of other dogs. DeMonbreun and Goodpasture (1932) were also able to propagate the tumors in this way. McFadyean and Hobday stated that the incubation period is from 28 to 42 days. DeMonbreun and Goodpasture found the incubation period to be about 30 to 32 days as a rule, but was somewhat longer in dogs showing malnurishment. The latter workers

passed tumor suspensions through Berkefeld N and W filters and found that the virus was present in abundance in the filtrates.

DeMonbreun and Goodpasture were unsuccessful in producing warts on the vaginal mucous membrane, on the mucous membrane of the conjunctiva, and on the skin of the abdomen, both with filtrates and with fresh unfiltered wart tissue. They also failed to infect the mouths of cats, rabbits, guinea pigs, and rats.

Clinical experience indicates that dogs which recovered from an attack of warts seldom or never are infected again. McFadyean and Hobday, DeMonbreun and Goodpasture found that it was impossible experimentally to re-infect dogs that had recovered.

Papillomatosis of Rabbits

In 1933 Shope showed that the common wart of the western wild cottontail rabbit was infectious and that the infectious agent was a virus. Experimentally, the disease may be transmitted by inoculating scarified skin areas with filtered or unfiltered tumor pulp. These tumors can be serially transmitted to the cottontail rabbit, but not in domestic rabbits. Shope (1935) was successful to infect this virus to both cottontail and domestic rabbits. Rous and Beard (1935) showed that, if the tumor-bearing domesticated rabbits were kept for long periods, considerable numbers

of the benign papillomas become transformed into malignant carcinomas. Kidd and Rous (1940), studying the matter further, showed that the same thing was true of such tumors produced by inoculation the non-domesticated rabbits. They believed that in the rabbit species in which the virus is foreign, there is virus variation which leads to malignancy. By gradual change in morphological characteristics, the malignant tumors arise from cells which are already neoplastic as a result of virus action. Shope (1933) found that the virus was readily filterable through Berkefeld filters of all grades of porosity but usually not through Seitz filters. The first detectable evidence of epithelial proliferation is seen from the sixth to the twelfth days, averaging 8 days, after inoculation. The filtrates often produce tumors after shorter incubation periods than those of the same suspensions unfiltered. This is interpreted as meaning that some inhibiting agent had been removed by filtration. Inoculation of the scarified skin is the only way by which the disease can be transmitted with a high degree of regularity. Subcutaneous, intramuscular, intraperitoneal, and intravenous inoculations usually fail to produce tumors.

Persons and Kidd (1943) described an oral papillomatosis of the rabbit. These are benign growths of the epithelium which were found in a high percentage of normal domestic rabbits in the New York City area. They are

readily transmitted by filtrates to other domestic rabbits. The virus readily passed Berkefeld V and N candles. The average incubation period when unfiltered virus is used is 15 days; with Berkefeld V filtrates, 19 days; and with Berkefeld N filtrates, 23 days. The virus is a very stable one. Tissues stored in 50 per cent glycerol at 4°C. retain their pathogenicity undiminished for 2 years and more. Those stored in the frozen state remain potent for long periods, and material dried, while frozen, remains potent for many months. The resistance to heat is rather remarkable. Heating at 65°C. for 30 minutes appears to exert little injury.

Intranuclear inclusions were found by Parsons and Kidd in about 10 per cent of the tumors in domestic rabbits. When present, they were located in the outer six to ten layers of epithelial cells. They varied greatly in size and shape. Some were hyaline and others showed a striped structure. They were basophilic and located near the center of the nucleus, the chromatin being marginated. Such bodies were not found in normal oral epithelium of domestic rabbits.

Human Papilloma

Wile and Kingery (1919) and Kingery (1921) discovered the viral etiology of warts by the production of verrucae on human skin by inoculation of a filtrate of wart sus-

pension. This evidence was supported by the observations of Strauss and co-worker (1949, 1950).

Melnick and co-worker (1952) took material from the papillomas in which the elementary bodies were observed. They also used control specimens of papillomas in which elementary bodies were not found. Both the papillomas in which the elementary bodies were observed and the control specimens were grounded with alundum and distilled water, centrifuged at 2,000 r.p.m. for five minutes, and the resulting supernatant fluid subjected to further centrifugation at 6,000 r.p.m. for 45 minutes. The sediment was resuspended in a small volume of distilled water (about 1 ml.). For electron microscopy, a small drop was placed on a collodion screen and shadow-cast with chromium or palladium before examination. Although isolated particles may be found in all specimens, the particles are arranged in crystalline-like clusters with such regularity that this arrangement appears to be characteristic. The particles are spherical and, when in crystalline array, average 52 mu. in diameter, with a range of 50 to 54 mu. When these particles are not in crystalline array, they average 68 mu. in diameter with a range of 56 to 80 mu. The papilloma virus particles are morphologically stable when stored in distilled water at 4^{O}C. for 10 months, and they have been recovered from tissue stored in the frozen state for one month. Preparations from other warts (without intranuclear

inclusion bodies) and from normal human skin have revealed no uniform particles, but only amorphous scattered clumps of matter, collagen fibers, and spherical particles of varying diameter.

Infectious Myxomatosis

Infectious myxomatosis of rabbits is a highly contagious and almost always fatal disease of domesticated rabbits which was first recognized in South America, later in Mexico and California. The disease often destroys whole rabbitries. It was first described in 1898 by Sanarelli who ascribed the disease to a virus since he could not see or cultivate any organisms in the lesions. Rivers (1926) was the first to call attention to another characteristic of these virus tumors, that is, a peculiar type of degeneration of the epithelial coverings. The epithelial cells are greatly swollen and vacuolated, and acidophilic bodies rapidly develop in their cytoplasm. These bodies contain blue-staining coccoid elements. The whole structure resembles the Bollinger bodies of fowlpox.

River and Ward (1937) found it possible to obtain suspensions of these elementary bodies, which they regard as the virus, in a relatively pure form. Not only are such suspensions highly pathogenic, but the bodies are specifically agglutinated by the serum of recovered or immunized animals. Virus can be obtained from the tumors,

internal organs, blood, and the discharges from the body openings. It is filterable through Berkefeld filters. It is believed that the elementary bodies, mentioned above, actually constitute the virus.

Shope Fibroma of Rabbits

Shope (1932) described a type of fibrous tumor of the cottontail rabbit which proved to be transmissible to other cottontail rabbits and to the domestic species by the injection of cellular suspensions and of Berkefeld filtrates. The tumor occurs subcutaneously in naturally infected cases. There may be one or several in the same animal. They are firm, spherical masses which can be moved about under the skin because they are only loosely attached. Sections show that the masses are made up of spindle-shaped, connective tissue cells, without evidence of inflammatory reaction.

Filtrate of tumor tissue when injected into the testicles of rabbits regularly cause the formation of similar tumors. Subcutaneous and intramuscular inoculations frequently, but not always, succeed. Intraperitoneal and intracerebral inoculations fail. The virus is found only in the tumors. It has not been demonstrated in the blood, visceral organs, or any of the secretions. In susceptible animals it stimulates a proliferation of the connective tissue at the point where it is deposited. There is no

evidence of inflammation or of necrosis in the lesions. The virus is readily filterable through Berkefeld V and N filters. It remains viable in glycerol for long periods of time.

The mode of natural transmission of the virus is not known. It is not transmitted from animal to animal by simple contact. Hyde and Gardner (1939) found that it was not transmitted from mother to young either through the placenta or through the milk.

Shope (1938) showed that in domestic rabbits in which fibroma tumors had formed and retrogressed, reinfection did not occur. He also found that these rabbits also had a high degree of resistance to the virus of myxomatosis.

Berry and Dedrick (1936) and Berry (1937) indicated that fibroma virus probably is an attenuated form of myxoma. The virus of myxomatosis was heated to 75°C., which completely inactivated it. Mixed with fibroma virus, however, the mixture produced typical myxomatosis when injected into rabbits, and this disease can be transmitted to other animals indefinitely. It was suggested that something in the heated myxoma virus had acted as a sort of hapten to lend greater virulence and malignancy to the fibroma virus.

Hodgkin's Disease

Gordon *et al.* (1934) were the first to start a system-

atic search for a virus in Hodgkin's disease. Before that time, others had speculated on the possible etiological role of virus in tumors in general. Their significance in certain tumors from animals was well known. Based mainly on clinical, inductive and some experimental evidence, several authors had considered that Hodgkin's disease might be caused by a virus. Since then, many investigators have searched for a viral agent in Hodgkin's disease.

Gordon (1934) reported that tissue from Hodgkin's disease, when injected into rabbits, produces an encephalitis. At first, the material which caused this response seemed to possess many viral characteristics. The lymph node extracts contained no bacteria; the agent was Seitz-filterable; it was inactivated by heat (80°C. for 30 minutes); and the encephalitis produced had an incubation period of from two to six days after inoculation. The encephalitis, however, could not be passed from rabbit to rabbit. Friedman (1934) demonstrated that the encephalitic agent could be isolated from certain normal tissues and has clearly demonstrated that the factor is not a virus, that it is doubtlessly an enzyme, and that it is probably associated with the eosinophilic leucocyte.

Bostick (1948) reported an increased mortality in embryonated chicken eggs inoculated with Seitz-filtered extracts of Hodgkin's disease lymph nodes. The controls

were normal tissues inoculated into the embryonated eggs. The Hodgkin's disease material was inoculated into the amniotic sac of 7-day incubated eggs and the lethal effect was recorded during the following ten days.

Grand (1944) noted the formation of small vesicles on chicken egg chorio-allantoic membranes infected with supernatant fluid from Hodgkin's disease tissue cultures. In 1949 he found an abnormal amount of cellular degeneration and liquefaction in tissue cultures of Hodgkin's disease tissue. He prefers to interpret this as being evidence in favor of the presence of an injurious agent specific for Hodgkin's disease cultures. Mottino (1949) also noted a liquefaction tendency that was greater in Hodgkin's disease than in other tumor tissue cultures. Although greater in Hodgkin's disease tissue explants, he preferred to consider it probably nonspecific and resulting from fibrinolytic enzymes liberated by the many reticular cells in Hodgkin's disease.

