ON THE CAUSATION BY FILTERABLE AGENTS OF THREE DISTINCT CHICKEN TUMORS.*

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PLATES 7 TO 12.

The study of chicken tumors has especial interest because the cause of two such growths has been found in filterable entities. The object of the present paper is to report experiments which show that yet a third neoplasm of the fowl is so caused, and to discuss at some length the methods and findings with all three. One of the growths,—Chicken Tumor I in our series of spontaneous chicken tumors,—is a pure spindle-celled sarcoma;¹ the second (Chicken Tumor VII) is an osteochondrosarcoma;² and the third (Chicken Tumor XVIII), of which the cause will here for the first time be reported, is a spindle-celled sarcoma of peculiar intracanalicular pattern.³ The three are very unlike, not only histologically but in their general behavior. Yet, as will be seen, the entities causing them have much in common and may profitably be considered together.

THE IMPORTANCE OF PRELIMINARY TRANSPLANTATION.

In our experience the transplantation of chicken tumors is of great importance for experiments looking to their cause. This is in part on account of the material afforded by successful transplantation, but it has its essential basis in the enhanced malignancy resulting therefrom. The findings have shown strikingly that the more malignant the growth the easier is the demonstration of its etiological agent. The fact that tumors caused by an agent dam-

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aged in some way, as for example by heat or by long sojourn in the dry state, are of relatively benign course suggests that the ready separation of the agent from growths of high malignancy is dependent on its enhanced activity.

THE TUMOR-PRODUCING ENTITIES ARE FILTERABLE.

Three methods, namely filtration, desiccation, and glycerination, have been chiefly used to demonstrate a cause for the chicken tumors, as distinct from the tumor cells. Specimen protocols will be given to illustrate the results with each method and these will be followed by a summary of the findings obtained by its employment. The growths produced have in every instance been examined with the microscope.

Filtration has proved by far the most uniformly successful method of study. Under suitable conditions the agents of all the growths will pass through Berkefeld filters that retain small bacteria (Bacillus prodigiosus, Bacillus fluorescens liquefaciens). Our technique has been described several times. It is again briefly given in the first of the two following protocols that show the filterability of the agent causing Chicken Tumor XVIII.

Chicken Tumor XVIII, a Sarcoma of Intracanalicular Pattern. Filtration Experiment 1.—The tumor material came from fowl 257 of the second transplantation generation, series A. Fresh neoplastic tissue to the amount of 7.5 gm. was ground fine with sand, taken up in 250 c.c. of Ringer’s solution at 40°C. and a 24 hour culture on slant agar of Bacillus fluorescens liquefaciens was added. Shaking was done in a machine for forty-five minutes, then brief centrifugation, and portions of the supernatant fluid were filtered by suction through one or another of three Berkefeld filters; (a) a medium sized V (No. 3), and (b) and (c) two small N cylinders (No. 5). Filtration was continued for about thirty minutes. The fluids from filters a and b were united as filtrate AB. That from filter c will be called filtrate C. To each was added a little sterile diatomaceous earth (Kieselguhr) and then 14 c.c. of AB and 7 c.c. of C were injected intramuscularly in the right and left pectoral regions respectively of five normal brown Leghorn fowls. All remained free of tumor until 170 days after the inoculation, when in one a small mass, 2.5 by 1.5 cm. in diameter was noted at the site of injection of filtrate AB. Part of this was removed and found to have


5 It may not be amiss to point out that in epidemic poliomyelitis, a disease caused by a filterable virus or parasite, the initial infection of monkeys with human nervous tissues is accomplished with difficulty, while after the virus has
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the characteristic structure of Chicken Tumor XVIII. The fowl was shortly afterwards lost through accident. The other chickens were kept some months longer but never developed tumors.

Plates of the filtrates (1 c.c. to the tube of agar) remained sterile, whereas plates of the unfiltered fluid (a few drops to the tube of agar) showed innumerable colonies of *Bacillus fluorescens liquefaciens*. At the time of the experiment bits of the tumor which furnished material for it were transplanted into three chickens. Two of these developed tumors within a month. The third remained healthy.

