PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

III. Attempts at Isolation of the Active Principle*

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(Received for publication, March 12, 1932)

Since the demonstration of the transmissibility of chicken tumors by agents separable from the malignant cells, the relationship of this group of neoplasms to those of the mammalian type has been a question of first importance. At first it was generally considered that the chicken tumor agents belonged to the virus group of organisms. This conclusion led to some doubt that the chicken tumors represented the same sort of disease as mammalian neoplasms, for there was no evidence that the latter were infectious processes. This difference, illustrated principally by the failure to transmit mammalian tumors by other means than actual grafts of living tumor cells,[1] deserves consideration on the basis of the fact that virus diseases are as a rule easily transmitted experimentally to susceptible animals. Yet even mice, which are particularly receptive to inoculated tumors, show no response to injection of tumor extracts free from living tumor cells.

The factual basis on which the chicken tumor agents are classified as viruses requires close examination in our opinion, for there are some properties of this group difficult to reconcile with such an assumption. The variety of types of chicken tumors, each transmitted faithfully

* This investigation was carried out under the Rutherford Donation.

[1] There are no very extensive reports in the literature on the failure to transmit mammalian tumors by agents separable from the cells, although the subject has been exhaustively investigated. In this laboratory many attempts have been made to separate an agent from mouse, rat and rabbit tumors, by various methods of extraction, filtration and desiccation, with hundreds of animals inoculated with the various products. Not a single instance of transmission has been encountered when the tumor cells were definitely eliminated.

by its agent, the limitation to special breeds of fowls in the early transfers, and the fact that the agents cause specific cells to differentiate into definite types of tissue suggest that the process represents a perverse physiological phenomenon. On the basis of this conception it has seemed justifiable to set up as a working hypothesis the possibility that the tumor agents are of endogenous origin, representing abnormal activities of the forces which normally control growth and differentiation of tissues. A series of investigations to test this idea have been carried out, in which the properties of the tumor agents have been contrasted with those of typical viruses on the one hand, and with cell products such as enzymes on the other.

Two studies of the series have been published. The first (1) reported the fact that the tumor agent is fixed or inactivated *in vitro* by the mesodermal tissues of susceptible fowls, but not by the epithelial tissues, while none of the tissues of non-susceptible animals affect the agent. In this respect the agent resembles many cell products which are fixed by the specific substrate on which they act. As a contrast to this finding, the viruses were not fixed *in vitro* by tissues most susceptible to infection, but had their infectivity greatly increased by the contact (2). The second investigation (3) was on the quantitative and qualitative action of ultraviolet light on the tumor agent. Bacteria, viruses and phage have been shown to be injured in much the same degree by given wave lengths in this general range of the spectrum. On the other hand, the chicken tumor agent is not only far more resistant to these wave lengths, but there is a striking qualitative difference in that the most active wave lengths for the agent do not correspond to those for the other group. This result is interpreted as indicating a common factor in the bacteria-virus-phage group and it suggests that inactivation of the tumor agent is due to a destruction of a substance having an entirely different spectrum from the group mentioned and therefore of a different chemical character.

The present study is an attempt to gain further information on the nature and properties of the chicken tumor agents by the relatively direct method of isolation and purification. This will be followed by a publication on the antigenic properties (4) of the agent and another presenting the evidence of an inhibiting factor associated with the agent (5).

Fractionation of Active Tumor Filtrates

As a first step in this investigation we have fractionated active tumor extracts in order to determine whether the activity is associated

with one of the recognized proteins, as is the case with most if not all of the effective cell products.[2] Three means have been used in attempts to obtain different fractions from the extracts; namely, reduction of the salt content and the increase of hydrogen ion concentration by direct addition of acid or with proper buffered solutions.

