STUDIES OF THE TRANSMISSIBLE AGENT OF THE ROUS CHICKEN SARCOMA I

PRECIPITATION WITH BASIC PROTEINS*

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Since 1911, when Rous (1) found that a chicken sarcoma could be transmitted by a cell-free filtrate, many investigators have attempted to isolate and thereby identify the causative agent of this tumor.[1] Because of the lability of the agent, the usual methods for isolation of the more stable biological substances could not be used. The earlier investigations have been reviewed by Claude and Murphy (2). More recently several investigators (3, 4) have applied the technique of the ultracentrifuge. Though very active sediments were obtained, the material appeared to be far from homogeneous. Two of the present authors have reported (5) that tumors could be produced with lipid extracts of dried tumor tissue. These were still active after filtration through a Berkefeld W filter. Neither by chemical nor by biological methods were proteins demonstrable in these extracts. However, because of the extreme activity of the customary filtered water extracts, the possibility exists that proteins may have been present in such lipid extracts in amounts sufficient to produce tumors but incapable of detection. Further studies in this laboratory have shown that extraction of the desiccated tumor tissue with organic solvents in the cold, for the brief period of time necessary to preserve activity, did not measurably reduce the tumor producing quality of the residue. In addition, quantitative measure of the active agent in the filtered lipid extracts compared very unfavorably with the amount of active material in cell-free water extracts of the same dried tumor. In view of these findings it seemed advisable to seek a more efficient method of isolation, in order to study the chemical nature of the active agent.

One of the difficulties in working with a Rous sarcoma extract is its very

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[1] Chicken Tumor I of the first series of spontaneous growths studied by filtration methods.

high viscosity which is caused by a polysaccharide. Because of this, previous workers were compelled to use very dilute extracts in order to obtain a Berkefeld filtrate and even when they were successful in filtering the extract in a reasonable length of time, tremendous losses in nitrogen and in activity occurred. Since it would seem that a more concentrated solution would facilitate the isolation of the active agent, the first problem that arose was that of lowering the viscosity of the tumor extracts.

In 1937 Meyer *et al.* (6) isolated from pneumococcus an enzyme that hydrolyzed the polysaccharide of umbilical cord, vitreous humor, and synovial fluid. Kabat (7) recently found that the same enzyme hydrolyzed the polysaccharide which causes the high viscosity in extracts of fowl leucotic tumors. We have found that treatment of a Rous sarcoma extract with the polysaccharide enzyme isolated from pneumococcus (8), markedly reduced its viscosity. It was then possible to filter very concentrated extracts with comparative ease and without reduction of the amount of nitrogen or the degree of activity. We have also found that the polysaccharide enzyme which Chain and Duthie (9) isolated from testis will also remarkably lower the viscosity of the tumor extracts.

Since the active agent has an acid isoelectric point (the components of a Rous sarcoma extract migrate toward the anode in an electrical field at pH 7)[2] and Bawden and Pirie (10) indicated that the tobacco mosaic virus can be precipitated with basic proteins, we have investigated the possibility of concentrating and isolating the Rous sarcoma agent by the use of basic proteins, namely papain (isoelectric point 8.5–9) (11) and thymus histone (isoelectric point 9). In conjunction with the basic protein precipitation we have applied the electrophoretic technique to separate the basic protein from the precipitated components of the Rous sarcoma extract.

Materials and Methods

The Rous Chicken Sarcoma I was used. All tests of biological activity were made by the following quantitative technique. Tenfold dilutions were made of each fraction to be tested and four 1 cc. samples of each dilution were injected intramuscularly into 2 to 3 week old chicks. All fractions were inoculated during the experimental day except those studied in the electrophoresis apparatus. As routine the chicks were killed 4 weeks after the injections and the presence and size of the tumors noted. The successful inoculations are given in the tables as fractions of the total number of inoculations. Usually we tried to reach an end-dilution so that we could compare the activity of different fractions by noting the dilution containing the smallest amount of nitrogen which would give rise to tumors.

A weighed amount of fresh tumor tissue was ground in a mortar with sand and then

[2] Data from electrophoresis experiments being conducted in this laboratory.

extracted with 0.02 M phosphate buffer, pH 7.2 to 7.4 at 0–5°C. for 30 minutes, by mechanical stirring. The suspension was rapidly warmed to 37°C., polysaccharide enzyme added, and the suspension further extracted by stirring. After 10 minutes the suspension was rapidly cooled and centrifuged at 5000 R.P.M. for about 30 minutes in the cold. The supernatant fluid was then filtered through a Berkefeld V filter, and a portion of the filtrate was assayed.