Reiman et al. (1950) studied in detail the effect of Hodgkin's disease tissue and normal serum on different types of normal, abnormal, Hodgkin's disease and neoplastic tissue cultures. They concluded that Hodgkin's disease cells and serum give rise to abnormal tissue cultures. Among the changes are increased fat granules in cells, more liquefaction and numerous free cell forms, also, larger numbers of nuclei in the giant cell formations and

a decreased span of life of Hodgkin's disease cells.

Gordon (1934) was the first to propose that Hodgkin's disease tissues contain elementary bodies. Elementary and inclusion bodies were next described by Grand (1944, 1949). In tissue cultures, brilliant cresyl blue was used for staining inclusion bodies which were irregular in size and shape in the Hodgkin's disease lymphocytic cells and Reed-Sternberg cells. Similarly stained cultures of normal, inflamed and lymphosarcomatous lymph nodes did not show such inclusions. In fixed tissues, Giemsa's and Seller's stains showed the inclusions in the Hodgkin's disease material and not in the controls. In cultures of normal lymph nodes, Grand noted that inclusions began to appear in the cells within 15 minutes after the addition of the supernatant fluid from a Hodgkin's disease tissue culture. By 24 hours, these inclusions were even more striking and resembled those found in Hodgkin's disease. Supernatant fluid from other tissues than Hodgkin's disease did not cause inclusions to develop in the normal lymph node cultures. Hoster et al. (1950) studied the macromolecular particles from various types of lymph nodes. They used a ten-step differential centrifugation procedure and then examined the particles under the electron microscope. Lymph nodes that were normal or non-neoplastic were compared with Hodgkin's disease lymph nodes and lymphosarcoma lymph nodes. They studied the frequency of

the various sizes of particles in these tissues. In Hodgkin's disease, the predominant particle size was 10 to 20 mu. which differed significantly from the particle sizes found in non-neoplastic tissues.

Gordon (1937) studied a flocculation reaction with the elementary bodies from Hodgkin's disease tissue and 18 anti-Hodgkin's disease antisena made by injection of rabbits. He obtained only variable results.

Grand (1950) inoculated rabbits with purified suspension of Hodgkin's disease lymph nodes. The nodes were prepared for inoculation by differential centrifugation. The final purified suspension was injected intravenously into normal rabbits for 5 to 10 days. Later, rabbit serum was collected and inactivated at 55°C. for 30 minutes. It was tested for the presence of agglutinins for purified Hodgkin's disease lymph node extract. The readings were made under the darkfield microscope. Agglutination of very small particles was noted in the Hodgkin's disease material. Agglutination was absent in similar tests using normal, lymphosarcoma and leukemia lymph node extracts.

Bostick (1950) employed as an antigen from amniotic fluid harvested from eggs in which filtered Hodgkin's disease extract had been serially passed. Complement-fixation properties were studied from two aspects. In the first instance, Hodgkin's disease amniotic fluid was

used as the experimental antigen, and, as a control antigen, non-Hodgkin's disease serially-passed amniotic fluid. Under these conditions, 66 per cent of the Hodgkin's disease patient sera fixed complement with the Hodgkin's disease amniotic fluid, whereas, only 35 per cent of the control patients' sera produced the same result. In the second instance, the Hodgkin's disease amniotic fluid was inoculated into rabbits. Their sera were later tested for complement-fixation properties by comparing the results obtained with normal amniotic fluid antisera. Not all rabbit sera possessed complement-fixing properties. In some rabbit sera however, the ability to fix complement was consistently demonstrated when using as antigens the serially passed and filtered amniotic fluid derived from cases of Hodgkin's disease.

Bostick (1950) passed serially (at least 4 passages) Seitz-filtered extracts of Hodgkin's disease lymph nodes in the amniotic sacs of embryonated chicken eggs. The harvested Hodgkin's disease amniotic fluid was tested for virus interference properties. This was done by inoculating Hodgkin's disease amniotic fluid into 7 day incubated chicken eggs, and after three more days of incubation, a challenging dose of influenza Lee virus was inoculated into the amniotic sac. After 18 hours additional incubation, the amniotic fluid was harvested separately from each egg and tested for the amount of Lee virus present by

means of the hemagglutination test. The amniotic fluid derived from Hodgkin's disease showed interference capacity on many occasions but not on all. Sometimes, the influenza virus inhibition was complete. More often, it was simply decreased as compared to of carefully tested control material. In tests with Hodgkin's disease amniotic fluid, 60 per cent showed an ability to interfere. There was also some degree of reversal, i.e. greater hemagglutinative titers in the Hodgkin's disease series than in the control.

Lundback and Lofgren (1950) inoculated ground lymph node emulsions from Hodgkin's disease, Hodgkin's sarcoma, and a lymphoma into the amniotic sac of 7 day incubated chicken eggs. Serial allantoic passages were maintained. The harvested allantoic fluids showed hemagglutination titers of from 2.51 to 3.11 (log units). Control material did not react. The hemagglutinating agent fixed complement with mumps antisera. In 1952 they found that the hemagglutinating agent was mump contamination.

Finally Bostick (1952) concluded that Hodgkin's disease is caused by an infectious viral agent, which itself is undetectable by the usual methods. It might however, have a demonstrable effect on the growth of known viruses.

Plant Tumours

There are several virus diseases of plants in which leafy outgrowths from the veins are produced, such as Kroepek disease of tobacco, Smith's rosette of tobacco, and tobacco mosaic in *Nicotiana paniculata* or *N. tomentosa*, Fiji disease of sugar cane, Wallaby-ear of corn, Clubroot of tobacco and Wound-tumor disease. (Black, 1952)

MATERIALS AND METHODS

III MATERIALS AND METHODS

The tumors used in this work were obtained from both animals and man. The tissues were removed at operation and held at refrigerator temperature for periods up to 24 hours. Representative samples of each tissue was placed in fixative for histological sections, the remaining tissue was used for egg inoculations and tissue culture.

The tumors and their origin are listed below.

Tumor I	Fibroma from human ovary.
Tumor II	Papillary adenocarcinoma from human rectum.
Tumor III	Scirrhous carcinoma from human mammary gland.
Tumor IV	Leiomyoma from human uterus.
Tumor V	Squamous cell carcinoma from human vulva.
Tumor VI	Fibrosarcoma from human.
Tumor VII	Serous papillary adeno-carcinoma from human ovary.
Tumor VIII	Melanoma from canine soft palate.
Tumor IX	Sweat gland adenoma from canine subcutaneous tissue.

Tumor X	Adenocarcinoma from canine anal region.
Tumor XI	Squamous cell carcinoma from external canthus of the eye of cow.
Tumor XII	Fibrosarcoma from canine subcutaneous tissue.
Tumor XIII	Lyphomatosis from fowl.
Tumor XIV	Identify lost.
Tumor XV	Carcinoma of breast from human.
Tumor XVI	Osteochondro-adeno-carcinoma from canine.
Tumor XVII	Adenocarcinoma from canine subcutaneous tissue.

(1) ULTRACENTRIFUGE CONCENTRATION OF MATERIAL FOR:

a. Tissue culture work.

b. Hemagglutination reaction.

c. Virus interference.

In this work each tumor was washed three times with sterile physiological saline solution. Penicillin and streptomycin were added in concentration of 500 u. per ml. and 50 mg. per ml. respectively. After adding the antibiotics, the tumor was kept in the refrigerator at least three hours. Fragments of the tumor were used for tissue culture, the remaining tissue was then masserated by grinding with 90 mesh alundum. Hank's solution was added to make 20 per cent suspension. The suspension was centrifuged in a refrigerated centrifuge (5°C.) for 20 minutes at 2,000 r.p.m. After centrifugation the supernatant fluid was removed and used to inoculate the allantoic sac (0.1 ml.) of 6 to 7 day old ambryonated chicken eggs. After 4 to 5 days incubation allantoic fluid was harvested from these eggs and stored in the deep freeze (-25°C.).

Just before use the allantoic fluid was thawed and clarified by refrigerated centrifuge for 15 minutes at 3,000 r.p.m. The supernatant fluid was then concentrated one-third by volume by ultracentrifugation (Spinco Model E.) at 115,000 X gravity for one hour. The concentrated allantoic fluid was inoculated into the allantoic sac of embryo-

nated chicken eggs and constituted the second passage. Five such passages were made each time with concentrated material.

Normal allantoic fluid from eleven-day old chick embryos, was used for the control and it was also concentrated one-third by volume by ultracentrifugation as mentioned above.

(2) TISSUE CULTURE WORK

A. Normal Growth of Tumor Tissue in Tissue Culture.

Tissue culture consists of removing tissues under sterile conditions from a living organism and incubating them in an environment (nutriment, aeration, temperature) conducive to growth. Variations in the technique have been developed in accordance with the type of cellular activity desired. The most successful methods deal with the multiplication of individual cells, and with the maintenance of cells in organized groups for a study of certain phases of their function.

After the tumor was washed with physiological saline solution and refrigerated with antibiotics for three hours, it was then removed from the cold and cut into pieces about half to one mm. mesh. Pieces of tumor were embedded into the chicken plasma coagulum and liquid nutrient medium was added. Erlenmeyer flask and slide culture techniques

were used.

Chicken plasma was prepared by aseptically bleeding a chicken from the heart. Heparin solution (1:2,000 in physiological saline solution) in 0.4 ml. amount per 20 ml. of blood was used as anticoagulant. The blood was centrifuged at 2,000 r.p.m. for 15 minutes and the plasma was removed by a capillary pipette. A sterility test was performed by dropping a few drops of plasma into nutrient broth. The plasma was kept in a frozen state at -25°C. and was thawed and clarified by refrigerated centrifuge at 2,000 r.p.m. for 20 minutes for use.

Hank's (1944) solution was used as part of the nutrient medium. Hank's stock solution A was prepared as follows:

NaCl	40	gm.
KCl	2	gm.
$MgSO_4.7H_2O$	1	gm.
dissolve in 200 ml. distilled H_2O		
$CaCl_2.2H_2O$	0.75	gm.
dissolve in 30 ml. distilled H_2O		

Mix the two solutions together and make up to 250 ml. with distilled water.