Filtration Experiment 2.—The material was furnished by fowl 531 of the third transplantation generation, series C. The tumor was of especially rapid growth. Three Berkefeld filters were employed: (a) a medium sized V cylinder (No. 3), and (b) and (c) two small N cylinders (No. 5). *Bacillus fluorescens liquefaciens* was used as before; and the filters proved impermeable to it. To each of the filtrates, A, B, and C, a little finely ground, sterile, diatomaceous earth was added previous to their separate injection in amounts of 5 c.c. each, into the muscle of the lower leg and breast of eight fowls. Two of the fowls developed progressively growing tumors which appeared, in the one case at sites A and C two months after the time of injection, in the other at sites A and C about five months after it. Both died of the tumor some seven and one half months after inoculation. In addition to the large primary growths there were metastases in the lungs, and in one case secondary growths about several joints (figure 1), a finding not infrequent when Chicken Tumor XVIII is propagated by transplantation. The hip joint was diffusely involved in sarcomatous tissue and fusiform swellings enclosed most of the sternal and vertebral ribs at their junction, forming what might be called a sarcomatous rosary. Whether in the present case these represent primary localizations of the tumor-producing virus or true metastases cannot be said. Microscopically all the growths had the characteristic structure of Chicken Tumor XVIII (figure 2).

In a third fowl a tumor nodule appeared within two months at the site of filtrate B, slowly enlarged to a diameter of 4 cm., and slowly retrogressed again. The only transplantations attempted from the filtrate tumors were performed with this growth. They gave negative results. The other fowls injected with filtrate remained healthy.

The association of a foreign body with the filtrate to bring about a tissue derangement renders much more likely the production of tumors. The influence of the factor has been carefully studied in the case of Chicken Tumor I. In all the experiments we have made use of powdered diatomaceous earth (*Kieselguhr*), which elicits, as Podwyssozki has shown, an intense reactive connective tissue proliferation with the formation of giant cells. Histologically this has been transmitted through several monkeys, it increases markedly in virulence for those animals and can be transferred with far greater certainty.

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reactive proliferation bears no resemblance to the tumors with which we have worked (figure 10).

\textit{Chicken Tumor XVIII. Filtration Experiment 3.}—Filtrates were prepared in the usual way, through filters which the control with \textit{Bacillus fluorescens liquefaciens} showed to be bacteria-tight. Three Berkefeld cylinders were used: (a) a large unmarked cylinder (No. 2), (b) a medium V (No. 3), and (c) a small N (No. 5). The filtrates were separately examined for the test bacterium, and injected. To half of each was added enough sterile, powdered \textit{Kieselguhr} to give a slight cloudiness. For this very little was required. Six fowls were injected, receiving on one side in the thigh and pectoral muscle respectively two filtrates with \textit{Kieselguhr}, at corresponding points on the other side the same filtrates in equal or slightly greater amount, without \textit{Kieselguhr}. Of the five fowls that survived three months one developed after the sixtieth day small tumor nodules, where the filtrates A and C had been injected in amounts of 10 c.c. and 3 c.c. respectively with \textit{Kieselguhr}. The corresponding sites, where slightly larger quantities of the filtrates without admixture had been put, remained free of growths. The tumors measured 4 cm. and 3 cm. in diameter respectively when the fowl was killed three months after injection. They had the characters of Chicken Tumor XVIII. The other fowls, kept long under observation, remained healthy.

\textit{Chicken Tumor VII, an Osteochondrosarcoma. Filtration Experiment 2.}—Six normal fowls were injected in the pectoral muscle on the right side with 6 c.c. each of an active Berkefeld filtrate, in the right thigh muscles with 4 c.c. of the same filtrate to which had been added a little \textit{Kieselguhr}. It has been repeatedly found that the thigh muscles furnish a relatively poor site of inoculation. Nevertheless, in four of the fowls rapidly growing cartilaginous tumors of multicentric origin appeared in the thigh (figures 3 to 8), whereas a pectoral tumor developed in only two of the four. It took the form of a discrete cartilaginous nodule in the track of the injecting needle. The other two fowls remained healthy. One of the tumor fowls furnished material for the experiment which follows and also for a successful attempt to dry the etiological agent. Some of the growths contained so much bone that the saw was required to lay them open.

\textit{Filtration Experiment 4.}—Portions of a tumor extract prepared as usual and with the usual bacterial control were passed through one of the following Berkefeld cylinders: (a) and (b) two small N cylinders (No. 5), and (c) a medium sized V (No. 3). The tumor material came from one of the fowls of filtration experiment 2. Filtrates A and B were injected separately in amounts of 10 c.c. into the leg muscles of three normal fowls. Filtrate C with and without admixture of \textit{Kieselguhr} was injected into the pectoral regions of the same fowls. Nineteen days later one had developed a tumor nodule 1.3 cm. in diameter at the spot where 5 c.c. of filtrate C with \textit{Kieselguhr} had been injected. The nodule rapidly enlarged to a diffuse mass which led to the death of the fowl after six weeks in all. At this time a nodule had just appeared in the other pectoral region where 10 c.c. of filtrate C without admixture had been put. At autopsy another small discrete nodule was found in the left leg as the result of 10 c.c. of filtrate A without \textit{Kieselguhr}. The other two fowls remained healthy.
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Chicken Tumor I, a Spindle-Celled Sarcoma. Injury Experiment 15.—An active Berkefeld filtrate was prepared as usual and to half of it a little Kieselguhr was added. 9 c.c. of the mixture was injected into the pectoral muscles on one side of eight fowls, and on the other as control an equal amount of the plain filtrate. The results are shown in text-figure 1. It will be seen that where there was Kieselguhr tumors arose much more rapidly, and when palpable were already diffuse. In this instance nearly all the fowls responding with a tumor to the filtrate plus Kieselguhr eventually developed growths where the filtrate alone had been placed. Ordinarily this happens in only a small percentage.

