PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

V. Antigenic Properties of the Chicken Tumor I*

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In the early investigations into the nature of the etiologic agent of fowl tumors, immunity phenomena were given some attention. The first observation was that the manifestations of resistance to these tumors in fowls, whether natural or acquired, are similar to those established for mammalian tumors, the principal feature being a local cellular reaction about the introduced graft (1). As was also established for mammals, the transfer of large amounts of blood or serum from resistant animals had no influence on the growth of established tumors in susceptible ones. Later results indicated that two types of immunity exist, one directed against the etiologic agent as distinct from that directed against the malignant cell (2). That directed against the agent of each kind of tumor seemed to be the more specific (3). Rous, Robertson and Oliver (4) undertook to develop antibodies in other species by the injection principally of finely ground tumor tissue or of the blood of fowls in the last stages of the disease. Rabbits failed to show any neutralizing antibodies, but the sera of the injected geese did inactivate the agent and prevent the induction of tumors in chickens. Later Mottram (5) found evidence of antibodies in a few fowls in which tumors had retrogressed. Mueller (6), using cell suspensions of the chicken tumor, was able to induce antibodies in rabbits and ducks, while Gye and Purdy (7) working with tumor filtrates have reported on protective antibodies induced in ducks and goats. Andrewes (8) has found natural inhibitors for the tumor agents in the sera of two normal fowls and some evidence that such "antibodies" develop in the blood of chickens with a slow-growing fibroma which are effective against the agents of more malignant tumors.

The studies to be reported here were undertaken along with attempts to isolate and purify the active principle of the chicken tumor (9),

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their primary object being to obtain light on the nature of the tumor agent through a better understanding of its antigenic properties.

Method.—When the source of the agent was fresh tissue, 25 gm. of tumor were ground with sand and extracted with 500 cc. of fluid. After passage through a Berkefeld filter, the extract was concentrated to 1/5 of its original volume in alundum thimbles lined with 8 per cent soluble cotton membrane. When a desiccate of the tumor was the source, 1 gm. of the powder was extracted with 60 cc. of the fluid. After centrifugation the extract was filtered through paper.

Rabbits were used throughout. The general method of immunization was to give 5 cc. of the antigen intravenously at 2 day intervals until each animal had received 6 injections or 30 cc. of the antigen. From 12 to 14 days after the last injection the animals were bled from the heart and the serum collected. The precipitating and neutralizing power of the sera were tested within a few hours after withdrawal of the blood. The precipitin tests were run with dilutions of the antigen to 1:1 to 1:320. For the neutralizing power of the sera, chickens were injected intradermally with a mixture of 0.5 cc. of serum to 0.2 cc. of a concentrated fresh tumor extract with control injections of tumor extract alone and with normal rabbit serum.[1]

Comparison of Antigenic Properties of Tumor Extracts and Protein Fractions of Extracts

In the first group of tests the antigenic properties of chicken tumor filtrates, prepared by three different methods of extraction, were compared with the acid-precipitable protein (10) of each extract.

The usual method of preparing tumor filtrates is to extract the tumor with Ringer's solution or normal saline solution. In our experience, however, the results have been somewhat better if the tissue is extracted with distilled water, keeping the suspension slightly alkaline during the procedure. A third method used, with the expectation of securing more of the nuclear protein in the solution, was to extract the tumor material with 5 per cent salt solution.

A given amount of each extract was divided into two equal portions, one to be used for testing the antigenic properties of the extract as such, and the other as the source of the protein fraction. The latter was secured by the addition of N/10 lactic acid until a clear-cut precipitate was formed. The point at which this occurred varied somewhat with different extracts, but was generally between pH 4 and 4.4. The precipitates were washed with distilled water and then dissolved

[1] The neutralizing power of the sera was tested in all experiments within a few hours of the withdrawal of the blood from the immunized rabbits. Therefore our tests do not indicate whether or not complement is necessary for the reaction.

in N/200 NaOH and sufficient water added to bring the volume up to that of the original extract. The supernatant fluids, after removal of the precipitate, were adjusted to pH 7.2 and also used as antigens.