Hank's stock solution B was prepared as follows:

$Na_2HPO_4.12H_2O$	0.76	gm.
KH_2PO_4	0.30	gm.

dextrose 5 gm.

distilled H_2O added to 250 ml.

Both stock solutions were sterilized by autoclaving at 10 lb. pressure for half an hour. Both stock solutions were stored at refrigerated temperature.

The phenol red solution in 4 per cent concentration was made by dissolving 4 gm. of phenol red in 1.3 ml. of N/2 NaOH solution and finally making up the volume to 100 ml. with distilled water. The indicator solution was sterilized by autoclaving at 10 lb. pressure for half hour.

Sodium bicarbonate solution, sterilized by Seitz-filtation, was a 1.4 per cent solution.

Hank's solution (stock solutions A and B) was made up just prior to use as follows:

Stock solution A	1.0 ml.
Stock solution B	1.0 ml.
Distilled Water	18.0 ml.
Phenol Red (4%)	0.02 ml.

The mixture was autoclaved at 10 lb. pressure for 10 minutes. After the solution was cooled, the following solutions were added.

Penicillin (500 U. per ml.)	0.2 ml.
Streptomycin (50 mg. per ml.)	0.1 ml.

About 0.4 ml. of $NaHCO_3$ (1.4%) was added to adjust the pH to 7.4 to 7.8.

Embryo tissue extract was prepared by using eleven-day old chicken embryos. The eggs were opened and the embryos removed. The eyes and legs were removed from the bodies which were then minced with a pair of scissors. The pieces of tissue were crushed by a pestle and mortar. Fifty-per cent Hank's solution was added as a diluent and the mixture was then stored over night in the refrigerator. The following day the suspension was centrifuged in a refrigerated centrifuge at 2,000 r.p.m. for 30 minutes. The supernatant fluid was removed by capillary pipette and stored at -25°C. Just prior to use the embryo tissue extract solution was thawed and clarified by centrifugation in the cold at 2,000 r.p.m. for 15 minutes.

The liquid nutrient medium consisted of two parts of chicken serum, two parts of Hank's solution and one part of embryo tissue extract.

Method of Flask Culture

Twenty-five ml. Erlenmeyer flasks were used in this work. In the preparation of solid medium, 1.3 ml. of Hank's solution and 0.5 ml. of chicken plasma were placed in the flask. Then 0.2 ml. of embryo tissue extract was added to produce coagulation. The flask was then allowed to stand on the flat surface. After the plasma was clotted, pieces of tumor tissue were embedded into this solid medium with a sterile pointed needle. The flask was allowed

to stand ten minutes and then one ml. of liquid nutrient medium was added and the culture was incubated at 37°C.

The liquid nutrient medium was changed every 3 to 4 days by removing the old medium with capillary pipette. One ml. of Hank's solution was then added and allowed to stand for 10 minutes. This Hank's solution was then replaced by 1 ml. of fresh liquid nutrient medium.

The growth of the tumor can be observed by an inverted microscope eye piece or by dissecting microscope. If the growth is luxurious a halo zone surrounding the tumor tissue can be seen by naked eyes.

Slide Culture Method

The slide culture method was the double coverslip method as described by Maximow (1925). Briefly it consisted of using a 75 by 45 mm. micro-concavity slide with a spherical concavity of 35 mm. in diameter and 5 mm. deep. Large No. 2 cover glasses (70 by 43 mm.) and small No. 1 (20 by 20mm.) cover glasses were combined. The small and large cover slides were held together by placing a drop of distilled water on their intersurfaces. The culture was prepared on the small square cover glass by placing one drop of Hank's solution, one drop of chicken plasma and one drop of embryo tissue extract in the middle of the small cover glass. The tumor tissue was placed upon the clotted medium and was embedded with a sterile pointed needle. A

drop of liquid nutrient medium was placed upon the culture.

The coverslip bearing the culture was inverted and pressed down over the depression in the slide and was sealed in place with paraffin and the culture was incubated at 37°C.

The liquid medium was changed every 2 to 3 days by dipping the small square coverslip with culture into Hank's solution and letting it stand about 10 minutes. The culture was then taken out and put on the large micro cover glass. One drop of fresh liquid medium was dropped on to the culture. The culture was then inverted and pressed down over the depression in the slide and was sealed in place with paraffin. By the cover slide method the growth of tumor tissue can be examined under the microscope and photomicrographs made.

B. The Effect of Tumor Passage Allantoic Fluid on the Growth In Vitro of Normal Tissue.

Normal chicken heart tissue from one day old chicks was used for this experiment. The heart was removed aseptically from the chick and the blood was washed off with sterile physiological saline solution and finally stored in Hank's solution in the refrigerator for further use.

Carrel $D_{3.5}$ flask and slide culture methods were used for the cultivation of the heart tissue. After a few experiments, the slide culture method was omitted in favor of the Carrel flask method. The flask technique for the cultivation of tissues in plasma depended on the preparation of two-phase system within the flask, one being a permeable, and semi-solid plasma coagulum in which the tissues were embedded, the other, a fluid phase that may be introduced and removed at intervals of 3 to 4 days. The semi-solid plasma coagulum was made by placing 1.3 ml. of Hank's solution, 0.5 ml. of normal chicken plasma and 0.2 ml. of embryo tissue extract into the flask. The flask was shaken to mix the content and then the flask was allowed to stand on a flat plane for the clotting of the plasma.

The plasma obtained from normal chickens was found to have different degrees of growth stimulating abilities for the normal tissue. Therefore the plasma from several chickens was pooled before use. This pooled plasmas was

used on all tissue in this experiment so that conditions remained identical. Three pieces of heart tissue from a one-day old chick were embedded in the plasma coagulum one cm. agart. One flask was made for each tumor to be tested. The liquid-phase consisted of one part of the concentrated passage allantoic fluid and one part liquid nutrient medium. The nutrient medium consisted of two parts of Hank's solution, two parts of chicken serum and one part of embryo tissue extract. The liquid-phase for the control consisted of one part of the concentrated normal allantoic fluid and one part of liquid nutrient medium.

The growth of the fibroblasts from the heart tissue was observed daily under the microscope. The eye piece of the microscope was fitted with a disc with 5 mm. squares enscribed upon it to record the area of growth occuring in each piece of tissue. The counting was done using a dissecting microscope with 15 X magnifications. The growth index (Ludford and Barlow, 1944) of each piece of tissues was calculated as follows:

$$\text{The growth index} = \frac{\text{Total area of culture - Area of original explant}}{\text{Area of original explant.}}$$

(3) HEMAGGLUTINATION REACTION AFTER PASSAGE OF TUMOR MATERIAL IN CHICKEN EMBRYOS.

A. Tested with Normal Chicken Red Cells

Hemagglutination, the Hirst phenomenon (1941) as used for detecting viruses, was used in this work in an attempt to identify any agent recovered from egg passage of tumor material.

Buffered saline solution was prepared by the method of Wheeler, Luhby and Scholl (1950). One volume of M/15 phosphate buffer solution (pH 7.3) was added to nine volumes of 0.85 per cent sodium chloride solution. M/15 phosphate buffer solution pH 7.3, was made by adding 7.66 ml. M/15 Na_2HPO_4 solution to 2.33 ml. M/15 KH_2PO_4 solution. This buffered saline solution was used in all tests described.

The concentrated passage allantoic fluid of each tumor and each passage was tested for hemagglutinative properties, with 0.5 per cent suspension of normal chicken red cells. The red cells were washed three times with buffered saline solution then 0.5 per cent suspension was made for the test. The passage allantoic fluid was diluted serially in two-fold dilution with physiological saline solution. Twenty-five hundredths ml. of each dilution and 0.25 ml. of physiological saline solution were placed into a tube (12 by 75 mm.) and then 0.25 ml. of 0.5 per cent suspension of chicken red cells was added. The tubes

were shaken to mix the content. The test was read at 15, 30 and 45 minutes intervals by examining the pattern of cells formed on the bottom of the tubes. Concentrated normal allantoic fluid was tested in the same way and served as a control. The red cells from one-day old chicks were also used but no significant differences in hemagglutinating properties was found in the different ages of chickens.

B. Tested with Chicken Red Cells Sensitized with Newcastle Disease Viruses.

Certain viruses and Vibrio cholerae filtrates were found by Burnet, McCrae, and Stone (1946) to modify the virus hemagglutination of human red cells. Normal chicken red cells were washed one with buffered saline solution and made up to a 10 per cent suspension. The source of the Newcastle disease virus was allantoic fluid. The virus was used for sensitizing the chicken erythrocytes.

Two parts of 10 per cent suspension of chicken erythrocytes were added to one part (having a hemagglutination titer of 1:320) of Newcastle disease virus. This mixture was kept in the refrigerator (4°C.) for one hour. After refrigeration, the red cells were washed by centrifugation in the refrigerated centrifuge at 1,500 r.p.m. for 15 minutes. The supernatant fluid was discarded. The cells were resuspensed in buffered saline solution making a 10 per cent suspension by volume. The red cells were then washed until the supernatant fluid failed to agglutinate a 0.5 per cent suspension of chicken red cells. Finally a 10 per cent suspension, by volume, was made in buffered saline solution and the suspension incubated at 37°C. in a water bath for three hours. The incubation eluted the virus from the red cells. After incubation the red cells were washed by centrifugation in the ordinary centrifuge at 1,500 r.p.m. for 15 minutes. The

red cells suspension was washed until the supernatant fluid failed to agglutinate a 0.5 per cent normal chicken erythrocytes suspension. The sensitized chicken red cells were then made to a 0.5 per cent saline suspension and were ready for use.

The passage allantoic fluid from tumors was diluted serially in two-fold dilution with physiological saline solution. Twenty-five hundredths ml. of each dilution and 0.25 ml. of physiological saline solution were placed into a tube (12 by 75 mm.) and then 0.25 ml. of 0.5 per cent suspension of sensitized chicken red cells was added. The tubes were shaken to mix the content. The test was read at 15, 30 and 45 minutes intervals by examining the pattern of cells formed on the bottom of the tubes. Concentrated normal allantoic fluid was tested in the same way and served as a control.