SUMMARY ON FILTRATION.

The growths engendered by a Berkefeld filtrate have the distinctive characters of the strain of tumor which furnished the material for filtration (figures 1 to 9). In the case of Chicken Tumors I and VII growths so caused have themselves been successfully used for the preparation of filtrates, as for example in one of the experiments just cited. They have also been found to be transplantable. The results with Chicken Tumor XVIII are relatively meagre owing to the long period of latency after the injections,—more than five months in one case,—and to the slow growth of the filtrate tumors. The agents causing Chicken Tumors I and VII often pass the filter in large amount, as shown by the multicentric development of tumors in the region injected, but even when the element of tissue derangement is present, Chicken Tumor XVIII usually appears from one, or at most a few, centers. This is more probably due to low average virulence or resistance in the agent than to difficulties in filtration, for the findings show that all the agents pass or are held back by filters of about the same texture. They pass most V cylinders, many designated as N, but they are usually retained by the fine textured W cylinders. As might be expected from these results the agent of Chicken Tumor I,—the only one thus tested so far—fails to pass Chamberland bougies.

Not infrequently filtrates prepared from malignant material under the best conditions prove entirely innocuous. This is in most cases due to the narrow limits within which the agents are filterable. Two possibilities suggest themselves as accounting for these limits, first that the agents are formed bodies, second that if unformed they are associated with substances which clog the pores of the filters. Mucinous substances are so abundant in Chicken Tumor I
that coarse filters are soon completely stopped by very dilute extracts of the growth; but with VII and XVIII there is no such complication. Extracts of these tumors run rapidly through the filters and yet are often inactive. The agents then would seem to be of relatively large size among the filterable causes of disease.

The tumor resulting from a local injection of filtrate appears at the site of inoculation. As already mentioned the importance of tissue derangement for the action of all three causative agents is very great. The experiments show that when this factor is supplied by the addition of Kieselguhr to a filtrate the percentage of fowls that develop tumors is much increased and the growths themselves appear sooner and enlarge more rapidly (text-figure 1). The Kieselguhr, injected alone, does not cause tumors. A limpid filtrate injected in the breast muscle finds its point of action in the track of the injecting needle (figure 9), and there results a discrete growth from one center. Oftentimes, as ordinarily prepared, the filtrate contains a few particles from the interior of the filter, and the growth may then arise from several centers. But when powdered Kieselguhr has been added the growth is multicentric and appears all at once as a mass of coalescing foci. The microscopic findings show that the sarcomatous change in the reactive tissue about the Kieselguhr is not diffuse but punctate, and the growth of the little tumors is largely expansive (figure 10). In fowls inoculated intravenously with a filtrate of Chicken Tumor I the tumors have been found to arise at sites of tissue derangement. Their incidence is approximately doubled when Kieselguhr has been introduced into the blood stream.

As already mentioned the reactive tissue called forth by Kieselguhr contains large numbers of giant cells. These are of ordinary foreign body type and enclose the Kieselguhr fragments. With the replacement of the reactive tissue by tumor the giant cells are destroyed (figure 10) and the Kieselguhr is set free. It can be found here and there among the sarcoma cells, which appear in no way affected by it.

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Text-FIG. 1. CHICKEN TUMOR I. INJURY EXPERIMENT 15. Eight normal Plymouth Rock fowls, Nos. 932 to 939, were injected in one pectoral region with a Berkefeld filtrate of Chicken Tumor I, in the other with an equal quantity of the same filtrate to which had been added a little powdered diatomaceous earth (Kieselguhr). Examinations were made every week thereafter and the size of the tumors charted. It will be seen that where filtrate plus Kieselguhr was injected tumors appeared more frequently and much earlier, and were diffuse when first noted in sharp contrast to the discrete nodules on the other side.

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DESICCATION.