The material tested in this experiment included the following: water, Ringer's solution and 5 per cent salt solution extracts of chicken tumors, their acid-precipitable protein fractions and the supernatant fluid after removal of the precipitate. The tumor-producing property of such extracts and precipitates, as tested on chickens in each experiment, is shown in Table I. In 12 experiments the antigenic properties were determined by the injection of 48 rabbits. In each case the precipitating and neutralizing powers of the antisera developed were tested. The results are shown in Text-figs. 1 and 2.

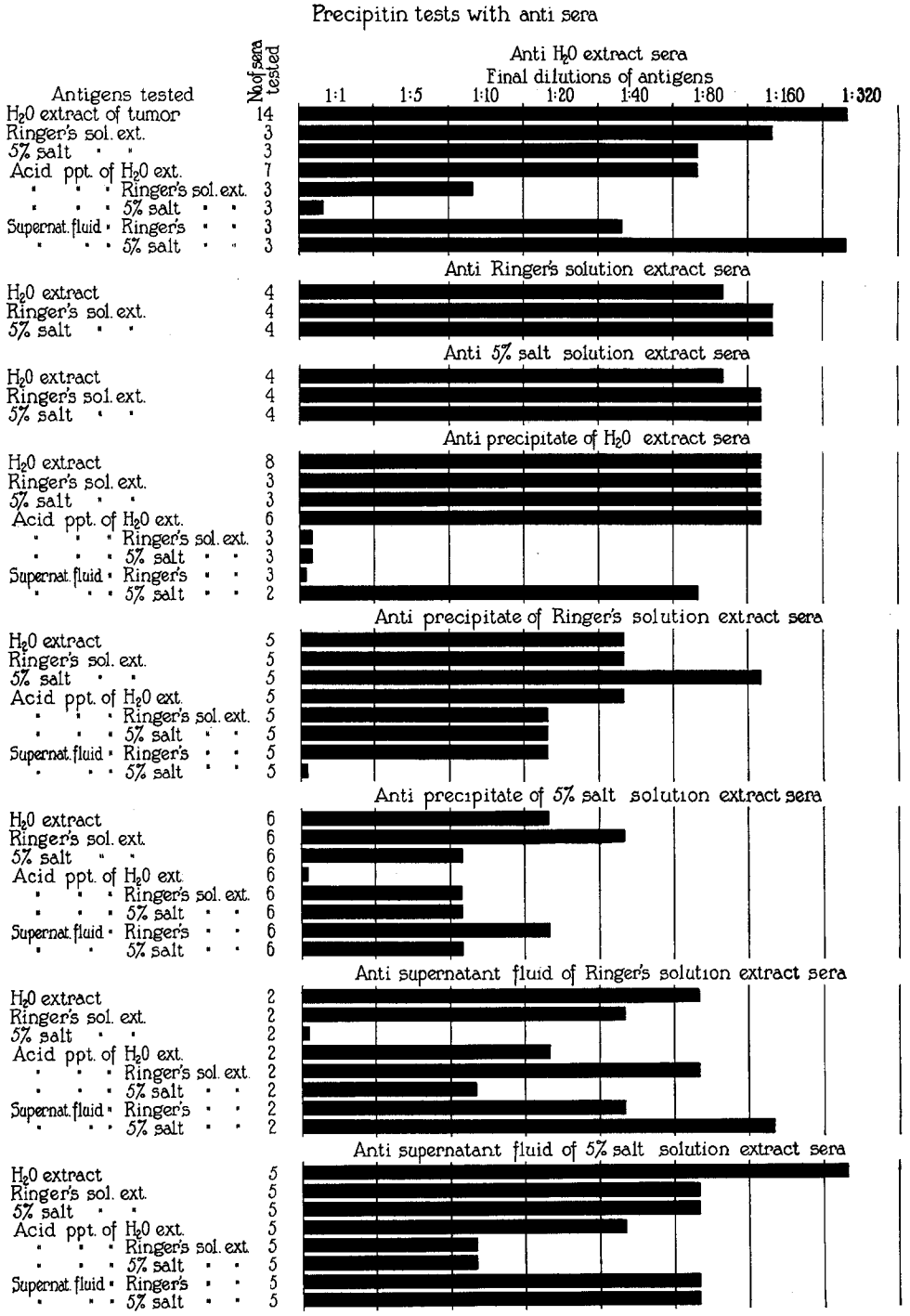
TABLE I

Material inoculated	From fresh tumor		From desiccated tumor	
	No. of tests	Tumors	No. of tests	Tumors
		per cent		*per cent*
Water extract	8	100	14	100
Protein fraction of water extract	14	100	11	91
Supernatant fluid after precipitation	12	0	8	0
Ringer's solution extract	12	92	8	100
Protein fraction of Ringer's solution extract	14	21	11	27
Supernatant fluid after precipitation	12	0	8	0
5 per cent salt extract	12	25	8	100*
Protein fraction of salt extract	14	0	11	0
Supernatant fluid after precipitation	12	0	8	0