To a known volume of the filtrate a neutral solution of the basic protein was added. The amount of papain solution (12) added was determined by the following method. In several centrifuge tubes each containing 1 cc. of filtrate, varying amounts of the papain solution were added. The precipitates were centrifuged and the supernatant fluids tested with more papain. Originally we selected as an end-point the tube in which no precipitation occurred on further addition of papain. As increasing amounts of papain were added to the filtrate the character of the precipitate changed. In the tubes which contained the lower concentration of papain the precipitate was white, readily suspended in water, and dissolved in 3 per cent NaCl; while in the tubes with the higher concentration of papain, the precipitate was light brown, gummy, and could not be readily suspended in water or dissolved in 3 per cent NaCl. Since we found that the supernatant fluids from the former were not appreciably more active than the supernatant fluids from the latter, we abandoned the maximum precipitation in favor of the addition of that amount of papain solution which would give a maximum precipitate yet a white and easily suspendible one.

The precipitate was centrifuged in the cold, washed several times with ice cold water, and finally dissolved in a known volume of 3 per cent NaCl solution. This solution was centrifuged in the cold, and a small amount of undissolved material discarded. The NaCl solution was designated as unpurified papain-agent-complex and its biological activity compared with that of the tumor filtrate and of the supernatant fluid from the original precipitation with papain. The papain-agent-complex was further purified in the following manner. Cold water was added to the NaCl solution, until a tenfold dilution occurred, and the precipitate which formed was centrifuged off, dissolved in a small volume of 3 per cent NaCl, and reprecipitated by dilution with cold water. Finally the precipitate (purified papain-agent-complex) was dissolved in 3 per cent NaCl and its tumor-producing activity compared with that of the original filtrate and of the unpurified papain-agent-complex.

The active agent of the filtrate could also be precipitated with histone isolated from calf thymus (13). However, the histone-agent-complex did not lend itself to purification, for it was extremely insoluble and for this reason we confined our attention to papain as a precipitant. The activities of the filtrate, of the supernatant fluid, and of the suspension of the histone-agent-complex were compared quantitatively.

Splitting of the papain-agent-complex into its two main parts (basic protein and components from tumor) was accomplished in a Tiselius electrophoresis cell.

The nitrogen was determined by the micro Kjeldahl method.

Viscosity measurements were made with an Ostwald viscometer at 25°C.

EXPERIMENTAL

All the experimental findings described are typical results of many experiments.

1. Comparison of Viscosity of Tumor Extract with Viscosity of Tumor Extract Treated for 15 Minutes at 37°C. with Varying Amounts of Polysaccharide Enzyme Isolated from Pneumococcus and Testis.—This is expressed as the relative viscosity compared to a 0.9 per cent NaCl solution.

It is evident from Table I that the polysaccharide enzyme from pneumococcus and testis reduced the viscosity of Rous sarcoma extracts.

2. Comparison of Activity of Enzyme Treated Extract with That of Untreated Extract.—

5 gm. of dry tumor tissue were extracted with 150 cc. of 0.02 M phosphate buffer. After centrifugation, the extract contained 1.54 mg. N/cc. 10 cc. of extract were treated with 0.6 mg. of polysaccharide enzyme and filtered through a Berkefeld filter. The filtrate contained 1.50 mg. N/cc. and its activity was compared with that of the untreated extract. In another experiment the biological activity of the extract containing 1.45 mg. N/cc. was compared with that of the enzyme treated filtrate containing 1.37 mg. N/cc.

TABLE I

Concentration of enzyme isolated from pneumococcus	Relative viscosity of tumor filtrate
mg./cc.	
0	3.0
0.01	1.4
0.025	1.2
0.03	1.2
0.05	1.1
Concentration of enzyme isolated from testis	**Relative viscosity of tumor filtrate**
mg./cc.	
0	3.0
0.01	1.5
0.025	1.2
0.05	1.2

One can see from Table II that the enzyme treated material was as active as the untreated extract.

3. Precipitation of Active Agent with Papain.—

(*a*) *Comparison of Biological Activity of Original Filtrate, Crude Papain-Agent-Complex, and Supernatant Solution.*—50 gm. of fresh tumor tissue were extracted with 200 cc. of phosphate buffer and the extract was treated with 1.5 mg. of enzyme and filtered through a Berkefeld filter. Papain in solution was added to the filtrate and the washed precipitate dissolved in 3 per cent NaCl solution. The activities of the original filtrate, the papain precipitate, and the supernatant fluid were compared.

One can see from Table III, that the papain-agent-complex was at least as active as the original filtrate, the latter being active to a concentration of 7×10^{-5} mg. N, the complex 2×10^{-5} mg. N, and the supernatant

TABLE II

Incubation with Polysaccharide Enzyme

Dilution	Untreated extract 1.54 mg. N/cc.		Enzyme treated extract 1.50 mg. N/cc.	
	No. tumors / No. inoculated	Average size tumor	No. tumors / No. inoculated	Average size tumor
1	4/4	++++	4/4	++++
1:10	4/4	+++	4/4	+++
$1:10^2$	4/4	+++	4/4	++++
$1:10^3$	2/4	++	2/4	+++
$1:10^4$	1/4	+	2/4	+
	1.45 mg. N/cc.		1.37 mg. N/cc.	
1	2/2	++++	2/2	++++
1:10	4/4	+++	4/4	+++
$1:10^2$	4/4	++++	4/4	++++
$1:10^3$	4/4	+	4/4	++
$1:10^4$	0/4	–	0/4	–