Since many microorganisms withstand drying and the tissue cells of the higher animals do not, workers have frequently used desiccation in attempts to separate an etiological agent from mammalian tumors. The literature need not be discussed since it deals only with negative results. The agents causing two of our chicken tumors, namely the pure spindle-celled sarcoma (Chicken Tumor I), and the osteochondrosarcoma (Chicken Tumor VII), resist drying. No special complications are met with in obtaining an active dry form of the agent of Chicken Tumor I, but in the case of Chicken Tumor VII successful results have been obtained only by the desiccation of frozen material. A few specimen protocols will be given.

Chicken Tumor I. Desiccation Experiment 2.—The tumor material, obtained from fowl 243 of the ninth transplantation generation, series A, was ground fine and spread thin in a desiccator containing sulphuric acid. A partial vacuum only was produced. After forty-eight hours the scales of dry tissue were pulverized in a mortar and replaced in the desiccator. After sixty-seven hours in all the powder was taken up in Ringer's solution by grinding, in the proportion of 2 gm. to 70 c.c. 5 c.c. of the turbid, viscous fluid were injected into each breast of seven normal hens. Six showed rapidly growing tumors at the end of the month. The seventh remained healthy. One of the fowls furnished tumor material for the experiment which follows.

Desiccation Experiment 4.—A fowl of experiment 2, with a large tumor, was killed and the neoplastic material dried as before, for forty-eight hours; ground; replaced in the desiccator for seventy-two hours more; and then sealed in glass and kept in the dark at about 2° C. Fifty-four days later 2 gm. of the material were taken up in 25 c.c. of Ringer's solution, and the thick suspension was inoculated in amounts of 5 c.c. into each breast of five normal hens. Three of these had developed tumors two weeks later, and one growth was already large. In a fourth fowl the tumor appeared later. The fifth remained quite healthy.

Some of the dried material was left sealed and in the cold for seven months. 3 gm. of it were then taken up in 20 c.c. of normal salt solution and 1 c.c. of the mixture was injected into one breast of eight fowls. Of these two had developed small tumors after forty days. The others remained healthy. The growths were typical Chicken Tumor I.

Chicken Tumor VII. Desiccation Experiment 3.—Fowl 549, carrying a large growth engendered by a filtrate (filtration experiment 2) was killed, and the tumor, which consisted of cartilage and precartilaginous, sarcomatous tissue, was ground fine and placed in a cold compartment at several degrees below 0° C., where in the course of forty-eight hours it gradually froze solid. While still frozen it was put in a cold desiccator over sulphuric acid, and the air exhausted until the manometer showed a pressure of less than 1 mm. of mercury. The desiccator was then placed immediately in an ice chest. At the end of three days the material was taken out and ground. It had the form of a light, brittle cake
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of nearly the bulk of the fresh tissue, was cottony in texture, and ground with great difficulty. Twenty-four hours after grinding, 2 gm. of the powder were taken up in 25 c.c. of sterile water and of the thick fluid 5 c.c. were injected into the left breast of each of five normal fowls. Within a month one developed a growth measuring 4 by 9 cm. It was killed and portions of the tumor were used for the experiment that follows. The tumor consisted of a spindle-celled sarcomatous tissue undergoing cartilaginous differentiation in the way characteristic of Chicken Tumor VII. The other fowls, kept under observation several months, remained healthy.

**Desiccation Experiment 4.**—The material came from the susceptible fowl of the previous experiment. It was ground, frozen, and dried while still frozen, by the method just outlined. It remained in the freezing chamber three days, in the desiccator twenty-four hours, and was then powdered and placed in the ice chest for twenty-four hours more. Eight fowls were injected in the left breast with 5 c.c. each of a thick fluid made by taking up 2 gm. of the powder in 40 c.c. of distilled water. In the other breast glycerinized tumor tissue was placed at the same time (glycerin experiment 2). Within three weeks all except one of the injected fowls had developed a tumor mass at the site of inoculation of the dried material, the largest measuring 3.4 by 7.5 cm. Four of the fowls were allowed to die of their growth, the other three being killed and examined at various times. All the growths presented the character of Chicken Tumor VII, with much cartilage and in several instances bone. The eighth fowl remained healthy.

In this experiment there was the complicating factor of tumors developing synchronously from glycerinated material. But that the tumors which arose where dried material had been injected were engendered by this is certain, not only from the local character of the neoplastic disease in its early stages, but from many other instances that might be cited in which the dried tissue of Chicken Tumor VII has been successfully used to cause the growth.

**SUMMARY ON DESiccATION.**

The growths caused by dried tissue of Chicken Tumors I and VII appear at the site of injection and themselves furnish material which has a tumor-producing activity when dried. The etiological agent of Chicken Tumor I undergoes a gradual attenuation when the dried tissue is stored in the dark at a temperature slightly above 0° C., but after seven months is still capable of producing tumors. In the case of Chicken Tumor VII no attempt has been made to store dried material for longer than three weeks. At the end of this time it still produced tumors.

