# PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

## IV. Association of an Inhibitor with the Active Principle*

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In earlier papers the nature of the chicken tumors has been discussed and some doubt expressed as to the basis on which the etiologic agents are considered to be filterable viruses (1). The idea has been advanced that these active principles may possibly be of endogenous origin, representing abnormal manifestations of the forces which normally control growth and differentiation of cells. Accepting this point of view as a working hypothesis, a series of tests has been devised with the expectation that the hypothesis may be either established or discarded. The present paper is a report of the evidence we have thus far obtained that indicates the presence of an inhibitor and represents an amplification of a previous publication[1] (2). Sittenfield, Johnson and Jobling have also published some evidence of the presence of such a factor in chicken tumors (3).

In an extensive series of experiments in which an attempt was made to isolate the tumor agent, it was noted that it could be precipitated out from the tumor extract along with certain of the proteins (4). Among other tests applied to the active precipitate was the Feulgen microchemical staining reaction for nucleoprotein. As a parallel to this, the precipitates were also tested with Mallory phosphotungstic stain, which differentiates intercellular material, cell protoplasm and nucleus. A correlation between the staining reactions of the precipitate and the tumor-producing activity of the extract showed that the more active material gave a strong Feulgen test and a clear yellow-red color with the Mallory. With less active material the Feulgen reaction was not so pronounced, and the Mallory gave a deep maroon-red. Finally with filtrates or extracts of dry tumor, having a

* This investigation was carried out under the Rutherford Donation.

[1] While this article was in press the paper by Sittenfield, Johnson and Jobling appeared.

very low grade of activity, the Feulgen reaction was faintly positive, while the Mallory showed blue-staining material predominating in the precipitate. These empirical observations, which seemed to indicate that the more active extracts contained a higher ratio of nuclear material, together with the fact that an extract of desiccated chicken tumor often has a low tumor-producing power while the residue is quite active, led to the following experiment.

### *Serial Extraction of Dry Tumor*

On the assumption that the blue-staining material found in the Mallory test might be in some way responsible for the low grade activity of the tumor extract in which it is most abundant, we have attempted to eliminate it.

*Experiment.*—1 gm. of a finely powdered tumor desiccate was extracted with 60 cc. of distilled water, by first rubbing the desiccate into a smooth paste in a mortar and then thoroughly mixing it by drawing it back and forth in a syringe. The mixture was centrifuged and the supernatant fluid filtered through filter paper. Chickens were inoculated intradermally with 0.2 cc. of the extract and with 0.1 cc. of the residue. The remaining residue was extracted again with 60 cc. of water, thoroughly mixed by pumping back and forth in a syringe and then centrifuged. The supernatant fluid was passed through filter paper and 0.2 cc. of this second extract and 0.1 cc. of the residue injected intradermally. This procedure was repeated eight times and each extract and each residue tested for its activity. The results in tumor production are shown in Text-fig. 1, which represents the average of 7 experiments, and in Text-fig. 2, giving one of several tests in which the first extract was inactive.

The nitrogen content of the extracts, indicating the amount of protein present, is shown in Text-fig. 3, which also shows the phosphorus content and the amount of reducing substance, figured as glucose. These figures are based on the average from 3 experiments. Over 60 per cent of the soluble protein, as indicated by the nitrogen present, is found in the first extract, while the third extract—which is the most active in tumor production—has only about 1/4 as much. The fourth extract, which is almost as active as the third and far more active than the first, has only 0.08 mg. of nitrogen per cc. The reducing substance decreased at almost the same ratio. These figures are based on analyses carried out by Dr. O. M. Helmer.