Electrodialysis.—About 30 gm. of fresh tumor tissue were ground and thoroughly extracted with 600 cc. of water and filtered through a Berkefeld candle. This filtrate was rapidly concentrated to about 1/10 its original volume in thimbles lined with an 8 per cent collodion membrane. The concentrate was subjected to electrodialysis according to the Bronfenbrenner (6) method. The precipitate

TABLE I

Experiment No.	Material	Time of dialyzing	Precipitate		Fluid from dialysate		Control positive	pH of dialysate
			No. of inoculations	Positive	No. of inoculations	Positive		
		min.		*per cent*		*per cent*	*per cent*	
1	Berkefeld filtrate	7	4	100	4	0	100	4.5
2	" "	5	4	50	4	0	50	4.3
3	" "	3	4	50	4	0	100	
4	" "	5	4	0	4	0	100	4.5
5	" "	5	6	100	6	0	100	4.4
6	" "	5	12	91.7	12	0	100	4.7
7	H_2O extract dry tumor	5	6	66.6	6	0	50	
8	Concentrated serum tumor chicken	8.	3	66.6	3	0		

which occurred was clumped and usually adhered to the positive pole, and could easily be separated from the fluid. It was then washed, dissolved and injected intradermally into chickens. The fluid was neutralized and also injected.

In the first group of experiments the electrodialysis was continued until an amperage corresponding to that of distilled water was reached, the time required varying from 45 minutes to 2 hours. The heavy precipitate secured and the fluid were both inactive. In a new group of experiments the time of electrodialysis was shortened to 15 minutes,

[2] Sugiura and Benedict (*J. Cancer Research*, 1927, **11**, 164) have reported that the tumor agent can be salted out from a filtrate with the globulin fraction.

and finally it was determined that a clear-cut precipitate occurred when the amperage reading reached 0.1, which required approximately 3 to 8 minutes of dialysis. The precipitates were sticky, mucoid material, becoming stringy and tough on exposure to air. The remaining fluid was clear. The accompanying Table I gives the results of inoculations of the precipitate and the fluid, and the reaction of the latter. One experiment is included in which the serum from a tumor-bearing chicken was treated in the same fashion as the filtrates.

It was considered that the adherence of the precipitate to the positive pole was due to the sticky properties of the precipitate and not the effect of electric charge. The fact that the precipitate came down when the pH of the fluid reached about 4.6 (due to a more ready elimination of the alkaline salts) suggested that the precipitation might be due to the increased hydrogen ion concentration rather than the reduction in the salt concentration of the solution. Test-tube experiments showed that the addition of acid to the concentrated tumor filtrates produced a precipitate similar in its physical properties to that resulting from electrodialysis. The activity of such precipitates was next investigated.

Acid Precipitation of Tumor Extracts.—Preliminary tests in which the concentrated tumor extracts were precipitated with N/10 HCl indicated that the activity of the material was reduced by this treatment, so a variety of weaker acids were tried, including phosphoric, tannic, tartaric, citric and lactic acids. N/4 citric and N/10 lactic acids gave the most satisfactory results, and these were used in the majority of our experiments.

The source of other material was either a concentrated Berkefeld filtrate or a water extract of dried Chicken Tumor I. To a measured quantity of the tumor extract the acid was added drop by drop. As the precipitate formed, it would adhere to a stick twisted in the solution. Some filtrates showed, in addition to this stringy precipitate, an amorphous one which when centrifuged left a clear supernatant fluid. The point at which a clear-cut end-point was reached varied somewhat with the different preparations, but the range was usually between pH 4.2 and 4.8. The precipitates were dissolved in weak alkali, the fluid portion neutralized and the tumor-producing activity of the two tested.

The results of 9 experiments with 42 test inoculations of the precipitate with lactic acid gave 88 per cent of tumors. In the first 3 experiments, in which the pH was not accurately controlled, there was some activity left in the supernatant fluid, but in the later experi-

ments the injection of this fluid gave uniformly negative results. Citric acid used for precipitation gave approximately the same results.

When these active precipitates were dissolved in alkali and reprecipitated with acid, the activity was retained, but as these procedures were repeated there was a gradual reduction in activity. By the fifth reprecipitation the average number of takes was reduced to 15 per cent for the whole group, but in several experiments the fourth precipitate still yielded 100 per cent good tumors. The reduction in activity was undoubtedly due in part to the time required to complete the experiment, for frequently 8 to 9 hours elapsed from the preparation of the extract until the last inoculation was made. It is known that the activity of a tumor extract decreases at room temperature.