* The tumors in this group were only a fraction of the size of those developing from the injection of the other extracts.

As a control to these tests, rabbits were immunized in the same fashion with water extracts of chicken muscle, liver, kidney, testicle and nucleoprotein of the blood, 2 rabbits being used for each preparation. The antisera to liver, kidney and testicle showed slight precipitins for tumor extracts, but there was no evidence of these bodies in the other sera. With the possible exception of the anti muscle and anti testicle sera, there was no evidence of neutralization of the tumor agent in the protection tests. The figures for these two may be significant, but compared with the definite evidence obtained with the antisera developed against the tumor extracts the results with them are not striking.

The significant points shown by this group of experiments are that precipitating antibodies may be developed in rabbits against a water or Ringer's solution extract of chicken tumors, and that the protein frac-



TEXT-FIG. 1. The amount of extension of the lines into the spaces under the dilutions indicates the intensity of the precipitin reaction for the given dilution of antigen.

tion carrying the activity of either of these extracts is equally effective in calling out precipitins in rabbits. The neutralizing power is shown not only by the figures for complete inhibition but also by those for partial inhibition, as indicated by the size of the tumor in the positive inoculations. The results with 5 per cent salt solution are significant

Neutralization of tumor extracts by anti sera

Sera inoculated with tumor filtrate	No. of sera tested	No. of inoculations	Per cent neutralization	No. of tumors	Average size of tumors
Anti H_2O tumor extract	10	34	82.4	6	0.9 × 0.6 cm.
Anti Ringer's solution extract	4	10	90.0	1	1.3 × 1.2 "
Anti 5% salt solution extract	4	10	40.0	6	1.0 × 0.8 "
Anti precipitate of H_2O extract	7	30	76.7	7	0.8 × 0.8 "
Anti precipitate of Ringer's solution extract	7	20	80.0	4	1.0 × 0.8 "
Anti precipitate of 5% salt solution extract	6	20	25.0	15	1.9 × 1.5 "
Anti supernatant fluid of Ringer's solution extract	4	9	33.3	6	1.5 × 1.0 "
Anti supernatant fluid of 5% salt solution extract	5	11	45.5	6	1.9 × 1.5 "
Normal rabbit serum	12	39	2.6	38	2.3 × 1.4 "

TEXT-FIG. 2. All inoculations were made intradermally and each fowl received besides the test inoculations an injection of untreated tumor extract for control. The measurements were recorded when the tumor from the control inoculation had attained a certain size.

only because there is some evidence that neutralizing antibodies may be developed by extracts which themselves have a low grade tumor-producing activity. The same lack of relationship between the tumor-producing properties of the antigen and antibody response is seen in the fact that the neutralizing power of the sera developed against the protein precipitate of a Ringer's solution extract is just as effective

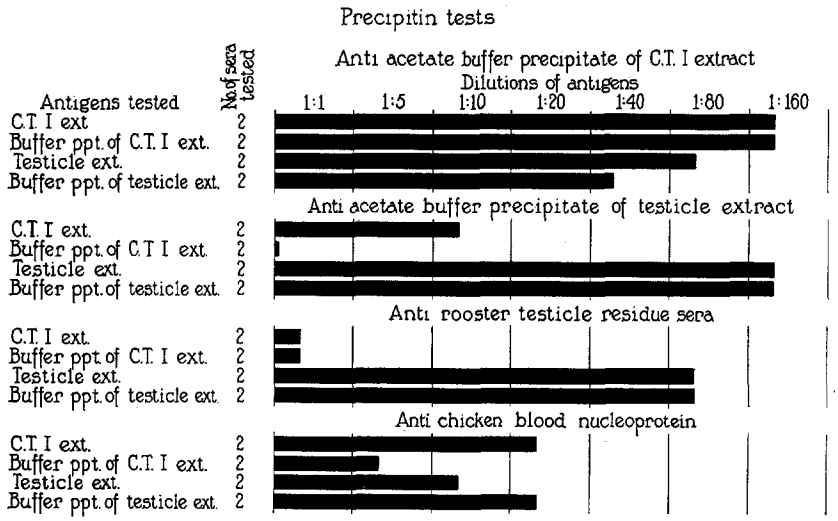
as that of the sera developed against the precipitate of the water extracts. Yet these two antigens show a marked difference in tumor-producing power. The antisera developed against normal chicken tissue extracts have no very significant precipitating or neutralizing antibodies toward the tumor extract.