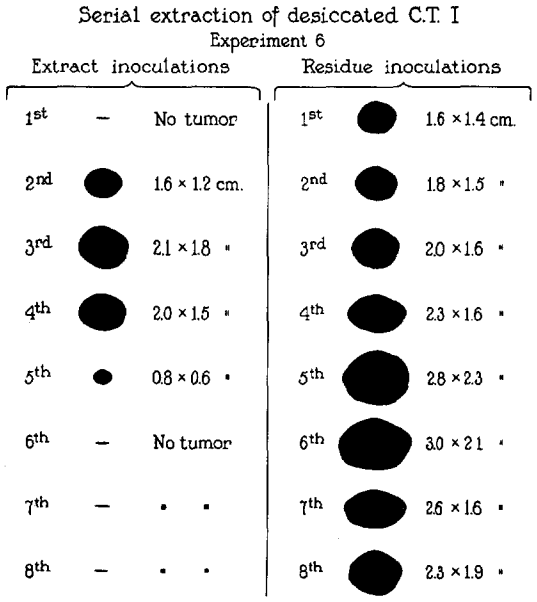
As will be seen from Text-fig. 3, the third and fourth extracts are more active than the first and second. This might be taken to indi-

## Serial extraction of desiccated C.T. I

Composite chart of 7 experiments

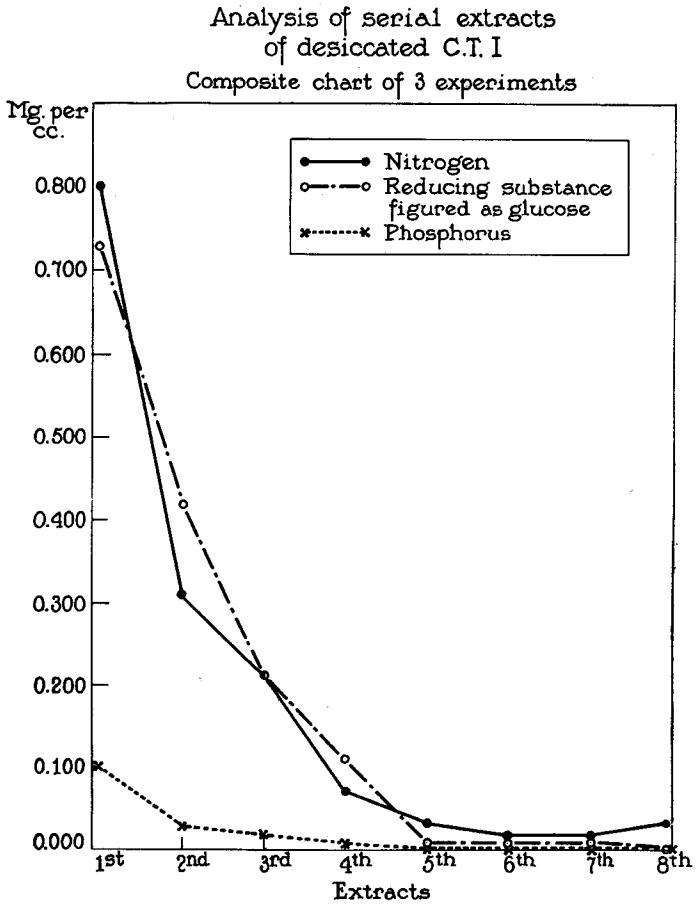
| Extract inoculations | | | | Residue inoculations | | | |
|---|---|---|---|---|---|---|---|
| Extracts | No. of inoculations | Per cent tumors | Average size of | Residues | No. of inoculations | Per cent tumors | Average size of |
| 1st | 17 | 47 | 8 tumors ● 0.7 × 0.6 cm. | 1st | 15 | 93.3 | 14 tumors ● 1.4 × 1.2 cm. |
| 2nd | 17 | 100 | 17 " ● 1.6 × 1.2 " | 2nd | 14 | 92.8 | 13 " ● 1.8 × 1.5 " |
| 3rd | 16 | 93.7 | 15 " ● 1.9 × 1.4 " | 3rd | 14 | 100 | 14 " ● 2.0 × 1.6 " |
| 4th | 16 | 93.7 | 15 " ● 1.8 × 1.4 " | 4th | 14 | 100 | 14 " ● 2.6 × 1.9 " |
| 5th | 16 | 37.5 | 6 " ● 1.1 × 0.9 " | 5th | 13 | 100 | 13 " ● 2.5 × 2.2 " |
| 6th | 16 | 25 | 4 " ● 0.9 × 0.7 " | 6th | 14 | 100 | 14 " ● 2.6 × 2.1 " |
| 7th | 10 | – | – – | 7th | 10 | 100 | 10 " ● 2.3 × 1.7 " |
| 8th | 10 | – | – – | 8th | 10 | 100 | 10 " ● 2.4 × 2.0 " |