Precipitation in Buffered Solutions.—In the hope that a better separation of the active fraction could be obtained, precipitation from buffered solutions was next attempted.

The procedure adopted after preliminary tests was to add 2 cc. of a concentrated tumor filtrate to 10 cc. of a N/100 buffered solution. A range from a pH of 4 to 4.8 was set up, and the first tube showing a clear-cut precipitation was used for the test. The precipitate was dissolved in weak alkali, the supernatant fluid concentrated to an equal volume and each tested for its activity by inoculation.

The first buffer used was sodium citrate, which generally gave a precipitate at pH 4. The results were very irregular in that the activity was destroyed or there was no clear-cut separation. Sodium acetate proved more satisfactory. With it the precipitate occurred between pH 4.2 and 4.6. In 9 experiments with 35 test inoculations the precipitate yielded tumors in 68.6 per cent, while the concentrated supernatant fluid in 34 tests gave 17.6 per cent of tumors.

The indications from these three groups of experiments were that the tumor agent was carried down by a precipitate formed at a fairly definite pH. While precipitates secured by the three methods were similar in gross character, it was desirable to know whether they represented the same fraction, and if so whether they consisted of a definite protein.

Chemical Nature of Active Precipitates.—Chemical analysis of the precipitates secured by the three methods showed little differences. The average content of nitrogen and phosphorus from a large number

of analyses is given in Table II. On hydrolysis a reducing substance was found present in all the precipitates, representing about 15.25 per cent figured as glucose. The ratio of nitrogen in precipitates to that of intact extracts varies with the method of preparation of the extract. With the concentrated Berkefeld filtrate from 80 to 90 per cent of the nitrogen goes into the precipitate, while with extracts of tumor desiccate, which have a higher nitrogen content, the amount carried by the precipitate may be as low as 60 per cent of the total amount. If the mixtures are kept slightly alkaline during extraction, the percentage of phosphorus is increased. There is little change in the physical properties or chemical constituents of the fraction repeatedly dissolved in alkali and reprecipitated with lactic acid. It was considered that the fraction was either a mixture of proteins or a protein of unusual constitution. Study of this point was rendered unnecessary by the results of the following experiments.

TABLE II

Method of precipitation	N	P
	per cent	*per cent*
Electrodialysis	12.47	0.27
Lactic acid	12.64	0.29
Sodium acetate buffer	13.33	0.22

Separation of the Tumor Agent from the Bulk of the Proteins

As further attempts to isolate and purify the protein associated with the tumor agent failed because the necessary procedure inactivated the material, other methods of accomplishing the purpose were sought. We had previously undertaken to adsorb the active principle from the filtrate on aluminum hydroxide and then to release it by treatment with an alkaline fluid, but at the time the results were considered too irregular to justify an extension of the work along this line. Other investigations have shown that a variety of substances adsorb or inactivate the tumor agent (7). Leitch has shown that the active material may be released after adsorption on kaolin and more recently Fränkel has reported some success in releasing it after adsorption on aluminum hydroxide (8), but his results were irregular

in that the released agent was not highly active and sometimes failed to induce tumors. It was considered worth while to reexamine the possibility of utilizing this method for our purpose.

Method.—For the tumor agent we used either a Berkefeld filtrate of a fresh extract concentrated in a collodion membrane or an extract of tumor desiccate. The solutions were kept at a pH of about 7.2 during the process of preparation by the addition of N/100 NaOH. Type C aluminum hydroxide was prepared according to the method described by Willstätter and Kraut (9). 20 cc. of the tumor extract were mixed with 20 cc. of the aluminum hydroxide suspension. After thoroughly shaking, the mixture was centrifuged and the supernatant fluid, which for convenience will be referred to as the aluminum supernatant, was decanted. The deposit was washed several times with distilled water, and the washing concentrated to 20 cc. in an 8 per cent collodion membrane. Enough of the washed aluminum deposit was set aside for inoculation and the remainder was shaken for 5 minutes with 20 cc. of M/15 Na_2HPO_4 at a pH of 8, centrifuged and the supernatant fluid drawn off. This will be referred to as the released material.