C. Tested with Chicken Red Cells Modified with Trypsin.

Morton and Pickel (1949) found that red cells treated with crude and crystalline trypsin solutions increased their sensitivity to the incomplete anti-Rh antibody. Therefore trypsin modified erythrocytes were used with the tumor passage allantoic fluid. Whole chicken blood was washed with buffered saline solution. Nine volumes of four per cent red cells suspension were added to one volume of one per cent crude trypsin (Difco 1:250 brand) in physiological saline solution. The mixture was incubated at 37°C. in a water bath for 10 minutes. The cells were centrifuged by refrigerated centrifuge 1,500 r.p.m. for 15 minutes and washed once with physiological saline solution. A 0.5 per cent red cells suspension was used for the test.

The passage allantoic fluid from tumors were diluted serially in two-fold dilution with physiological saline solution. Twenty-five hundredths ml. of each dilution and 0.25 ml. of physiological saline solution were placed into a tube (12 by 75 mm.) and then 0.25 ml. of 0.5 per cent suspension of modified chicken red cells was added. The tubes were shaken to mix the content. The test was read at 15, 30 and 45 minute intervals by examining the pattern of cells formed on the bottom of the tubes. Concentrated normal allantoic fluid was tested in the same way and served as a control.

(4) TESTS FOR INTERFERENCE BETWEEN TUMOR PASSAGE ALLANTOIC FLUID FOLLOWED BY NEWCASTLE DISEASE VIRUS:

A. In Vivo.

It has been established by many workers, Price (1940), Vilches and Hirst (1947), Ginsberg and Horsfall (1949) that in certain circumstances the presence of one virus will interfere with the growth of a subsequently inoculated one. Although the usual laboratory test may not indicate the passage of an agent in the allantoic fluid, an in vivo test may show a difference between passage allantoic fluid and normal allantoic fluid.

Concentrated passage allantoic fluid from tumors was first inoculated into 6 to 7 day old embryonated chicken eggs. This was followed in 3 to 4 days by an injection of 0.05 ml. of Newcastle disease virus. Both injections were given into the allantoic cavity. The Newcastle disease virus used was adjusted so that a hemagglutination unit titer of 1:40 was contained in 0.05 ml. Twenty-four hours following the virus injection, the allantoic fluid was harvested. This fluid was then titrated by using the hemagglutination test and the titer of the tumor passage allantoic fluid was compared to the titer obtained in the controls. The controls consisted of concentrated normal allantoic fluid which was injected into embryonated eggs prior to Newcastle disease virus.

B. In Vitro.

The purpose of this part was to try to demonstrate interference by concentrated passage allantoic fluid from tumors with the absorption of Newcastle disease virus onto guinea-pig erythrocytes. The guinea-pig erythrocyte was found to absorb Newcastle disease virus similarly to chicken red cell however, it was eluted very slowly when compared to the chicken red cell.

Guinea-pig red cells were washed once with buffered saline solution. One part of concentrated passage allantoic fluid from tumors was added to two parts of 10 per cent buffered saline suspension of guinea-pig erythrocytes. The mixture was held in the refrigerator (4°C.) for one hour. After refrigeration the mixture was centrifuged in a refrigerated centrifuge at 1,500 r.p.m. for 15 minutes and the supernatant fluid was removed and discarded.

The treated red cells were resuspensed to make a 10 per cent suspension in buffered saline solution. Two parts of this suspension were added to one part of Newcastle disease virus (titer 1:1280). The mixture was held in the refrigerator (4°C.) for one hour. After refrigeration the cells were recentrifuged. The supernatant fluid was then tested for the presence of Newcastle disease virus by the regular hemagglutination reaction. The Newcastle disease virus hemagglutination titer in the supernatant fluid was then compared to the control. Concentrated normal allantoic

fluid and untreated red cells served as controls.

RESULTS

IV RESULTS

Tissue Culture Work

A. Normal Growth of Tumor Tissue.

Nine of the tissues from neoplasms grew well (Tumor I, Tumor II, Tumor V, Tumor VIII, Tumor IX, Tumor X, Tumor XI, Tumor XIII, and Tumor XVIII) and were maintained for several months *in vitro*. Figures 1 through 4 show the tissue culture growth of these tumors. Eight tumors failed completely to grow. There was no correlation when comparing the type of neoplasm or its ability to grow *in vitro*. Cultivation of the tumor tissue was more often successful when the tumor tissue could be obtained immediately after removal, however, some tissues were capable of growth after periods of storage at refrigeration temperature (4°C.).

B. The Effect of Tumor Passage Allantoic Fluid on the Growth (*in vitro*) of Normal Tissue.

When normal chicken heart was grown in tissue culture the rate of growth of the tissue could be estimated by making daily counts of the area of growth. When using normal heart tissue and growing this tissue in a medium containing ultracentrifuged concentrated tumor passage allantoic fluid, the results obtained varied a great deal. Table I gives the average growth index with the 17 tumors studied. Graphs I through IV show a composition of these average growth indexes. It may be seen from Graph I that

concentrated allantoic material, after five egg passages was capable of stimulating the growth of normal heart tissue culture cells. The growth index of Tumor II was nearly twice that of the control culture of cells.

Allantoic fluid passage material from tumors III and XII also gave growth indexes greater than the control culture however, the indexes are so close to that of the control that it is doubtful if they could be considered significant.

The majority of allantoic fluid material added to the normal tissue produced a retardation upon the growth of the tissue. Lower than average growth indexes were obtained from egg passage allantoic fluid from tumors I, IV, VI, VII, VIII, IX, X, XIII, XIV.

Figure 5 shows a photograph of the normal chicken heart to which ultracentrifuge concentrated normal allantoic fluid has been incorporated into the medium. When comparing figure 5 and figures 6 and 7, it can be seen that a great deal of variation exists. Figure 6 illustrates normal chicken heart culture to which passage allantoic fluid from tumor II was used as nutrient in the medium. This tumor material gave the greatest average growth index (Graph I). Figure 7 illustrates the results obtained when passage allantoic fluid from tumor XVI was used in the nutrient medium. Figures 5, 6 and 7 were photographed at the same magnification to better illustrate these results.

Table I

Average Growth Index of One Day Old Chicken Heart In Tissue Culture When Tumor Passage Allantoic Fluid Were Added.

	Time In Days						
Tumors	1	2	3	4	5	6	7
I	.67	3.55	3.73	4.62	5.41	5.31	6.46
II	.81	8.36	16.62	24.64	33.45	37.29	40.36
III	1.05	8.00	15.9	18.2	22.4	23.9	24.4
IV	.53	3.1	5.85	7.31	7.66	8.48	8.38
V	1.53	6.45	11.44	16.2	19.0	22.58	23.23
VI	.77	3.99	5.44	5.8	6.83	7.44	7.49
VII	.8	4.4	9.8	12.9	17.2	19.7	19.6
VIII	.75	5.07	9.18	11.76	14.36	16.8	17.62
IX	.91	2.8	3.73	5.01	5.79	5.57	5.26
X	1.25	7.0	9.25	10.41	12.25	13.33	14.91
XI	0	4.0	5.33	10.6	16.65	20.5	25.0
XII	.88	7.91	10.58	18.0	23.06	25.6	28.98
XIII	.98	5.08	7.15	8.34	9.97	11.87	14.5
XIV	.5	2.1	1.76	1.3	1.5	3.6	9.26
XV	1.05	3.61	3.30	6.33	6.54	6.87	10.20
XVI	.44	3.14	1.75	2.18	2.29	2.55	4.11
XVII	.38	4.66	5.89	5.88	6.0	7.11	8.17
Normal Concentrated Allantoic fluid (Control)	.92	5.28	10.04	16.05	20.78	24.39	26.61

FIGURES 1 AND 2.

Figure 1. Tumor-I in tissue culture, 23 days old. (Magnification about 1200X)

Figure 2. Tumor-II in tissue culture, 20 days old. (Magnification about 1200X)

Figure 1.

Figure 2.

FIGURES 3 AND 4

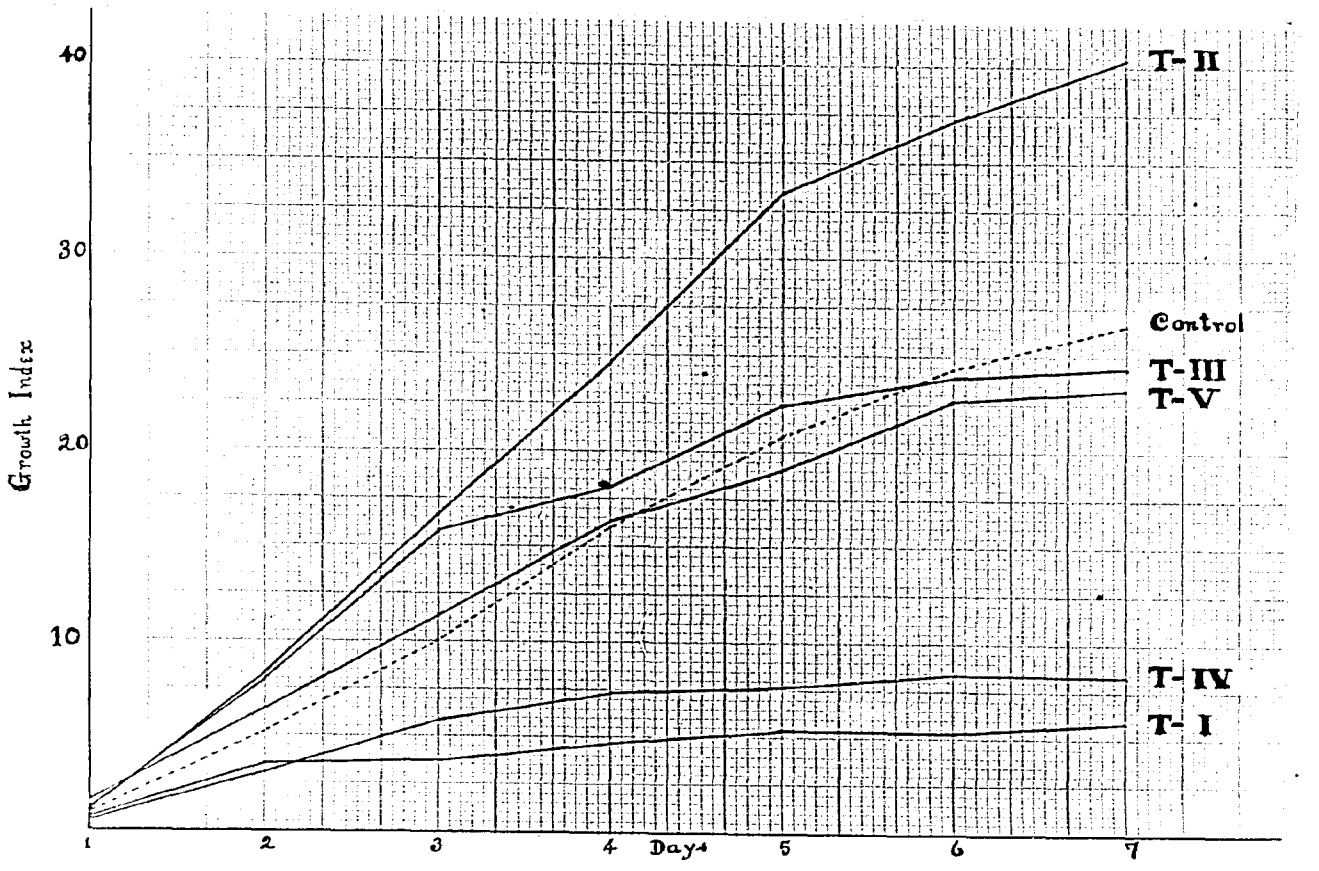
Figure 3. Tumor-T in tissue culture, 5 days old.
(Magnification about 1200X)