Antigenic Properties of Buffer Precipitates of Tumor Extracts

As another step in the attempts to isolate the agent from the tumor extracts, it was hoped that a clearer-cut separation could be obtained if the precipitation were carried out with buffered solutions (10). The study in parallel of the antigenic properties of these precipitates is recorded here, primarily because of the results with normal tissue extracts which are used as controls.

Experiment.—The water extracts of dried tumor and of fowl testicle were prepared as described above. To secure the precipitates, these extracts were added in the ratio of 1 cc. to 5 cc. of M/100 acetate buffer at a pH of about 4.2. The precipitates were dissolved in N/200 NaOH. The residues, after extraction of the testicle material, were suspended in Ringer's solution and also used as antigens. As a further control the nuclear material from chicken red blood cells was obtained by laking the blood and washing out the hemoglobin. Thus the antigens tested in this experiment included buffer precipitates from the water extracts of tumor and testicle tissue, the water-soluble testicle residue and the nucleoprotein of the chicken blood. 2 rabbits were used for each antigen. The results of precipitin and neutralization tests are given in Text-figs. 3 and 4.

It is of interest to note that the sera developed against the tumor extract precipitates had precipitins for both testicle extract and its precipitate, while the anti testicle precipitate and testicle residue sera showed no reaction with the tumor precipitate. The antibody response to the blood protein was slight, but about equal for the tumor and testicle extracts. The neutralizing power of the anti tumor sera is definitely shown by the number of negative results from inoculation with the sera and fresh tumor extracts, as well as by the small size of the tumors in successful inoculations. The other sera were without definite neutralizing power, with the possible exception of that developed against the testicle residue, which yielded when injected with an active extract 33 per cent of negative inoculations, while the tumors



TEXT-FIG. 3. The lines show the extent of the principal reaction. The degree of penetration into the spaces for the dilutions indicates the intensity of the reaction for that particular dilution.

Neutralization of tumor extracts by anti sera

Sera inoculated with tumor extract	No. of sera tested	No. of inoculations	Per cent neutralization	No. of tumors	Average size of tumors
Anti buffer precipitate of tumor extract	2	6	66.6	2	1.1 × 0.8 cm.
Anti buffer precipitate of testicle extract	2	14	7.1	13	1.7 × 1.4 "
Anti testicle residue	2	6	33.3	4	1.0 × 0.8 "
Anti chicken blood protein	2	14	7.1	13	1.7 × 1.4 "
Normal rabbit serum	3	9	0.0	9	2.4 × 1.9 "
Tumor extract (control)	—	10	—	10	1.8 × 1.5 "

TEXT-FIG. 4. The method of recording the relative sizes of the tumors in these experiments was the same as that described under Text-fig. 2.

from the positive inoculations were somewhat smaller than the controls.

Antigenic Properties of the Purified Tumor Agent

The various attempts to determine the nature of the tumor agent resulted in a method of securing very active products with low protein content or even almost free from protein. The first observation in this line was that a water extract of dry tumor had a relatively low tumor-producing activity. The residue extracted a second time gave a more active material, while a third extract of the material was still more potent in the production of tumors (11). The principal significance of this observation was that over 60 per cent of the soluble nitrogen-containing compounds were removed in the first extract, while the most active extract, the third, had less than 12 per cent and the fourth, which was still very active, contained only about 0.08 mg. of nitrogen per cc.