For testing the activity of the various products, they were inoculated intradermally in chickens, every fowl receiving 0.2 cc. of each test material. These included the following: (*a*) original tumor extract, (*b*) the supernatant fluid after the aluminum hydroxide with its adsorbed material had been separated from the extract, (*c*) concentrated washing, (*d*) the aluminum hydroxide after washing and (*e*) the material released from the aluminum by shaking with Na_2HPO_4.

In later experiments the technique was modified in one particular to avoid unnecessary dilution of the material. The 20 cc. of the aluminum hydroxide were first centrifuged and the excess fluid discarded before the addition of the tumor extract.

A large number of experiments was carried out by these methods, the results of 9 of which are given in Text-fig. 1.[3] In addition a great many more, in which the animal inoculated was to test the activity of the released material for chemical study, yielded similar results.

[3] By using the intradermal inoculations it is not only possible to secure more accurate measurements, but by having each fowl receive control and several test materials a better comparison of the growth rate of the induced tumors can be arrived at. The period of observation was from 3 to 5 weeks. To eliminate the variations due to the difference in potency of the extracts and the susceptibilities of the individual chickens we selected the measurement taken at the time when the control tumors had reached a certain size. The more susceptible fowls with a very active extract may reach this point in 2 weeks, while the more resistant ones will require 4 weeks. This system has been utilized in arriving at the figures given in all of the charts.

From the results shown in Text-fig. 1 it is evident that a certain amount of the active material is either directly adsorbed on the aluminum or is carried down along with some adsorbed substance, as Fränkel has reported. That the amount of the agent carried down with the aluminum hydroxide represents only a fraction of the total amount present in the tumor extract is shown by the results of inoculations, for not only is the percentage of tumors induced by the released substance low, but the tumors obtained are of relatively small size. The increased potency of the agent remaining in the supernatant fluid of the tumor extract after removal of the aluminum hydroxide is unquestionably due to the elimination of an inhibiting substance, as

Release of chicken tumor agent
after adsorption on aluminum hydroxide
(9 experiments)

Material inoculated	No. of inoculations	Per cent tumors	No. of tumors	Average size of tumors
Tumor extract	20	100	20	1.4 × 1.2 cm
Eluate	41	60.9	25	1.3 × 1.0 "
Supernatant fluid	20	100	20	2.3 × 1.8 "

TEXT-FIG. 1. All inoculations were made intradermally, distributed so that each fowl received at least 1 injection of each test material. The size of the tumors in each individual was recorded when that arising from the control inoculation had reached about the size indicated in the above figure.

will be shown in another paper. However, allowing for this factor, the activity is such as to indicate that the concentration of the agent in this supernatant fluid has been little reduced by the aluminum adsorption.

Effect on the Tumor Agent of Variation of the Quantity and Type of Adsorbent

The selection of adsorbent and the ratio of adsorbent to extract as used in the foregoing experiments was more or less arbitrary. Improvements in method were sought by additional experiments in which the ratios used above were varied, and also Willstätter's three other types of aluminum hydroxide were tested.

Variation in Ratio of Adsorbent to Extract.—Tumor extracts prepared as described above were mixed with aluminum hydroxide Type C in the following proportions: 1:3, 1:2, 1:1, 1:1/2, 1:1/4 and 1:1/10. The mixtures were thoroughly shaken, centrifuged and the supernatant fluids injected intradermally in chickens. The untreated extract was also injected into each chicken for control. The results of these experiments with the proportions of 1:1 to 1:1/10 are shown in Text-fig. 2. 5 such experiments were carried out, in which 19 test inoculations were made of each test ratio. There were a few tests with higher ratios of aluminum hydroxide, in which the tumors produced by the supernatant fluid were considerably smaller.