Figure 4. Tumor-II in tissue culture, 10 days old
(Magnification about 1200X)

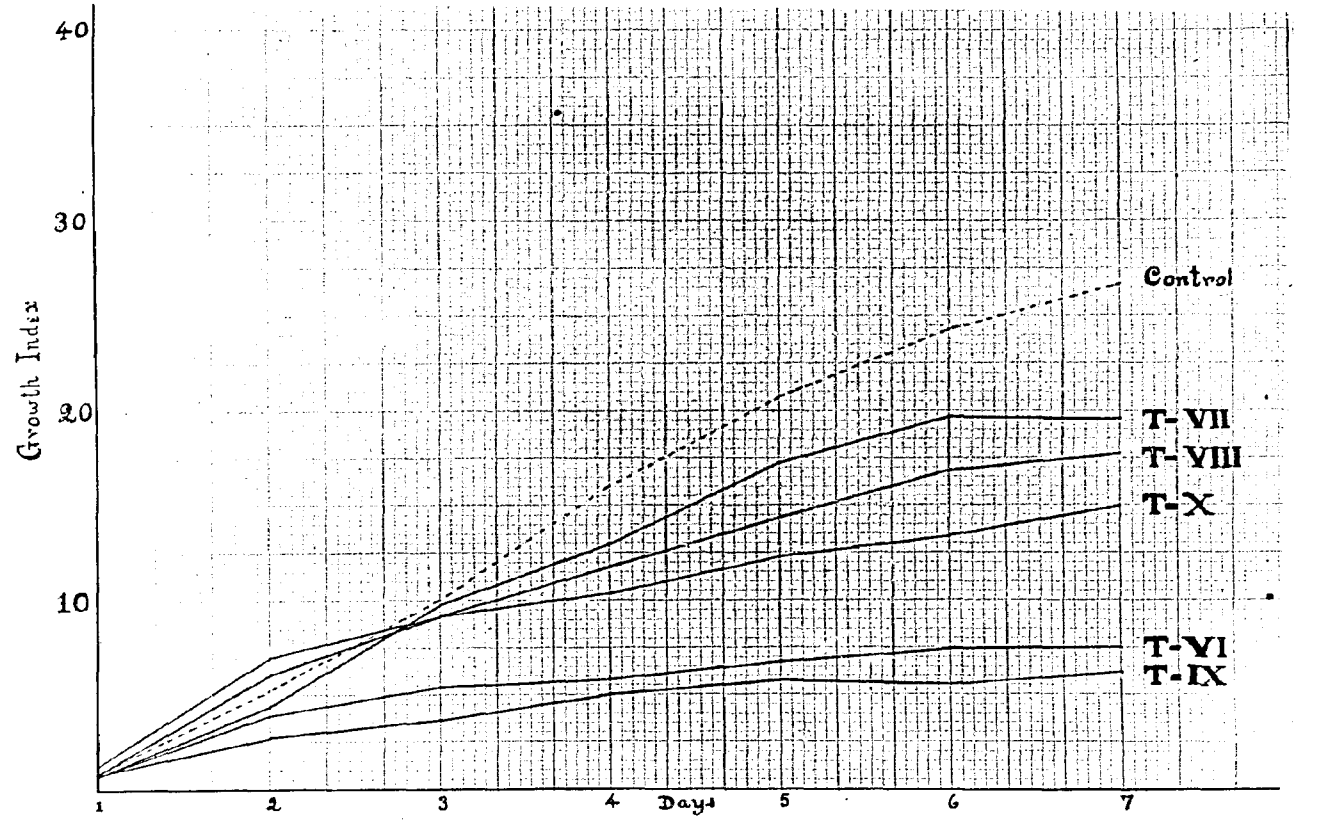
Figure 3.

Figure 4.