Another method of removing the bulk of incidental protein in the tumor extracts is by adsorbing them out on colloidal aluminum hydroxide (Willstätter Type C). With the proper ratio between the amount of aluminum hydroxide and tumor extract it has been found that practically all of the proteins are taken down with the colloid, leaving a highly active material in the supernatant fluid. The details of this method are given in another paper (12). The fact that guinea pigs are not sensitized by this material indicates the extremely small amount of protein remaining. The principal contamination of this supernatant fluid proved to be a material resembling chondroitin-sulfuric acid. This can be removed by combining it with a basic protein and then precipitating out the new compound without reduction in the tumor-producing activity. The principal products developed in this study were investigated for their antigenic properties.

Experiment.—In this group of experiments the antigenic properties of the following products were investigated: first and third extracts of tumor desiccate, the supernatant fluid after adsorption of a tumor extract with aluminum hydroxide[2] (which will be referred to as aluminum supernatant), the supernatant fluid of the above extract after precipitating out the chondroitin material with gelatin (which will be called the gelatin supernatant), and finally the chondroitin-gelatin precipitate. The system of injecting the rabbits was the same as that used in the

[2] The aluminum supernatants used in these experiments were prepared by Dr. O. M. Helmer.

Neutralization of tumor extracts by anti sera

Material inoculated	No. of sera tested	No. of inoculations	Per cent negative	No. of tumors	Average size of tumors
Anti tumor extract serum + tumor extract	10	32	53.2	15	1.2 × 1.0 cm.
Anti tumor extract serum + aluminum supernatant fluid	8	20	70.0	6	0.9 × 0.8 "
Anti aluminum supernatant fluid serum + tumor extract	14	68	73.6	18	0.8 × 0.6 "
Anti aluminum supernatant fluid serum + aluminum supernatant fluid	14	65	84.6	10	0.8 × 0.7 "
Anti 3rd extract serum + tumor extract	4	15	66.6	5	0.9 × 0.8 "
Anti 3rd extract serum + aluminum supernatant fluid	4	9	77.7	2	0.4 × 0.3 "
Anti gelatin supernatant fluid serum + tumor extract	15	29	24.1	22	1.9 × 1.3 "
Anti gelatin supernatant fluid serum + aluminum supernatant fluid	6	15	6.6	14	1.9 × 1.4 "
Anti gelatin precipitate serum + tumor extract	12	34	11.7	30	1.8 × 1.4 "
Anti gelatin precipitate serum + aluminum supernatant fluid	6	17	0.0	17	2.0 × 1.2 "
Normal serum + tumor extract	13	29	0.0	29	2.3 × 1.8 "
Normal serum + aluminum supernatant fluid	10	21	0.0	21	2.3 × 1.7 "

TEXT-FIG. 5. For explanation of tumor measurements see Text-fig. 2

preceding experiments. The activity of each product used in the immunization was tested on chickens.

Tests showed that the sera from animals immunized with the full tumor extract had precipitins for the extract as such and gave a doubtful reaction with the third

extract, but no evidence was obtained of precipitins for the aluminum supernatant and gelatin supernatant fluids. The antisera for the third extract gave a doubtful reaction with the third extract and had no precipitins for the extract as such or any of the other preparations. The sera of animals injected with aluminum supernatant, gelatin supernatant and gelatin precipitate showed no precipitins for any of the preparations, nor did these sera give flocculin when tested with the Ramon technique. The results of the neutralization test are shown in Text-fig. 5.

In 10 experiments the complement-fixing power has been tested on 11 sera developed against the water extract of the chicken tumor, 21 sera developed against the supernatant fluid of a tumor extract after the major portion of the protein had been adsorbed out on aluminum hydroxide, 9 sera from rabbits injected with extracts after the removal of the viscous material and 9 against the viscous precipitate. The method employed in the immunization was that described above, and the standard Wassermann method was used for determining the complement fixation.

The experiments suggest that the precipitins in the sera developed against the tumor extract have no direct relation to the neutralizing property. The basis for this statement is to be found in the fact that these sera fail to produce precipitation or flocculation in the highly active aluminum supernatant fluid of a tumor extract. Furthermore the antisera developed against this active material showed no precipitins with the aluminum supernatant fluid or the full tumor extract, although they have a high neutralizing power for the tumor agent. The sera of the rabbits injected by Rous, Robertson and Oliver with the tissues of the chicken tumor had strong precipitins for chicken serum, yet had no evident effect on the tumor-producing agent (13). Altogether it would seem that the precipitins result from the injection of incidental proteins of the tumor not directly associated with the tumor-producing agent. The failure in practically all of the tests of the most highly purified product to induce either precipitins or antibodies for the tumor agent may require further investigation. As this material has a tumor-producing activity at least equal to that of the full tumor extract, it does not seem probable that the absence of antibody response can be attributed to the failure to inject into the rabbit sufficient tumor agent.