On the whole the results given in Text-fig. 2 are of relative importance only, the tests being necessarily crude, since there is considerable variation in the activity of the extracts and the adsorbing power of

Activity of tumor extract after adsorption
with varying proportions of aluminum hydroxide

Ratio of tumor extract to aluminum hydroxide	1:1	2:1	4:1	Control
Number of inoculations	19	19	19	19
Average size of tumors (cm.)	2.07 × 1.46	1.73 × 1.35	1.67 × 1.20	1.60 × 1.20

TEXT-FIG. 2. The above results are based on the average size of tumors induced in 5 experiments in which all inoculations resulted in tumors. Each chicken received an intradermal inoculation of each of the test materials and the measurements recorded in the figure were based on those of all tumors in an individual at the time when the tumor from control inoculations had reached about the size indicated.

the aluminum hydroxide varies with time. However, even allowing for the two variable factors, the supernatant fluid of the 1:1 mixture was plainly the most active in tumor production in practically every experiment.

Action of Different Types of Aluminum Hydroxide.—Comparative tests have been made with Willstätter's four types of aluminum hydroxide.

The technical procedure was essentially the same as that described above. Mixtures were made of chicken tumor extract and of Types A, B, C and D aluminum hydroxide in a ratio of 1:1. The mixtures were shaken and centrifuged and the supernatant fluid injected intradermally in chickens. The injections were so

distributed that each of the chickens received an inoculation of the supernatant fluid from the four types of aluminum and a control inoculation of the original tumor extract. The tumors produced by the supernatant fluid from Types B and D aluminum hydroxide were little if any larger than the controls, while that from Type A was smaller. The results with the supernatant fluid from Type C in this experiment were similar to those reported in the previous experiments, in that the tumors were considerably larger than those produced by the original tumor extract.

While this experiment also is crude, owing to the fact that the amount of aluminum hydroxide by weight is not accurately indicated by volume of the different preparations, from the point of view of our objective, the separation of the tumor agent from contaminating material, the results indicate that Type C aluminum hydroxide is the most satisfactory preparation for this purpose. In subsequent studies this type was used exclusively.

Nature of the Tumor Extract after Adsorption with Type C Aluminum Hydroxide

The aluminum supernatant fluid is derived from either a concentrated Berkefeld filtrate from fresh Chicken Tumor I material or from a water extract of a desiccate of the tumor. When the filtrate of fresh tumor is used, the supernatant after removal of the aluminum hydroxide is a clear, colorless fluid with a high viscosity. If an extract of tumor desiccate is the source, the fluid is equally viscous and is generally opalescent, a property probably due to lipoids. A detailed chemical study of this material will be published later, but a summary of the preliminary work follows.

Nitrogen Content.—The nitrogen content of the concentrated filtrates and extracts of dry material shows considerable variation, but on the average of some 12 analyses is 0.527 mg. per cc. for filtrates and for the extracts of desiccates is 0.724. Of this amount 92.60 per cent is adsorbed on the aluminum hydroxide from the filtrates and 86.47 per cent from the extracts of dry tumor. Of the amount adsorbed on the aluminum about 27 per cent is found in the released material.

Reducing Substances.—On hydrolysis of the aluminum supernatant fluid a reducing substance is found, which, figured as glucose, amounts to 0.175 mg. per cc. This represents about 1/3 of the amount present in the full tumor extract, indicating that this substance is adsorbed in a smaller ratio than the nitrogen-containing

substances. The ratio of nitrogen to sugar in the full extract is 1 to 0.95, while in the aluminum supernatant fluid it is 1 to 3.48.

While the usual protein-precipitating agents, such as acetic, tannic, tungstic and trichloracetic acids, produce no precipitate in the aluminum supernatant, salts of the heavy metals such as lead, silver and mercury, and the basic dyes, safranine and neutral red, do give precipitates. The biuret, Millon, Adamkiewicz's and xanthoproteic tests are negative. Molisch and Tollens tests are positive.