TEXT-FIG. 1. In this series of experiments and those included in subsequent text-figures, the inoculations were made intradermally, each animal receiving from 6 to 8 inoculations. The measurements of the tumors of each fowl used in the charts were those made when the tumor from the control inoculations, or a selected one of the test tumors, had reached a certain size. This method gives more accurate data on the relative potency of the materials tested and largely eliminates the confusing variation due to differences in susceptibility in individual chickens.




TEXT-FIG. 2. For method of inoculation and comparative measurements see explanation of Text-fig. 1.

cate that the active principle is difficultly soluble and that more comes out with the repeated washings; but the fact that the residues after extraction become progressively more active leaves little doubt



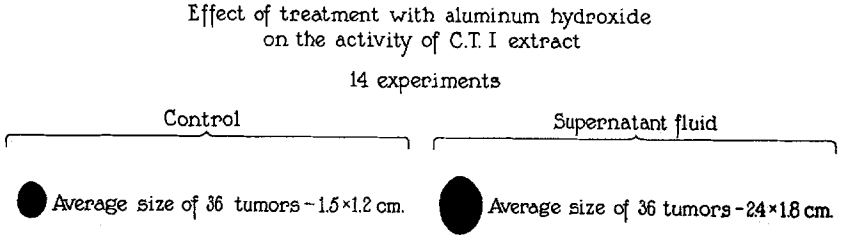

TEXT-FIG. 3

that some inhibiting substance is being removed with the extract. That the first extracts contain considerable amounts of the active principle is shown by the next experiments.

*Removal of Inhibitor by Adsorption on Aluminum Hydroxide*

In a preceding paper the results of treating extracts of the chicken tumor with aluminum hydroxide have been described in connection with purification of the tumor agent (5). The removal of approximately 90 per cent of the nitrogen-containing elements from the extract with the aluminum hydroxide was accomplished without loss of activity in the remaining fluid. In fact, the remaining fluid was more active than the original extract, in spite of the removal of a certain amount of the agent on the aluminum hydroxide adsorbate.

*Method.*—A concentrated Berkefeld filtrate of fresh chicken tumor or an extract of tumor desiccate was added to an equal volume of aluminum hydroxide (Willstätter Type C), prepared in the usual way (6). This was shaken until thoroughly mixed,




TEXT-FIG. 4. The method of recording measurements of tumors was the same as that used in the preceding text-figures. The control inoculation was a sample of the same tumor extracts subsequently treated with aluminum hydroxide.

centrifuged and the supernatant fluid drawn off. Chickens were inoculated intradermally with 0.2 cc. of this fluid in several areas, and also with equal amounts of the original extracts for controls. Weekly measurements were made. The average of the results of 14 experiments, in which 36 inoculations were made of both tumor extract and aluminum supernatant fluid, are shown in Text-fig. 4.

The fact that an appreciable amount of the agent is removed with the aluminum has been shown in a previous paper. In spite of this loss in concentration the fluids left after adsorption on aluminum are markedly more active in the production of tumors than the full extracts before adsorption. This seems to indicate that an inhibiting substance must have been carried with the aluminum fraction, leaving the reduced concentration of the agent in the supernatant fluid more active, unhampered by an inhibitor.

### *Inhibiting Substance in Slower-Growing Tumors*

While the Chicken Tumor I is extremely rapid in its growth, occasionally a slower-growing tumor is encountered, or the tumor appears