Complement Fixation Tests with Anti Chicken Tumor Sera

In addition to the tests of the rabbit sera for precipitins and for neutralizing power, the presence of complement-fixing antibodies has been investigated.

Each serum was tested in two amounts, 0.2 cc. and 0.1 cc., against undiluted water extracts of chicken tumor and aluminum supernatant fluid, and also with these two antigens diluted 1:10 and 1:5.

The 11 anti tumor extract sera, in both amounts tested against the undiluted extract as antigen, gave complete fixation, but there was no fixation with this antigen when diluted or with the undiluted or diluted aluminum supernatant fluid used as antigen. The 21 anti aluminum supernatant fluid sera gave no fixation with aluminum supernatant fluid, but did with undiluted water extract of the tumor. The anti gelatin supernatant fluid sera and anti gelatin precipitate sera gave no fixation with the aluminum supernatant fluid, but 4 out of 9 of the former and 3 of the latter did give fixation with the undiluted water extract of the tumor.

It is evident from these results that there is not a sufficient amount of the antigenic factor present in the aluminum supernatant fluid to interact with the antibody and fix the complement. Yet, by the only test available, namely tumor production in chickens, the concentration of the tumor agent in the aluminum supernatant fluid is almost equal to that in the tumor extract as such. Therefore, it would seem that the antibody against the tumor agent is not demonstrable by the complement fixation test.

DISCUSSION

The interpretation of the results reported here offers some difficulties, in that there is no very close analogy with the antigenic properties of known disease-producing agents. It seems plain that the precipitins stimulated in rabbits by the injection of the intact tumor extracts are developed against the incidental proteins of the tumor and have no essential association with the antibodies capable of neutralizing the tumor-producing activity of the etiologic agent. The most effective neutralizing sera were those developed against the purified agent practically free from protein, and these sera showed no demonstrable precipitins or complement-fixing antibodies. This suggests the type of immune bodies developed against certain toxins and recalls the discussion as to whether precipitins in antitoxic (14) and anti enzyme sera are not incited by contaminating proteins. In the latter case the question seems to be answered by the work of Kirk and Sumner (15) who have found a parallel development of precipitins and neutralizing antibodies to crystalline urease, a result which might have been expected as the enzyme in this case is a protein according to these authors.

The failure of the more highly purified agent to induce any demonstrable antibodies, in our opinion, is probably not attributable to the reduction in concentration. There is no doubt that some of the agent is lost in each step in purification, but this loss must be small for there is no evidence of reduction in tumor-producing power, a property which is definitely influenced by comparatively slight dilution. An analogy suggested by this result is the failure of the purified specific substance of pneumococci to induce antibodies, but this substance is definitely precipitable in even very high dilutions with the antisera developed to the type pneumococci (16), while with the purified tumor agent there is no such reaction with the sera of animals immunized with the full tumor extract.

In contrasting the antigenic properties of the chicken tumor agent with those of the viruses, perhaps the most striking difference is the comparative ease with which neutralizing antibodies for the tumor agent can be developed in non-susceptible species and the doubtful results obtained with most of the viruses under similar conditions. In fact with many viruses, notably vaccine virus, protective antibody development is not only limited to susceptible species but it is doubtful if they develop then in the absence of an actual infection with manifest lesions of the disease (17).

SUMMARY

The injection of tumor extracts and their active protein fractions into rabbits induced the formation of precipitins and neutralizing antibodies. When the major portion of proteins in the tumor extract had been eliminated it induced the formation of neutralizing antibodies, but not of precipitins. The tumor agent, more highly purified by removal of the viscous fraction, did not induce precipitins, and only 2 out of the 15 sera gave any evidence of neutralizing bodies. After the removal of the major portion of protein, the extracts showed insufficient interaction with the sera to fix complement.

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