Numerous attempts have been made to obtain the agent of Chicken
Tumor XVIII in an active dry form, but as yet without success. In this connection it should be recalled that Chicken Tumor XVIII under the best conditions grows much more slowly than I and VII and that a filtrate of it gives rise only exceptionally to tumors and after a long latent period. The agents of Chicken Tumors I and VII when dried at low temperature are both very active; but the one easily survives drying at room temperature whereas the other fails to. Differences in the viability of organisms would well account for this.

The growths caused by dried material spring from multiple foci and are often diffuse when first palpable. The bits of dead tissue doubtless act to produce like Kieselguhr a tissue derangement favorable to the production of tumors.

GLYCERINATION.

The effect of glycerin has especial interest because of the ultramicroscopic organisms which retain their vitality when immersed in it. Loeb has observed its effect upon mammalian tumor tissue. He placed pieces of rat sarcoma in pure glycerin, and, seventeen to twenty-four hours later, washed them in salt solution and injected bits into other rats. In some instances a tumor developed. If it can be assumed that the glycerin thoroughly penetrated the tumor tissue the finding is significant. For this reason we have repeated and enlarged upon Loeb's experiment, previous to observations with the chicken tumors. Briefly, the findings show that pieces of the Jensen rat sarcoma 0.5 cm. thick, when kept for twenty-four hours in pure glycerin at a temperature of 1° to 2° C., will sometimes give rise to tumors on being cut up and implanted; but the same material, finely chopped and stirred into the glycerin, yields only negative results, even when well washed and injected in quantity. Rat sarcoma treated according to the latter method occasionally retains its viability for twenty-four hours in a 12.5 or 25 per cent. glycerin mixture with Ringer's solution, but never in 50 per cent. glycerin, nor for a longer period (four days) in the weak dilutions mentioned. Hence it seems then that the positive results with pieces of rat tumor kept in concentrated glycerin are dependent on incomplete penetration.

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Of the filterable entities that cause chicken tumors the two which withstand drying will also survive glycerination.

Chicken Tumor I. Glycerin Experiment 3.—13 gm. of fresh tumor tissue from fowl $582$ (16th generation $B$) were ground with sterile sand, and suspended in 40 c.c. of Ringer's solution. The larger tissue particles were got rid of by straining, and the sand was allowed to settle out. Of the thick suspension of tissue 10 c.c. were stirred into an equal amount of pure glycerin, shaken until well mixed and placed in the ice box. After seven days the tissue fragments, which had by this time settled, were drawn off through the base of the tube by breaking a sterile glass projection in its wall. In this way all contamination with particles which had escaped the glycerin's action was avoided. The tissue suspension was now washed with Ringer's solution, centrifuged, and of the pasty sediment 0.15 c.c. was injected into the right and left pectoral muscles respectively of three young Plymouth Rock fowls. Ten days later in one fowl a small sarcomatous nodule made its appearance at each injection site, and in a second after seventeen days a single nodule developed. The subsequent growth of the tumors was slow, but otherwise they proved characteristic of Chicken Tumor I. The third fowl remained healthy.

Glycerin Experiment 2.—Fresh tumor tissue was ground in a mortar with sand and taken up in Ringer's solution. By rapid centrifugation the suspension was rid of all but extremely minute tumor fragments. To portions of it pure glycerin was added to the amount of 50, 25, and 12 per cent. respectively of the total bulk. The mixtures were placed in wide tubes and kept in the dark at $1^\circ$ to $2^\circ$ C. After nine days some of the material was withdrawn by breaking a projection at the base of the tube, as in the experiment already described. Twenty-two days later a fowl injected in each pectoral region with 0.4 c.c. of the mixture in 50 per cent. glycerin, diluted to 4 c.c. with Ringer's fluid, had developed very large tumors having the character of Chicken Tumor I. In the three fowls injected with 12 and 25 per cent. mixtures, variously diluted, growths had also appeared at this time.

Some tubes of the 12.5 and 25 per cent. mixtures were left untouched and in the cold for thirty-one days. Their contents were then drawn off as usual. One fowl injected in each breast with 3 c.c. of 25 per cent. mixture, undiluted, developed tumors. These first became palpable after six weeks. Another fowl injected in the same way with 1 c.c. of the 25 per cent. mixture made up to 2 c.c. with Ringer's fluid likewise slowly developed tumors. Three fowls inoculated with the 12.5 per cent. mixture, diluted with an equal bulk of Ringer's solution, remained healthy.