Inhibitors from chicken tumor I

| Material inoculated | No. of inoculations | Per cent inhibited | No. of tumors | Average size of tumors |
|---|---|---|---|---|
| Heated extract of slow-growing tumor + active filtrate | 50 | 78 | 11 | 0.9 × 0.7 cm. |
| Active filtrate (alone) | 21 | 0 | 21 | 2.0 × 1.3 " |
| Heated extract of slow-growing tumor + aluminum supernatant fluid | 33 | 91 | 3 | 0.8 × 0.8 " |
| Aluminum supernatant fluid (alone) | 24 | 0 | 24 | 2.5 × 1.8 " |
| Heated extract of rapid-growing tumor + active filtrate | 37 | 0 | 37 | 1.9 × 1.7 " |
| Active filtrate (alone) | 26 | 0 | 26 | 2.2 × 1.8 " |
| Heated extract of rapid-growing tumor + active aluminum supernatant fluid | 9 | 0 | 9 | 1.8 × 1.4 " |
| Aluminum supernatant fluid (alone) | 6 | 0 | 6 | 1.6 × 1.3 " |
| Heated mucoid exudate from slow-growing tumor + active filtrate | 14 | 100 | 0 | — |
| Heated mucoid exudate from rapid — growing tumor + active filtrate | 10 | 0 | 10 | 2.0 × 1.3 " |
| Active filtrate (alone) | 20 | 0 | 20 | 1.9 × 1.3 " |

TEXT-FIG. 5. The figures given here are based on 250 inoculations. Those included in the inhibitor group were inoculations resulting in no growth. There is undoubted evidence of retardation even when tumors did arise from these test inoculations. The system of recording measurements of tumors was the same as that used in the preceding charts.

at times to pass through a phase of reduced malignancy. It was considered possible that these phases might be due to relative variations in the ratio of agent and inhibitor.

*Experiments.*—The desiccates of a number of slower-growing tumors were used in these experiments. An extract was prepared in the usual way, and the tumor-producing activity was destroyed by heating at 55°C. for 30 minutes. This inactivated material was mixed with an equal amount of an active extract from a fast-growing tumor, and 0.4 cc. injected intradermally into chickens. As a further test the action of the inactivated extract was tested on a highly potent fluid left after adsorption on aluminum hydroxide. The results of some 35 experiments, in which 250 test inoculations were made, are shown in Text-fig. 5.

The heated extract of slow-growing tumors completely neutralized the tumor-producing power of the active extracts in 78 per cent of cases, and partly neutralized the activity in the remaining 22 per cent. The more active supernatant fluid from tumor extracts treated with aluminum, inoculated together with the inhibiting extract, failed to induce tumors in 91 per cent of tests. The control injections of active extracts and aluminum supernatant fluid resulted in 100 per cent tumors. The mucoid exudate obtained from certain slow-growing tumors, showed after heating a similar inhibiting action on active extracts. No inhibiting action was noted with the heated extracts or the mucoid exudate obtained from rapidly growing tumors.

All attempts to release the inhibiting substance in detectable amounts after adsorption on aluminum hydroxide have thus far failed. Berkefeld filtrates of fresh tumors show little evidence of the presence of an inhibitor, owing perhaps to the relatively great dilution of this material.

The effect of heat on the inhibitor was next tested. The same methods were used as in the foregoing experiment, except that various samples of the extract were heated at 60°, 65°, 70°, 75°, 80°, 90° and 100°C. for 30 minutes each. The effect of these samples was tested on the tumor-producing power of an active extract. The results, as shown in Table I, demonstrated that little or no inhibiting power remains in the specimens heated to 65° and over.

From the above experiments it is evident that the slow-growing chicken tumors contain an inhibiting factor capable of neutralizing the tumor agent in its most active form. The inhibiting substance withstands 55°C. for 30 minutes, but is inactivated when heated above 65°C. The fact that the more active tumors do not contain sufficient amounts of the inhibitor to be demonstrable by this method, although

there is adequate proof that it is present, indicates that the degree of malignancy may depend in part on the ratio of agent to inhibitor. There is no doubt that the individual susceptibility of the inoculated fowl plays a part; but, when a number of extracts of different tumors of the same type are injected in the same fowl, the variation in potency is evident. If, as we have noted many times, this test is repeated on a number of chickens, some will be markedly more susceptible than others; but the relative activity of the different extracts will be manifest in all.