The biological tests for protein have been negative. 9 guinea pigs, injected intraperitoneally with 8 cc. each of intact tumor extract, showed no anaphylactic symptoms when given 14 days later from 2 to 10 cc. of aluminum supernatant fluid intravenously. Furthermore no sensitization was induced in 14 animals by the injection of even the equivalent of 40 cc. of highly active aluminum supernatant, as demonstrated by the absence of anaphylactic symptoms when a second injection of 2 to 10 cc. of the same material was given 12 days later. However, there is some sensitization induced in these animals to an intravenous injection of unpurified tumor extract. As a further indication of the very low protein content of the aluminum supernatant, the sera of rabbits repeatedly injected with the material, while showing neutralizing antibodies, gave no evidence of complement-fixing antibodies.

Removal of Viscous Material from Aluminum Supernatant Fluid

The properties of the aluminum supernatant fluid as outlined above indicate that the protein content is extremely small, and that the main constituent is probably a carbohydrate. The fact that the tumor extracts contain muco-protein, and that the aluminum supernatant fluid contains a viscous substance which behaves like an acid, suggested that the latter has properties similar to chondroitin-sulfuric acid. In attempts to eliminate it as a further step in purification, the direct removal by precipitation with such agents as the salts of heavy metals or basic dyes was found to destroy the tumor-producing activity of the solution. One of us (Claude) conceived the idea that the removal might be accomplished by combining the substance with a basic protein. Gelatin was selected for the reason that it has no antigenic properties and is not precipitated by acids under the conditions of the experiment.

Experiment.—It was found by preliminary tests that when gelatin is added to aluminum supernatant fluid and the pH of the mixture brought to between 4 and 4.8 with M/10 acetate buffer a precipitation is induced. No precipitation occurs in either the aluminum supernatant fluid or the gelatin solution alone when the acid is added. On the basis of these findings the following method was evolved

for use in the experiments: to 10 cc. of aluminum supernatant fluid of a chicken tumor extract, prepared as described above, was added 1 cc. of a 2 per cent solution of commercial gelatin (Gold Label). Sufficient M/10 acetate buffer at pH 4.7 was added to bring the solution to pH 4.8. After 10 minutes the fluid was centrifuged, the supernatant fluid filtered through filter paper, the precipitate washed with acetate buffer at pH 4.7 and dissolved in a sufficient amount of Ringer's solution to bring the volume up to that of the supernatant fluid.

The gelatin supernatant fluid proved to be water-clear and limpid. No precipitate was formed by neutral red or basic lead acetate. The presence of excess gelatin did not permit of any conclusion from the nitrogen and sugar determinations. The dissolved gelatin precipitate, when treated with neutral red or lead acetate, gave a precipitate of the same character as that derived from the aluminum supernatant fluid and a substance could be extracted having all the physical

Effect of removal of viscous material
from aluminum supernatant fluid of tumor extract
(21 experiments)

Material inoculated	No. of inoculations	No. of tumors	Average size of tumors
Aluminum supernatant fluid	36	33	2.2 × 1.8 cm.
Gelatin supernatant fluid	45	29	2.4 × 1.9 "
Gelatin precipitate	19	10	1.7 × 1.6 "

TEXT-FIG. 3. The same system of recording the sizes of tumors was used here as in the preceding text-figures.

properties of the viscous fluid referred to above. Guinea pigs sensitized by the injection of 8 cc. of full strength chicken tumor filtrate, aluminum supernatant fluid or gelatin supernatant fluid show no anaphylactic symptoms when given gelatin supernatant fluid intravenously. This statement is based on the results on 18 animals tested. There are symptoms however in animals sensitized with the gelatin supernatant fluid and subsequently injected with unpurified tumor extract. This result is similar to that with the aluminum supernatant fluid recorded in the preceding section.

The tumor-producing activity of the gelatin supernatant fluid and the dissolved precipitate was tested by intradermal injection of 0.2 cc. of each into chickens which also received an equal amount of the aluminum supernatant fluid from which these products had been derived. The results of 21 experiments in which the relative activity of these three materials was tested by inoculation of 36 chickens are shown in Text-fig. 3.