Higher percentages of glycerin have been used in the experiments with Chicken Tumor VII.

Chicken Tumor VII. Glycerin Experiment 3.—Cartilaginous tumor material was ground to a foamy pulp in a meat chopper and two mixtures were made with pure glycerin, in the amounts of (a) 2 c.c. tumor pulp and 8 c.c. glycerin, and (b) 4 c.c. tumor pulp and 10 c.c. glycerin. The actual quantities of tumor tissue were considerably less than those mentioned, as it had the form of a
soufflé. The mixtures were tubed and kept in the cold as usual. Sedimentation in the glycerin was extremely slow and shaking was done each day to keep the larger tissue fragments in suspension. After thirteen days the tubes were opened at the base, and the mixtures, made up to 20 c.c. with Ringer's solution, were injected in amounts of 5 c.c. into the right and left pectoral region, respectively, of three normal fowls. After two months one fowl developed a tumor that in another three months proved fatal. The growth was 6 cm. in diameter at this time and in addition to much cartilage contained bone. It had arisen at the site of injection of the mixture that contained the greater percentage of glycerin (8 c.c. to 2 c.c. of tumor). The other fowls remained healthy.

**Glycerin Experiment 4.**—Fresh cartilaginous tumor material was ground to a soufflé and three mixtures were made: (a) 10 c.c. of pure glycerin with an equal bulk of tumor material, (b) 10 c.c. of glycerin with 5 c.c. of tumor, and (c) 10 c.c. with 3 c.c. of tumor. These were kept in the ice box; shaken every day; at the end of ten days drawn off from below; and each made up to 20 c.c. with Ringer's solution and inoculated in amounts of 5 c.c. in one pectoral region of five normal fowls. Four of these died of intercurrent disease. Ninety-nine days after the injection the fifth had developed a tumor measuring 8 cm. at the inoculation site of mixture (c), and one of half this size where mixture (a) had been put. Both growths were characteristic of Chicken Tumor VII and both contained bone.

**SUMMARY ON GLYCERINATION.**

The experiments leave no doubt that the tumor tissue was thoroughly penetrated by glycerin. The agent of Chicken Tumor I retains some activity for at least seven days in 50 per cent. and for thirty-one days in 25 per cent. glycerin. The effect upon it of higher concentrations has not been tested. In the case of Chicken Tumor VII the amount of glycerin has ranged from 50 to about 90 per cent., but no attempt has been made to determine the period of survival of the agent. That it remains active in the high concentrations for at least thirteen days is shown by experiment 3. Glycerination undoubtedly has an attenuating action on both agents. The tumors develop in few hosts, and after a relatively long latent period, and often grow slowly. It has been repeatedly noted that the activity of the agents is best retained in high concentrations of glycerin. This might be thought due to differences in penetration of the tumor tissue attendant upon differences in concentration, were it not for the findings with rat sarcoma. Here concentrated glycerin proves the more injurious. Tissue autolysis in the dilute glycerin mixtures affords a better explanation of the results with the avian tumors. The most active agent, that of Chicken Tumor I,
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has been found to be quickly destroyed by autolysis of the tumor tissue.

Repeated attempts to preserve in glycerin the agent causing Chicken Tumor XVIII have been unsuccessful.

SEPARATION OF THE AGENTS BY OTHER METHODS.

Only in the case of Chicken Tumor I have still other attempts been made to distinguish the tumor-producing agent from the neoplastic cells. Ultraviolet rays kill the cells and leave the agent unharmed. The method requires careful control and is not available for the separation of large amounts of the agent. The resistance of the agent to heat is only very slightly greater than that of the cells. On the other hand, the agent withstands freezing and thawing which reduce the associated tumor tissue to a pulp.

DISCUSSION.

In the first attempts to isolate a causative agent from Chicken Tumors VII and XVIII exact precautions were taken to avoid a possible contamination with the agent of Chicken Tumor I, though such contamination had never been observed in the many routine transplantations of these growths. To avoid exposure of the material to the laboratory air it was ground in a large sterile box and otherwise protected. The results of the experiments showed that these precautions were unnecessary, for the character of the tumors engendered by the agents effectually proved that they were not the result of contamination. Each agent produces only growths of the kind from which it came. One stimulates connective tissue to proliferate and elaborate cartilage, ultimately to be replaced in greater or less part by bone (Chicken Tumor VII); another causes connective tissue to proliferate and form large undifferentiated spindle-celled masses (Chicken Tumor I); while a third engenders, like the second, a spindle-celled growth, but one containing much collagen, and characteristically fissured by blood sinuses, into which the growth shows a tendency to extend, resulting in a complex intracanalicular pattern (Chicken Tumor XVIII). The behavior of the