TABLE I

*Effect of Heat on the Chicken Tumor Inhibitor*

| Material inoculated | | No. of inoculations | No. positive | Positive |
|---|---|---|---|---|
| | | | | *per cent* |
| Active tumor extract plus | Inhibitor heated 30 min. at 55°C. | 8 | 2 | 25 |
| | " " 30 " " 60° " | 8 | 2 | 25 |
| | " " 30 " " 65° " | 8 | 3 | 37.5 |
| | " " 30 " " 70° " | 8 | 7 | 87.5 |
| Active extract alone (control) | | 8 | 8 | 100 |
| Aluminum supernatant fluid of active extract plus | Inhibitor heated 30 min. at 55° C. | 3 | 0 | 0 |
| | " " 30 " " 60° " | 3 | 2 | 66.6 |
| | " " 30 " " 65° " | 3 | 3 | 100 |
| | " " 30 " " 70° " | 3 | 3 | 100 |
| | " " 30 " " 75° " | 3 | 3 | 100 |
| | " " 30 " " 80° " | 3 | 3 | 100 |
| | " " 30 " " 90° " | 3 | 3 | 100 |
| | " " 30 " " 100° " | 3 | 3 | 100 |
| Aluminum supernatant fluid of active extract alone (control) | | 6 | 6 | 100 |

## DISCUSSION

From the point of view of the suggested hypothesis, according to which the tumor agent may be related to the normal growth-controlling mechanism of the cell, it might be expected that an inhibiting agent would also be present in the tumor. This is suggested by the fact that biological forces are generally balanced phenomena, the presence of an active force checked by a retarding one. There seems little doubt from the results reported here that an inhibitor does exist in the chicken tumors studied, more powerful in the extracts of slow-growing tumors, but definitely present in those of more rapid development.

Aside from individual variation in susceptibility of the fowls, the relative activity of any given tumor extract seems to depend on the proportion of agent to inhibitor. It is not unusual to have an inactive extract, which, after removal of something by adsorption on aluminum hydroxide, shows the presence of sufficient agent to produce vigorous tumors. These observations, taken with the fact that the inhibitor from the chicken tumor acts definitely on mouse sarcoma and is without effect on carcinoma (7), suggest that this agent is a specific factor, not an incidental proteolytic enzyme or accidentally injurious chemical substance.

The relationship of this inhibitor to the normal growth-balancing mechanism of cells is not established by the experiments reported here. Theoretically, if our hypothesis is correct, it should be possible to separate the inhibitor from active normal tissues, just as it should be possible to isolate the growth-stimulating agent. While there is evidence that the latter can be accomplished,[2] the methods thus far used have not yielded regular results. That an inhibiting substance can be secured from normal tissues for mouse tumors under certain conditions is established.[3] Perhaps these results with the chicken tumor agent, deemed to represent an adsorption on normal tissues *in vitro*, really represent neutralization by an inhibitor (8). The relationship of the inhibitor in the tumor to the "antibody" which Andrewes has demonstrated in the blood of chickens with slow-growing tumors has not yet been determined (9).

While the presence of an inhibiting substance in the chicken tumor is established, and it would appear to be a specific force, its true nature and its relationship to the causative agent on the one hand, and to the

[2] In a report to the International Cancer Conference, London, 1928, a reference was made to tumors induced by the injection of a fraction of an extract of normal chicken testicle. 4 experiments thus far have resulted positively and twenty-three tumors have been produced by this method. However, there have been many negative experiments. Whether these results indicate that the method is inadequate, giving only occasionally the growth factor in sufficient concentration or free enough from the hypothetical inhibitor to induce tumors, or whether there is some other explanation, are questions which cannot be answered at present.

[3] A preliminary report has been published in *Science*. The complete study will appear later in *The Journal of Experimental Medicine*.

balancing factor of normal cells on the other, are questions which must await further development.

SUMMARY

The presence of an inhibiting substance in the chicken tumor is shown by the fact that a desiccate of the tumor is more active after it has been washed two or three times with water, and that an extract of the tumor is more potent after some factor is removed by adsorption on aluminum hydroxide.

When the tumor-producing factor in an extract of a slow-growing tumor has been destroyed by heating at 55°C. it is found to have the property of neutralizing a highly active tumor extract. This inhibiting property is destroyed by heating over 65°C.

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