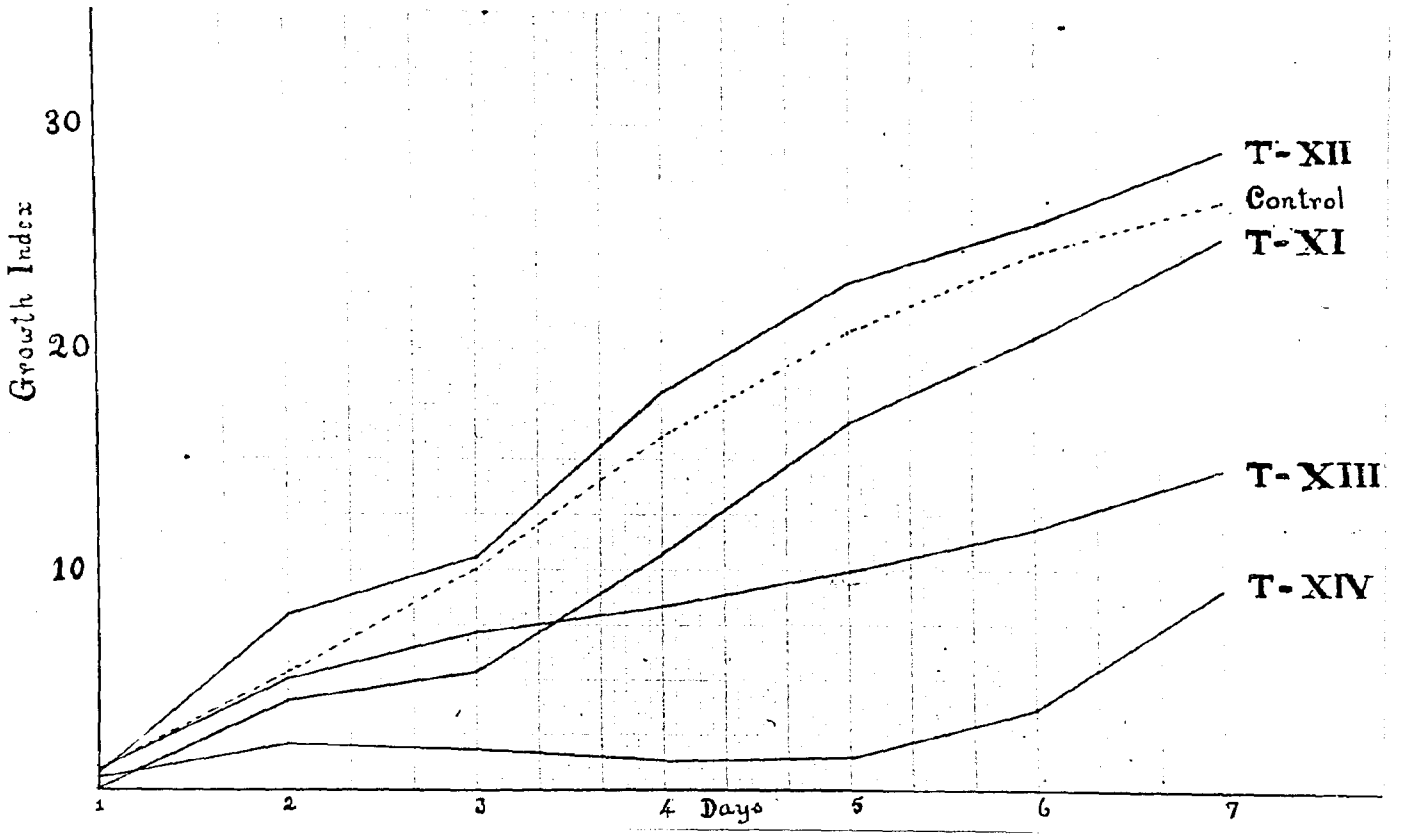
GRAPHS I, II, III, AND IV



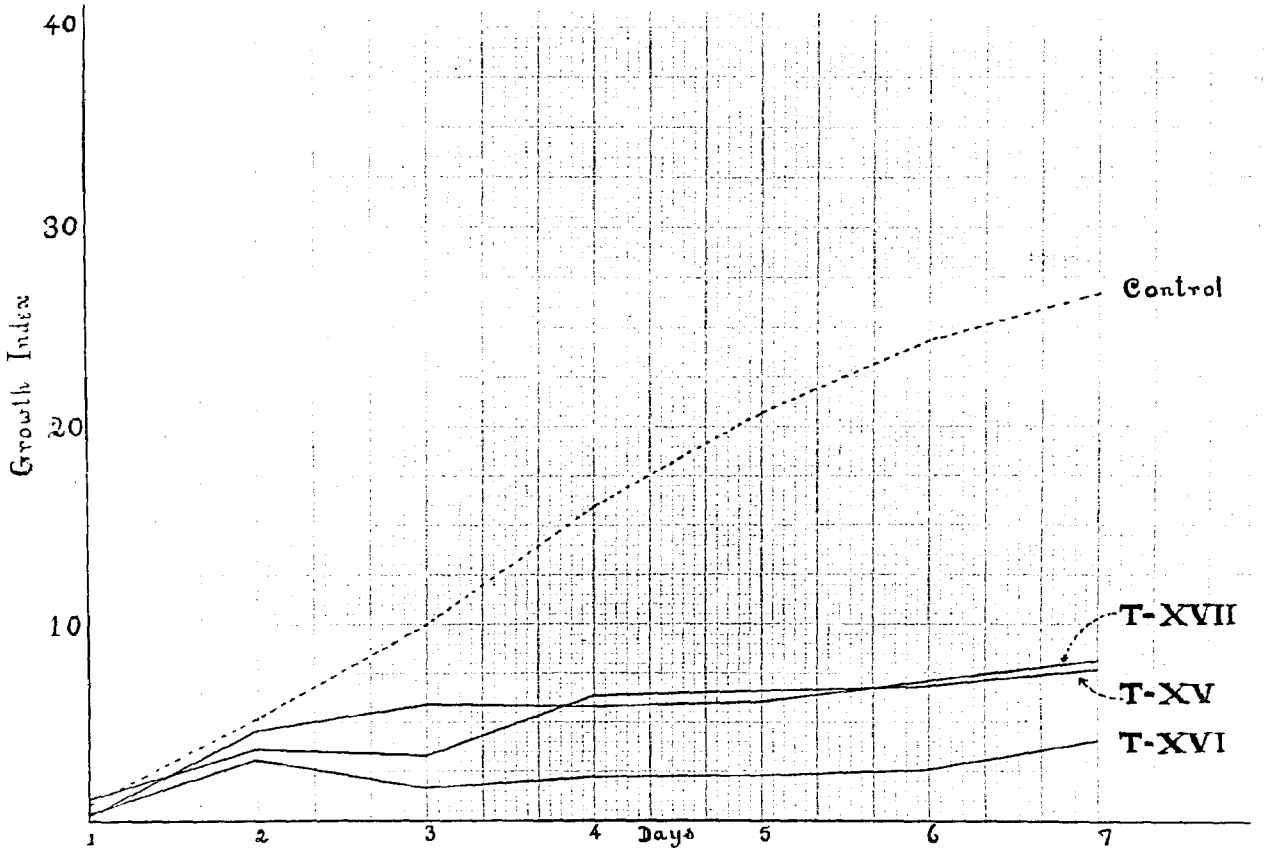
Graph I



Graph II



Graph III



Graph IV

FIGURES 5, 6, AND 7

Figure 5. Growth of Normal Chicken Heart in 7 day old Tissue Culture to which Normal Concentrated Allantoic Fluid (Control) was added. Mag. 140 X.

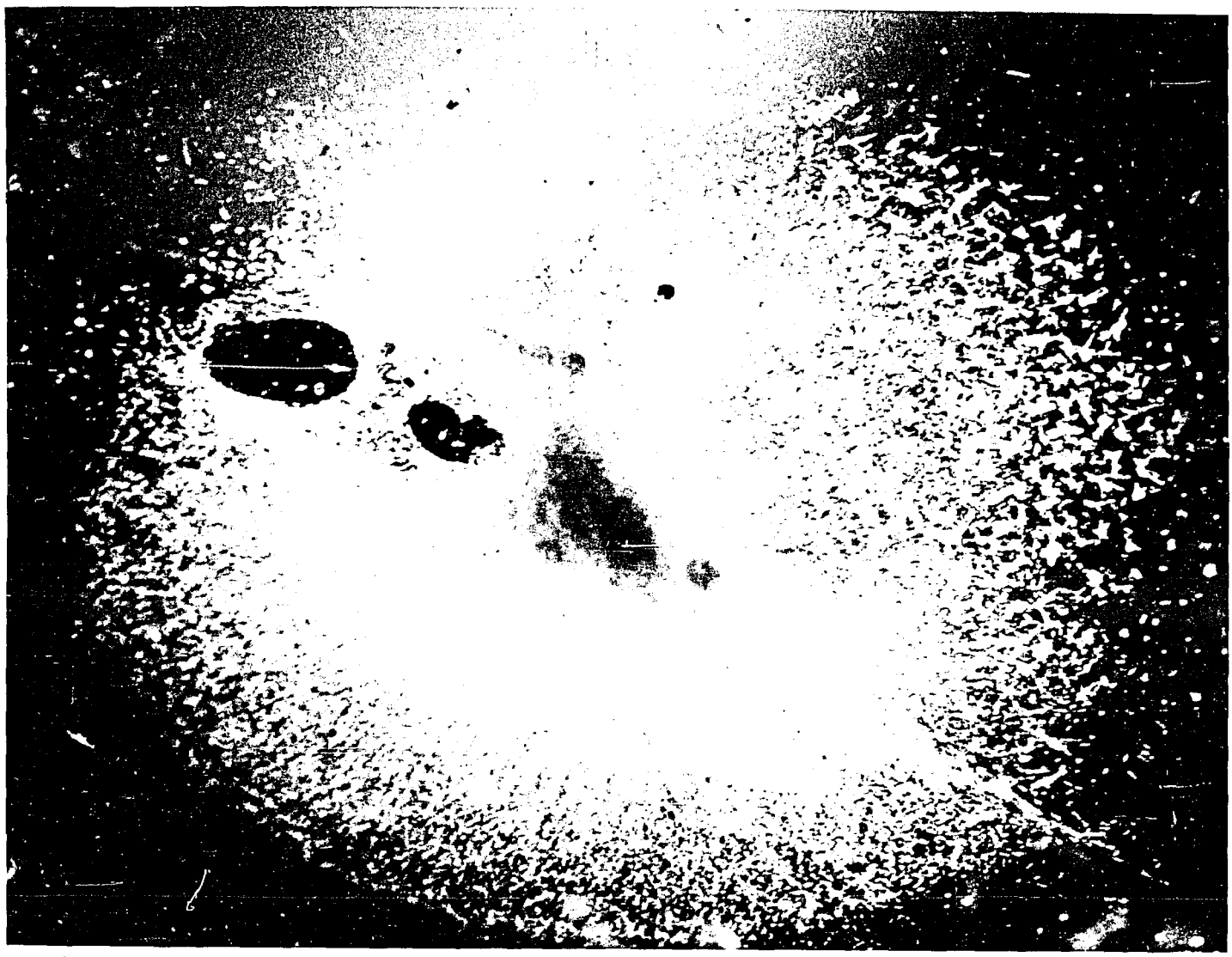


Figure 6. Growth of Normal Chicken Heart 7 day old Tissue Culture to which Concentrated Tumor Passage Allantoic Fluid from Tumor-II was added. Mag. 140 X.

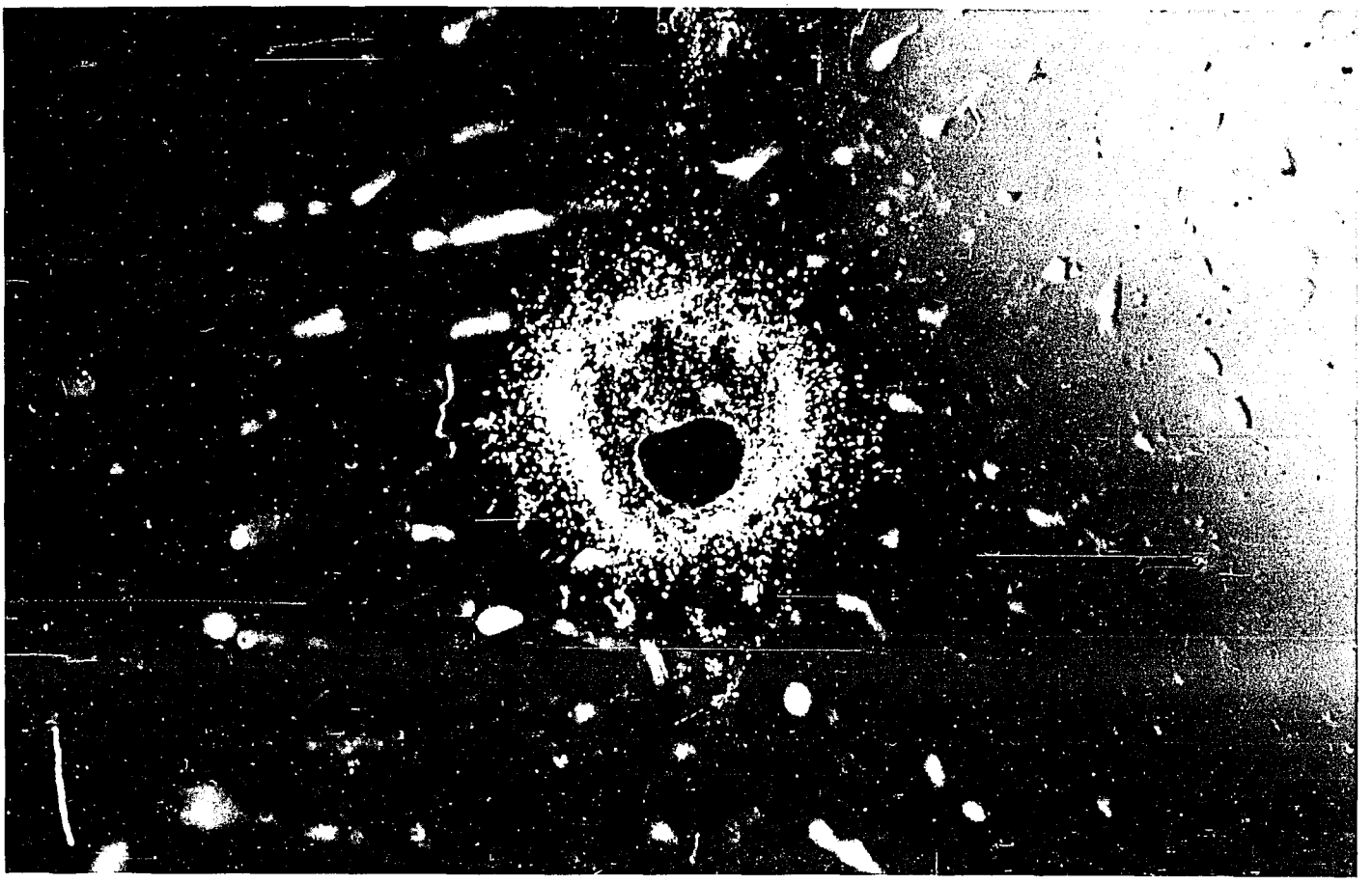


Figure 7. Growth of Normal Chicken Heart 7 day old Tissue Culture to which Concentrated Tumor Passage Allantoic Fluid from Tumor-XVI was added. Mag. 140X.