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several growths is as different as is their histology. Chicken Tumor I metastasizes to the lungs by preference, then to the further viscera; Chicken Tumor VII almost never gives metastases; and Chicken Tumor XVIII frequently disseminates to the muscles without other secondary localization. The individuality of the agents as exemplified in the neoplasms that they cause is not altered by attenuating them, yet it should be mentioned that Chicken Tumor XVIII, the fissured sarcoma, has recently shown a tendency, like some complex mouse tumors, to lose with repeated transplantation its histological peculiarities and become an undifferentiated spindle-celled sarcoma. Ultimately the agent causing it may produce tumors not very different from Chicken Tumor I.

The tumors engendered by the filterable agents become palpable only after a latent period,—which in the case of Chicken Tumor XVIII is usually several months. Chicken Tumors I and VII appear more promptly. Fowls which fail to develop the growths within one month after an injection of the agent remain free of them, as a rule. When large amounts of filtrate plus Kieselguhr, or of dried or glycerinated material, have been injected the resulting growths are diffuse when first observed, owing to proliferation from many foci, and they quickly become massive; but the period of latency—at least eight to ten days in the case of Chicken Tumors I and VII—is not appreciably shortened. That the greater part of this period is one of actual latency with tumor cells absent as yet, and is not merely an interval during which the tumor is growing but clinically imperceptible, has been determined by the early microscopic examination of sites where the agent is known to be present and active.

The injection of large amounts of the agent of Chicken Tumor VII results in tumors that grow progressively and soon lead to the death of the fowl; whereas the growths developing after implantation of a small bit of the tumor enlarge slowly and in most fowls become stationary and eventually regress. This difference might be thought due to the influence of dosage as affecting resistance,—a factor of much importance in the case of mouse tumors,—or it might conceivably result from the circumstance that growths induced by an agent are elaborated by the host's own tissues, while
such as result from transplantation represent tissue growing in a host to which it is strange. That the latter explanation is not fanciful has been shown by experiments with Chicken Tumor I demonstrating the existence in fowls of two types of resistance directed, the one against the tumor-producing agent, the other against the transplanted tumor cells. But in the case now being considered differences in dosage as affecting resistance are probably at the root of the matter. For by the injection of large amounts of the fresh tissue of Chicken Tumor VII in the form of a pulp, progressively growing tumors, such as result from massive doses of the agent, can be obtained.

The growths resulting from injection of a tumor-producing agent into the skeletal muscles are at first purely local in character, even when a filtrate is employed. In only one among many autopsies have growths been found which by their situation suggested a possible primary dissemination by the blood stream. This case is cited in one of the protocols (Chicken Tumor XVIII, filtration experiment 2). There is, of course, no reason why part of a filtrate, injected with a sharp needle, should not often pass directly into some blood vessel. But it has been shown with Chicken Tumor I that the direct intravenous inoculation of an active filtrate usually fails to produce tumors.

Many fowls are resistant to the tumor-producing agents in any of the forms that we have used. Glycerination markedly reduces the agents' activity and desiccation does so to a less degree, and roughly in proportion to the length of time that the material is kept after drying. Both filtrates and dried tissue prepared under the best conditions from malignant material are sometimes unaccountably inactive. More often the inactivity can be traced to the use of too concentrated extracts for filtration, too finely textured a filter, or too slow a process of drying.

The findings with the three tumor-producing agents have a striking similarity and it is difficult to avoid the conclusion that the three are of one class, whatever that class may be. All give rise to dis-

eases of neoplastic character, all act after a more or less pronounced latent period, the action of all depends to a striking extent on associated tissue derangement, and all pass through Berkefeld cylinders of about the same porosity, being held back by others of slightly finer grade. Two of them resist drying and can be preserved in glycerin. The third, which fails to retain its activity when so treated, causes tumors that are of relatively very slow growth (Chicken Tumor XVIII). Since in our experience the separation of a tumor-producing agent is largely a question of the growth's malignancy, it seems not improbable that with selective passage of the neoplasm an agent may eventually be obtained from it that is resistant to drying and glycerination.

The separation of etiological agents from three chicken tumors of such diverse character as those we have employed is strong evidence for the view that many other growths of the fowl have a like cause. It is hardly necessary to point out that were the latent period of Chicken Tumor XVIII, when produced by the specific agent, somewhat longer than the two to six months observed in the present investigation, or were the agent only very slightly more difficult to separate from the tissue by filtration, its presence would not have been demonstrated. Chicken Tumor XVIII would then have remained, with the sarcomata of the rat and mouse, among the transplantable tumors without a cause separable from tissue cells.