The results of these investigations show the possibility of eliminating one more impurity from the tumor extract without interfering with the activity. There is some difficulty in judging the comparative tumor-producing activity of the aluminum supernatant fluid and the gelatin supernatant fluid, because gelatin enhances the tumor-producing property of the agent.[4] The fact that an appropriate amount of the latter is adsorbed on the gelatin precipitate, and yet the amount left in the supernatant fluid is still capable of inducing tumors as large as or larger than those resulting from the injection of aluminum supernatant fluid, renders it very improbable that the viscous substance is involved in tumor production. Unfortunately the presence of protein cannot be determined on account of our inability to eliminate the excess gelatin; but the failure of this material to sensitize guinea pigs to a subsequent injection, and the fact that pigs sensitized to an unpurified tumor extract do not react to an injection of gelatin supernatant fluid indicates that an infinitesimal amount of protein is present or that the protein is non-antigenic. It is hoped that other methods can be developed for eliminating the viscous material which do not introduce factors interfering with direct chemical analysis.

DISCUSSION

The principal basis on which the chicken tumor agents are considered to be viruses is that they are deemed to be capable of self-perpetuation. It is established beyond doubt that these agents are definitely increased in amount with the propagation of the tumor. However, with increasing knowledge of their properties, certain apparently fundamental differences between this group and the animal viruses make it seem unlikely that they belong to the same order. Some of these have already been discussed. Such facts as the sharp difference in susceptibility to ultraviolet light, both quantitative and qualitative, alone suggested a wide gap between the tumor agents on one side and bacteria, viruses and phage on the other. The affinity between the tumor agent and susceptible tissues *in vitro* has no parallel among the parasites. We now have evidence for the association of the agent with a protein fraction, and for its possible dissociation from

[4] Unpublished observation by Murphy and Sturm, confirmed and extended by Claude.

the protein. In subsequent papers the presence of an inhibitor principle in tumor extracts and its peculiar antigenic properties will be brought out (10). Certain animal viruses can be carried down from a suspension with a precipitated protein (11), but so far none has withstood the wide range of pH variation and the vigorous chemical handling incident to repeated distribution in fluid and reprecipitation tolerated by the tumor agent. Viruses may be infective in dilutions so great that chemical tests fail to indicate the presence of protein, but the reduction of protein in a virus suspension is accompanied by evidence of reduction of the infective units present (12). On the other hand, tumor extracts cannot be diluted very much and still retain sufficient concentration to induce tumors (13).

In discussing a possible classification other than among the viruses we have previously used the term enzyme-like (14), a term meant to indicate the possible production of the active material by tissue cells. No closer analogy to enzymes is considered, for most of the evidence suggests that the tumor agents belong to a class not yet clearly defined. That a product of an abnormal cell can cause a normal cell of the same derivation to develop into an abnormal cell of the same type from which the product came, and in its new form be capable of producing more of the active material, has seemed to many a fantastic conception. At the time that this hypothesis was brought forward, there was no clear-cut example of such a phenomenon. Now it is known that a substance may be extracted from a type-specific pneumococcus which will cause avirulent, non-specific pneumococci to change to the virulent form of the same type from which the extract was obtained (15). In its new form the organism produces more of the active material and transmits the property to its descendants. If we can call this active substance an agent, and three distinct active chemical substances have been obtained from the pneumococci, there is undoubtedly evidence that such agents increase, or more precisely, are increased with the cultivation of the organism. Yet these agents are products of the virulent cells and are not viruses. The effect of these agents may properly be referred to as a mutation. As there are other perhaps less well known examples of this phenomenon among bacteria (16) lacking proper designation, the term transmissible "mutagen" has been suggested for the group (17). With present

knowledge it seems probable that the chicken tumor agents have a closer analogy to the mutagens than to the viruses.