Hemagglutination Reaction after Passage of Tumor Material.

A. Tested with Normal Chicken Red Cells.

When a hemagglutination test was made on allantoic fluid after the first passage, several produced hemagglutination, especially when the undiluted allantoic fluid was used. Most of the tumor passage material however, failed to produce hemagglutination and was consistantly negative. Tables II through VI give the results of testing all tumor passage allantoic fluid through the five serial egg passages. Tumor passage material from tumor IX (Table IV) gave hopeful results after the first egg passage in that the undiluted fluid and a 1:5 dilution were capable of agglutinating normal chicken erythrocytes. A 1:10 dilution of this material gave a doubtful reaction. A second passage in eggs with ultracentrifugation concentrated material from the first passage, also gave doubtful results with the undiluted allantoic fluid. Occasionally, hemagglutination was observed during the serial passage, however the reaction was never strong and was not consistent in the following egg passage.

Table II

Hemagglutination Tests With Normal Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
I	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
II	1	-	-	-	-	-	-	-
	2	+ *	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
III	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
IV	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	±	±	±	-	-	-	-

* + = strong hemagglutination
- = no hemagglutination
± = weak hemagglutination

Table III

Hemagglutination Tests With Normal Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
V	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	+	±	-	-	-	-	-
VI	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
VII	1	-	-	-	-	-	-	-
	2	±	±	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	±	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
VIII	1	±	±	±	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-

Table IV

Hemagglutination Tests With Normal Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
IX	1	+	+	±	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
X	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XI	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	±	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XII	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	±	-	-	-	-	-	-
	4	±	-	-	-	-	-	-
	5	±	-	-	-	-	-	-

Table V

Hemagglutination Tests With Normal Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
XIII	1	±	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XIV	1	-	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XV	1	-	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XVI	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-

Table VI

Hemagglutination Tests With Normal Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
XVII	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
Normal Concentrated Allantoic Fluid		-	-	-	-	-	-	-

B. Tested with Chicken Red Cells Sensitized with Newcastle disease virus.

When Newcastle disease virus was used to sensitize the chicken red cell prior to addition of ultracentrifuge concentrated tumor passage allantoic fluid, the results gave increased hemagglutination activity. Here, as with normal unsensitized cells the hemagglutination reaction was stronger with undiluted tumor passage allantoic fluid. The results of testing the 17 tumors with allantoic fluid from the 5th serial passages is shown in Tables VII through XI. Strong reactions were obtained when passage material from tumor VIII was tested for hemagglutination. Although the second passage allantoic fluid gave negative results for hemagglutination, the 3rd, 4th and 5th passages produced strong reactions. The 3rd and 4th passages were positive when tested undiluted and in 1:5 dilution. However, by the 5th passage only the undiluted material was capable of producing hemagglutination.

Table VII

Hemagglutination Tests With Newcastle Disease Virus Sensitized Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
I	1	o *	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	-	-	-	-	-	-	-
II	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	±	-	-	-	-	-	-
III	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	+	-	-	-	-	-	-
IV	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	+	±	-	-	-	-	-
	5	o	o	o	o	o	o	o

* + = strong hemagglutination
- = no hemagglutination
± = weak hemagglutination
o = The test was not performed.

Table VIII

Hemagglutination Tests With Newcastle Disease Virus Sensitized Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
V	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	+	-	-	-	-	-	-
	5	o	o	o	o	o	o	o
VI	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	-	-	-	-	-	-	-
	4	o	o	o	o	o	o	o
	5	-	-	-	-	-	-	-
VII	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
VIII	1	o	o	o	o	o	o	o
	2	-	-	-	-	-	-	-
	3	+	+	±	-	-	-	-
	4	+	+	-	-	-	-	-
	5	+	-	-	-	-	-	-

Table IX

Hemagglutination Tests With Newcastle Disease Virus Sensitized Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
IX	1	o	o	o	o	o	o	o
	2	+	-	-	-	-	-	-
	3	+	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	±	±	-	-	-	-	-
X	1	±	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	+	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	±	±	±	-	-	-	-
XI	1	±	-	-	-	-	-	-
	2	±	±	±	-	-	-	-
	3	±	-	-	-	-	-	-
	4	±	-	-	-	-	-	-
	5	±	-	-	-	-	-	-
XII	1	±	±	-	-	-	-	-
	2	+	±	-	-	-	-	-
	3	+	-	-	-	-	-	-
	4	±	±	±	-	-	-	-
	5	-	-	-	-	-	-	-

Table X

Hemagglutination Tests With Newcastle Disease Virus Sensitized Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
XIII	1	±	±	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	+	-	-	-	-	-	-
XIV	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	±	-	-	-	-	-	-
	4	±	-	-	-	-	-	-
	5	±	-	-	-	-	-	-
XV	1	-	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XVI	1	-	-	-	-	-	-	-
	2	±	±	±	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-

Table XI

Hemagglutination Tests With Newcastle Disease Virus Sensitized Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
AVII	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
Normal Concentrated Allantoic fluid		-	-	-	-	-	-	-

C. Tested with Chicken Red Cells modified with Trypsin.

The results of testing the ultracentrifuged concentrated tumor allantoic fluid passage material with trypsin modified red cells is given in Tables XII through XVI. As expected, the trypsin modification of the cells increased the sensitivity of the cells to agglutination. In general, many of the tumor passage allantoic fluids in many of the passages gave weak or doubtful reactions when tested as undiluted concentrate. However, passage material from tumors XI and XII (Table XIV) produced a marked agglutination, during at least one of the five egg passages. Tumor XI material in the second egg passage gave a strong hemagglutination reaction in the 1:10 dilution and a weak reaction in two greater dilutions. The third passage exceeded the second passage reaction in that a strong hemagglutination was found in the 1:40 dilution. By the 4th egg passage however, the hemagglutination reaction deteriorated to doubtful reaction in the undiluted material.

The concentrated allantoic passage material obtained from tumor XII produced hemagglutination in comparative manner.

Table XII

Hemagglutination Tests With Trypsin Modified Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantioc Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
I	1	o *	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	-	-	-	-	-	-	-
II	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	-	-	-	-	-	-	-
III	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o
	5	+	-	-	-	-	-	-
IV	1	±	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	o	o	o	o	o	o	o

* - = strong hemagglutination
- = no hemagglutination
± = weak hemagglutination
o = The test was not performed.

Table XIII

Hemagglutination Tests With Trypsin Modified Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
V	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o
	4	±	±	–	–	–	–	–
	5	o	o	o	o	o	o	o
VI	1	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o
	3	+	–	–	–	–	–	–
	4	o	o	o	o	o	o	o
	5	–	–	–	–	–	–	–
VII	1	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–
	4	+	+	±	–	–	–	–
	5	–	–	–	–	–	–	–
VIII	1	o	o	o	o	o	o	o
	2	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–
	4	–	–	–	–	–	–	–
	5	+	+	+	+	–	–	–

Table XIV

Hemagglutination Tests With Trypsin Modified Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
IX	1	o	o	o	o	o	o	o
	2	+	−	−	−	−	−	−
	3	±	−	−	−	−	−	−
	4	+	−	−	−	−	−	−
	5	+	+	+	−	−	−	−
X	1	±	−	−	−	−	−	−
	2	+	+	+	−	−	−	−
	3	+	+	+	+	−	−	−
	4	+	−	−	−	−	−	−
	5	±	±	±	−	−	−	−
XI	1	+	−	−	−	−	−	−
	2	+	+	+	±	±	−	−
	3	+	+	+	+	+	−	−
	4	±	−	−	−	−	−	−
	5	+	±	−	−	−	−	−
XII	1	±	±	−	−	−	−	−
	2	+	+	+	+	+	−	−
	3	+	±	−	−	−	−	−
	4	±	±	±	−	−	−	−
	5	−	−	−	−	−	−	−

Table XV

Hemagglutination Tests With Trypsin Modified Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
XIII	1	±	-	-	-	-	-	-
	2	+	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	+	-	-	-	-	-	-
XIV	1	-	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	±	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
XV	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	+	-	-	-	-	-	-
XVI	1	-	-	-	-	-	-	-
	2	±	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	+	-	-	-	-	-	-
	5	-	-	-	-	-	-	-

Table XVI

Hemagglutination Tests With Trypsin Modified Chicken Erythrocytes

Tumors	Passages	Undiluted	Tumor Passage Allantoic Fluid Dilution					
			1/5	1/10	1/20	1/40	1/80	Control
XVII	1	-	-	-	-	-	-	-
	2	+	-	-	-	-	-	-
	3	±	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	±	-	-	-	-	-	-
Normal Concentrated Allantoic fluid		-	-	-	-	-	-	-

Tests for Interference between Tumor Passage Allantoic Fluid Followed by Newcastle disease virus *in vivo*.

A. When the ultracentrifuged concentrated tumor allantoic fluid was injected into embryonated eggs and followed by Newcastle disease virus, there was no indication that any interference had occured. Table XVII gives results of two-fold dilution titration of the Newcastle disease virus found in eggs that had previously been injected with tumor passage allantoic fluid and finally Newcastle disease virus. Slight differences of one dilution tube occured but are not significant.

Tests on Interference between Tumor Passage Allantoic Fluid followed by Newcastle disease virus *in vitro*.

B. Table XVIII gives the results of tests done to determine if there was any interference between the absorption of tumor passage allantoic fluid and Newcastle disease virus upon the washed guinea pig red cell. As can be seen by the results, concentrated tumor passage allantoic fluid or concentrated normal allantoic fluid both reduced the original titer of the original Newcastle disease virus. There was no difference, however, between the tumor passage fluid and that obtained by normal concentrated allantoic fluid.

Table XVII

Tests For: Interference Between Tumor Passage Allantoic Fluid Followed By Newcastle Disease Virus, In Vivo.