CONCLUSION.

A causative agent has been separated from three chicken tumors of very different sort, namely a spindle-celled sarcoma, an osteochondrosarcoma, and a spindle-celled sarcoma peculiarly fissured by blood sinuses. This was accomplished after the tumors had been transplanted repeatedly and their malignancy enhanced. Each of the tumor-producing agents is a distinct entity in that it gives rise only to growths of the precise kind from which it has been derived. All pass through Berkefeld cylinders impermeable at the same test to small bacteria, and two of the three retain their activity in tumor tissue that has been dried or glycerinated. All are strikingly dependent for their action on derangement of the tissue with which they are brought in contact. The general findings strongly
Causation of Chicken Tumors.

suggest that the agents are of about the same size, and of the same natural class. It is perhaps not too much to say that their recognition points to the existence of a new group of entities which cause in chickens neoplasms of diverse character.

EXPLANATION OF PLATES.18

PLATE 7.

Fig. 1. Tumors caused by a Berkefeld filtrate of an extract of Chicken Tumor XVIII (a spindle-celled sarcoma fissured by blood sinuses) in Ringer's solution (filtration experiment 2). The sternum has been cut away and the body of the fowl eviscerated. In the left pectoral muscles is the large, pale, primary growth. On both sides at the junction of the sternal and vertebral ribs is the neoplastic rosary described in the text. That on the right has been cut through vertically. Its individual nodules have coalesced.

PLATE 8.

Fig. 2. A section of one of the growths produced by a filtrate of Chicken Tumor XVIII (filtration experiment 2). The fissuring with blood channels, sometimes accompanied by intracanalicular growth, is characteristic of this tumor. The numerous black points in the channels are the nuclei of the red blood corpuscles.

Fig. 3. Large osteochondrosarcoma produced by the intramuscular injection of 4 c.c. of the Berkefeld filtrate of an extract of Chicken Tumor VII (filtration experiment 2). The fowl was killed when comatose, eighty-seven days after the injection. Its emaciation should be noted.

PLATE 9.

Fig. 4. The growth shown in the preceding photograph, after it had been sawed open. Scattered amid the smooth, whitish cartilage is much bone with red marrow.

Fig. 5. An early stage of an osteochondrosarcoma produced by a filtrate of Chicken Tumor VII (filtration experiment 2). The fowl was killed when the tumor was first noted, eighteen days after injection. Here and there in the precartilaginous tissue, which has the general character of a spindle-celled sarcoma, the matrix of cartilage is in process of formation.

PLATE 10.

Fig. 6. Another portion of the growth illustrated in figure 5. The formation of cartilage is well advanced.

Fig. 7. A section of the growth shown in figures 3 and 4. The cartilage is in process of replacement by bone. Note the calcification and the abundant red bone marrow.

18 All the microscopic sections were stained with methylene-blue and eosin.
Fig. 1.

(Rous and Murphy: Causation of Chicken Tumors.)
FIG. 2.
(Rous and Murphy: Causation of Chicken Tumors.)
(Rous and Murphy: Causation of Chicken Tumors.)
(Rous and Murphy: Causation of Chicken Tumors.)
(Rous and Murphy: Causation of Chicken Tumors.)
FIG. 10.

(Rous and Murphy: Causation of Chicken Tumors.)
Peyton Rous and James B. Murphy.

PLATE XI.

Fig. 8. Another portion of the same growth. An unusual intermediate stage in the formation of cartilage.

Fig. 9. A spindle-celled sarcoma developing after the injection of 0.5 c.c. of a filtrate of Chicken Tumor I. No Kieselguhr had been added to the filtrate. The growth arose in the sheath of the outermost pectoral muscle, where the needle had been thrust through, and it was excised eleven days after the injection, at which time it measured only 0.15 cm. in diameter. There was no recurrence. The tumor is distinctly sarcomatous, sharply localized, and is enlarging to a considerable extent by expansive growth, as shown by the way in which the muscle fibers are pressed to one side. Here and there it has begun to infiltrate. About it there is a slight round-celled reaction.

PLATE XII.

Fig. 10. The border of an osteochondrosarcoma that developed after the injection of a filtrate, on the basis of a Kieselguhr reaction. The fowl was killed while the tumor was yet very small and before cartilage had been laid down in it. The reactive tissue has been compressed into strata by the expansive growth of the tumor. The tumor cells, which are of fibroblastic character and occupy the upper half of the picture, are invading and replacing the giant-celled, reactive tissue about the Kieselguhr. Numerous fragments of this latter can be seen. With the destruction of the giant cells the fragments are set free within the tumor. Here they induce no evident reaction.