SUMMARY

By two methods a protein fraction can be separated out from a Chicken Tumor I extract, which carries all the tumor-producing agent. The precipitate can be dissolved and reprecipitated a number of times without loss of activity. The agent can be largely dissociated from the protein as shown by the fact that aluminum hydroxide will adsorb the protein from an extract and leave the agent behind. This purified material has a very low protein content, if any, as shown by both chemical and biological tests.

REFERENCES

1. Duran-Reynals, F., and Murphy, Jas. B., *J. Exp. Med.*, 1929, **50,** 315.
2. Duran-Reynals, F., *J. Exp. Med.*, 1929, **50,** 327. Duran-Reynals, F., and Suñer-Pi, J., *Compt. rend. Soc. biol.*, 1928, **99,** 1908. Pijoan, M., *J. Exp. Med.*, 1931, **53,** 37. Hoffman, D. C., *J. Exp. Med.*, 1931, **53,** 43.
3. Sturm, E., Gates, F. L., and Murphy, Jas. B., *J. Exp. Med.*, 1932, **55,** 441. Murphy, Jas. B., Preliminary statement, *Rep. Internat. Conf. Cancer*, London, 1928, 33.
4. Murphy, Jas. B., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, **73,** 266 (preliminary note). Murphy, Jas. B., Sturm, E., Favilli, G., Hoffman, D. C., and Claude, A., *J. Exp. Med.*, 1932, **56,** 117.
5. Murphy, Jas. B., Helmer, O. M., Claude A., and Sturm, E., *Science*, 1931, **73,** 266 (preliminary note). Murphy, Jas B., *Tr. Assn. Am. Physn.*, 1931, **46,** 182. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56,** 107.
6. Bronfenbrenner, J. J., *J. Gen. Physiol.*, 1926, **10,** 23.
7. Lewis, M. R., and Andervont, H. B., *Bull. Johns Hopkins Hosp.*, 1927, **40,** 265; *Am. J. Hyg.*, 1927, **7,** 505; *Bull. Johns Hopkins Hosp.*, 1927, **41,** 185.
8. Leitch, A., *Rep. Internat. Conf. Cancer*, London, 1928, 20. Fränkel, E., *Lancet*, 1929, **2,** 538. Fränkel, E., and Mislowitzer, E., *Z. Krebsforsch.*, 1929, **29,** 491.
9. Willstätter, R., and Kraut, H., *Ber. chem. Ges.*, 1923, **56,** 149, 1117.
10. Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1932, **56,** 107. Murphy, Jas. B., Sturm, E., Favilli, G., Hoffman, D. C., and Claude, A., *J. Exp. Med.*, 1932, **56,** 117.
11. Andrewes, C. H., *J. Path. and Bact.*, 1930, **33,** 265.
12. Rivers, T. M., unpublished observation on vaccine virus.
13. Carrel, A., *J. Exp. Med.*, 1926, **43,** 647.

14. Murphy, Jas. B., *Am. Naturalist*, 1926, **60,** 227; *Rep. Internat. Conf. Cancer*, London, 1928, 33. Murphy, Jas. B., Helmer, O. M., and Sturm, E., *Science*, 1928, **68,** 18.

15. Griffith, F., *J. Hyg.*, 1927–28, **27,** 113. Dawson, M. H., *J. Exp. Med.*, 1930, **51,** 99, 123. Dawson, M. H., and Sia, R. H. P., *J. Exp. Med.*, 1931, **54,** 681. Sia, R. H. P., and Dawson, M. H., *J. Exp. Med.*, 1931, **54,** 701.

16. Burnet, E., *Arch. Inst. Pasteur Tunis*, 1925, **13,** 384; 1928, **17,** 128. Kuhn, P., and Woithe, *Med. Klin.*, 1909, **5,** 1709. Cantacuzene, J., and Bonciu, O., *Compt. rend. Acad.*, 1926, **182,** 1185. Favilli, G., *Sperimentale*, 1926, **80,** 395. Wollman, E., *Bull. Inst. Pasteur*, 1928, **26,** 1.

17. Murphy, Jas. B., *Tr. Assn. Am. Physn.*, 1931, **46,** 182.