Tumors	Undiluted	Dilution Titers of Newcastle Disease Virus									
		1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Control
I	+ *	+	+	+	+	+	+	−	−	−	−
II	+	+	+	+	+	+	±	−	−	−	−
III	+	+	+	+	+	+	+	−	−	−	−
IV	+	+	+	+	+	+	+	±	−	−	−
V	+	+	+	+	+	+	±	−	−	−	−
VI	+	+	+	+	+	+	+	±	−	−	−
VII	+	+	+	+	+	+	+	+	+	−	−
VIII	+	+	+	+	+	+	+	+	−	−	−
IX	+	+	+	+	+	±	−	−	−	−	−
X	+	+	+	+	+	+	±	−	−	−	−
XI	+	+	+	+	+	+	±	−	−	−	−
XII	+	+	+	+	+	+	+	±	−	−	−
XIII	+	+	+	+	+	+	+	+	±	−	−
XIV	+	+	+	+	+	+	+	+	−	−	−
XV	+	+	+	+	+	+	+	+	+	±	−
XVI	+	+	+	+	+	+	+	+	−	−	−
XVII	+	+	+	+	+	+	+	+	±	−	−
Normal Concentrated Allantoic fluid (Control)	−	+	+	+	+	+	+	+	±	−	−

* + = strong hemagglutination ± = weak hemagglutination
− = no hemagglutination

Table XVIII

Tests For: Interference Between Tumor Passage Allantoic Fluid Followed by Newcastle Disease Virus, In Vitro.

Tumors	Undiluted	Dilution Titers of Newcastle Disease Virus								
		1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Control
I	+ *	+	+	+	+	±	−	−	−	−	−
II	+	+	+	+	±	−	−	−	−	−	−
III	+	+	+	+	+	±	−	−	−	−	−
IV	+	+	+	+	±	−	−	−	−	−	−
V	+	+	+	+	+	−	−	−	−	−	−
VI	+	+	+	+	+	±	−	−	−	−	−
VII	+	+	+	+	+	+	±	−	−	−	−
VIII	+	+	+	+	±	−	−	−	−	−	−
IX	+	+	+	+	±	−	−	−	−	−	−
X	+	+	+	+	+	±	−	−	−	−	−
XI	+	+	+	+	+	−	−	−	−	−	−
XII	+	+	+	+	±	−	−	−	−	−	−
XIII	+	+	+	+	±	−	−	−	−	−	−
XIV	+	+	+	+	−	−	−	−	−	−	−
XV	+	+	+	+	±	−	−	−	−	−	−
XVI	+	+	+	+	±	−	−	−	−	−	−
XVII	+	+	+	+	+	+	±	−	−	−	−
Normal Concentrated Allantoic fluid (Control)	+	+	+	+	±	−	−	−	−	−	−
Newcastle Disease Virus (original)	+	+	+	+	+	+	+	+	+	+	−

* + = strong hemagglutination ± = weak hemagglutination
− = no hemagglutination

DISCUSSION

V DISCUSSION

The experiments described here were primarily designed to test the theories of Bostick (1952) and Lundback and Lofgren (1950), who had described agents isolated from tumors or neoplasms by embryonated chicken egg passage. However, the methods used in this work were such that any agent capable of multiplication or existing in an extract from tumor tissue, was given greater opportunity to do so. For instance, if the total allantoic fluid from an embryonated chicken's egg contained one infectious or hemagglutinating unit the material used for a second passage into embryonated eggs would therefore contain less than one unit, the inoculum (0.1 mm.) being only a fractional amount of the total allantoic fluid contained in an egg. To concentrate all possible infectious material contained in the allantoic fluid, a pool of allantoic fluid from each passage was made in the ultracentrifuge. This concentrated allantoic fluid, then, constituted the inoculum for the next embryonated egg passage.

It was essential that a more sensitive indicator system be used to test the presence of hemagglutinating agents since normal chicken erythrocytes failed to show agglutination in the preliminary tumor passage allantoic fluid material. Therefore, normal chicken red cells were

sensitized by contact with Newcastle disease virus or modified with trypsin prior to use in the hemagglutination tests. This has been shown to enhance their reaction in the presence of small amounts of viral agents.

Attempts were made to use the interference phenomenon to detect any agent contained in the material used for egg passage. Newcastle disease virus was used primarily because of the work of Lundback and Lofgren (1950) in which they showed that this virus was similar to the agent that they had isolated from tumors. Ultracentrifuged, concentrated tumor passage allantoic fluid was injected into embryonated eggs to be followed by Newcastle disease virus.

Neoplastic tissues were shown to be capable of growth in vitro. Nine cultures of these tumors could be maintained almost indefinitely; eight failed to grow. Although eight of the 17 tumors failed to grow in tissue culture, they were not excluded from the serial egg passages. It was felt that failure to grow in tissue culture did not exclude the presence of an agent, nor did it prove that the neoplastic cells were dead cells. Therefore, all tumors received at the laboratory were subjected the same experimental process.

When the concentrated tumor passage allantoic fluid was incorporated into the nutrient medium of normal chicken heart cultures, the effect was a retardation of the growth of the normal tissue. However, one outstanding

exception to this retardation, (Graph I) was found in allantoic fluid originally from tumor II. This neoplasm was from human rectum. Therefore, there seems to be doubt as to the origin of the agent since fecal virus might well have contaminated the tissue. On the other hand, there must be some reason for the marked stimulation given to the normal heart tissue by the concentrate of allantoic passage material from this tumor. This same allantoic passage material did not produce a hemagglutination reaction (Table II, VII, XII) when tested with normal or altered indicator cells. Even when considering the variation in growth area with normal cells, the effect produced in this one instance does not possibly seem to be due to chance alone.

The results obtained in the various experiments with normal and altered red cells in the presence of concentrated tumor allantoic fluid have one thing in common. It was frequently found that hemagglutination occurred when the undiluted concentrated fluid was used. This was especially true when sensitized or modified red cells were used in the hemagglutination reaction as illustrated in tables IX and XIV. Occasionally a hemagglutination reaction was found occurring in the concentrated material which could be diluted 1 to 5 and still produce a preceptible reaction, when controls were negative (Tumor XII, Table IX). These results were never found to persist

throughout the five serial passages in the egg.

The strongest hemagglutination reactions were persistently found when trypsin modified red cells were used with the allantoic passage fluid. Quite frequently, strong reactions were found in 1:40 dilutions of the allantoic fluid. As is shown in table XIV, tumor XI and XII. This was to be expected since trypsin modified cells are very sensitive to agglutinating agents (Morton and Pickel, 1949), viral or others.

Control material (Table XVI) for this experiment consisted of concentrated normal allantoic fluid. No evidence of agglutination occurred when the normal allantoic fluid was combined with the modified cells. It is impossible to answer the question as to why the passage allantoic fluid material produced hemagglutination whereas normal allantoic fluid did not. Both were concentrated by ultracentrifugation before use in the hemagglutination test. It might be that tumor XI (Table XII) contained an agent which increased in titer from the first through the third passage but had been diminished or lost when the fourth concentrate was tested. It also might be that serial passage of concentrated allantoic fluid carried with it, some component, normal to the fluid or its membrane, which would produce hemagglutination whentested with the modified erythrocytes. The evidence found herein does not warrant a claim of positive isolation of an infectious agent as such.

In an attempt to find a more sensitive means of testing for agents as causes of neoplasms, an interference *in vivo* test was devised. In theory, the concentrated passage allantoic fluid, provided it contained an agent capable of multiplying, may interfere with the growth of Newcastle disease virus in the allantoic sac. However, as table XVII indicates, there was no significant difference in Newcastle disease virus titers obtained from allantoic fluids whether or not the tumor passage material preceded the introduction of Newcastle disease virus. There is a possiblity, however, that a tumor agent was capable of multiplication in only select tissues or fluids of the embryonated egg, while Newcastle disease virus is capable of propagation or, at least, existing in all tissues and fluids of such eggs.

An experiment was also devised to test for the interference that might occur when concentrated tumor passage allantoic fluid was allowed to be in contact with erythrocytes followed by Newcastle disease virus. The fact that the virus may be eluted very slowly from guinea pig cells, when compared to what happens in the case chicken red cells are used, it seemed advisable to use guinea pig cells as an indicator. As can be seen from Table XVIII, the presence of tumor concentrated allantoic fluid did not alter the release of Newcastle disease virus from guinea pig cells. There were indications that considerable

amounts of Newcastle disease virus had been retained by the guinea pig cells. The original Newcastle disease virus hemagglutinating was 1:1280, while in the presence of the control material, containing normal concentrated allantoic fluid, the Newcastle disease virus titer was 1:20. The normal concentrated allantoic fluid and tumor passage allantoic fluid were comparable in titer.

Throughout these experiments, all the known methods available were used to enhance the growth and detection of any agent derived from neoplasms which would survive five serial passages in the embryonating hen's egg. In several experiments, results were obtained suggesting that the concentrated tumor passage allantoic fluid reacted when tested by the hemagglutination test, whereas concentrated normal allantoic fluid did not. However, no evidence was found that an agent such as a virus, was present or survived five passages in embryonating hen's egg.

SUMMARY

VI SUMMARY

1. Using tissue culture methods, nine of a total of seventeen tumors were cultivated *in vitro*. The neoplastic tissue was from animals and man. There was no correlation between origin or type of neoplasm, and growth *in vitro*.

2. Concentrated tumor allantoic fluid, after five serial passages in embryonated chicken eggs, retarded the growth of normal chicken heart tissue in cultures. One exception was encountered in which exceptional growth occurred.

3. When the concentrated allantoic fluid was tested for hemagglutination with normal chicken cells, no reaction occurred. Sensitizing cells with Newcastle disease virus or modification with trypsin produced hemagglutination in many cases. When the concentrated allantoic fluid was tested for hemagglutination with modified or sensitized cells between each passage, hemagglutination was frequently observed. None of the seventeen tumors studied contained an agent that was capable of producing hemagglutination through all five consecutive serial passages.

4. No interference, *in vitro* or *in vivo*, could be detected between concentrated allantoic fluid tumor passage material and Newcastle disease virus.

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