TABLE III

Precipitation with Papain

Dilution	Filtrate			Papain-agent-complex			Supernatant fluid		
	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor
	mg./cc.			*mg./cc.*			*mg./cc.*		
1:10	7×10^{-2}	3/4	++++	2×10^{-1}	3/4	+++	1×10^{-1}	0/4	–
$1:10^2$	7×10^{-3}	4/4	+++	2×10^{-2}	4/4	+++	1×10^{-2}	0/4	–
$1:10^3$	7×10^{-4}	3/4	++	2×10^{-3}	3/4	+++	1×10^{-3}	0/4	–
$1:10^4$	7×10^{-5}	1/4	+	2×10^{-4}	4/4	++	1×10^{-4}	0/4	–
$1:10^5$	7×10^{-6}	0/4	–	2×10^{-5}	1/4	+++	1×10^{-5}	0/4	–
$1:10^6$	7×10^{-7}	0/4	–	2×10^{-6}	0/4	–	1×10^{-6}	0/4	–

fluid was totally devoid of any activity. In other experiments the supernatant fluid produced one or two small tumors in the highest concentration.

(*b*) *Comparison of Activity of Original Filtrate, Crude Papain-Agent-Complex, and Purified Papain-Agent-Complex.*—To 50 cc. of Berkefeld filtrate obtained from 40 gm. of fresh tumor tissue, 25 cc. of a papain solution were added containing 3.9 mg. N/cc. The precipitate was washed twice with cold water, and then dissolved in 20 cc. of 3 per cent NaCl. The solution, designated as crude papain-agent-complex, was centrifuged and a portion taken for biological assay. The clear solution was diluted to 200 cc. with

ice cold water and the formed precipitate centrifuged off, dissolved in 15 cc. of 3 per cent NaCl, centrifuged, and then diluted to 150 cc. with water. The purified papain-agent-complex was finally dissolved in 10 cc. of 3 per cent NaCl and the activity assayed.

One can see from Table IV that the filtrate was active to a concentration of 1×10^{-4} mg. N, the crude papain-agent-complex to a concentration

TABLE IV

Purification of Papain-Agent-Complex

Dilution	Filtrate			Crude papain-agent-complex			Purified papain-agent-complex		
	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor
	mg./cc.			*mg./cc.*			*mg./cc.*		
1:10	1×10^{-1}	4/4	+++	1.7×10^{-1}	3/4	+++	1×10^{-1}	3/4	+++
$1:10^2$	1×10^{-2}	4/4	+++	1.7×10^{-2}	4/4	+++	1×10^{-2}	4/4	++
$1:10^3$	1×10^{-3}	4/4	+++	1.7×10^{-3}	4/4	+++	1×10^{-3}	4/4	+++
$1:10^4$	1×10^{-4}	3/4	++	1.7×10^{-4}	3/4	+++	1×10^{-4}	3/4	+
$1:10^5$	1×10^{-5}	0/4	–	1.7×10^{-5}	2/4	++	1×10^{-5}	1/4	++
$1:10^6$	1×10^{-6}	0/4	–	1.7×10^{-6}	0/4	–	1×10^{-6}	1/4	++

TABLE V

Precipitation with Histone

Dilution	Filtrate			Histone-agent-complex			Supernatant fluid		
	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor
	mg./cc.			*mg./cc.*			*mg./cc.*		
1:10	1.3×10^{-1}	4/4	++++	1.1×10^{-1}	4/4	+++	8×10^{-2}	0/4	–
$1:10^2$	1.3×10^{-2}	3/4	++	1.1×10^{-2}	2/4	++	8×10^{-3}	0/4	–
$1:10^3$	1.3×10^{-3}	4/4	+++	1.1×10^{-3}	2/4	+++	8×10^{-4}	0/4	–
$1:10^4$	1.3×10^{-4}	3/4	+++	1.1×10^{-4}	1/4	++	8×10^{-5}	0/4	–
$1:10^5$	1.3×10^{-5}	2/4	++	1.1×10^{-5}	0/4	–	8×10^{-6}	0/4	–
$1:10^6$	1.3×10^{-6}	0/4	–	1.1×10^{-6}	0/4	–	8×10^{-7}	0/4	–

of 1.7×10^{-5} mg. N, and the purified complex was active at least in a concentration of 1×10^{-6} mg. N.

4. Precipitation of Active Agent with Histone: Comparison of Activity of Tumor Filtrate with That of Histone-Agent-Complex and of Supernatant Fluid.—

50 gm. of fresh tumor tissue were extracted with 200 cc. of 0.02 M phosphate buffer, and treated with 0.75 mg. of enzyme. To the filtrate a histone solution was added and

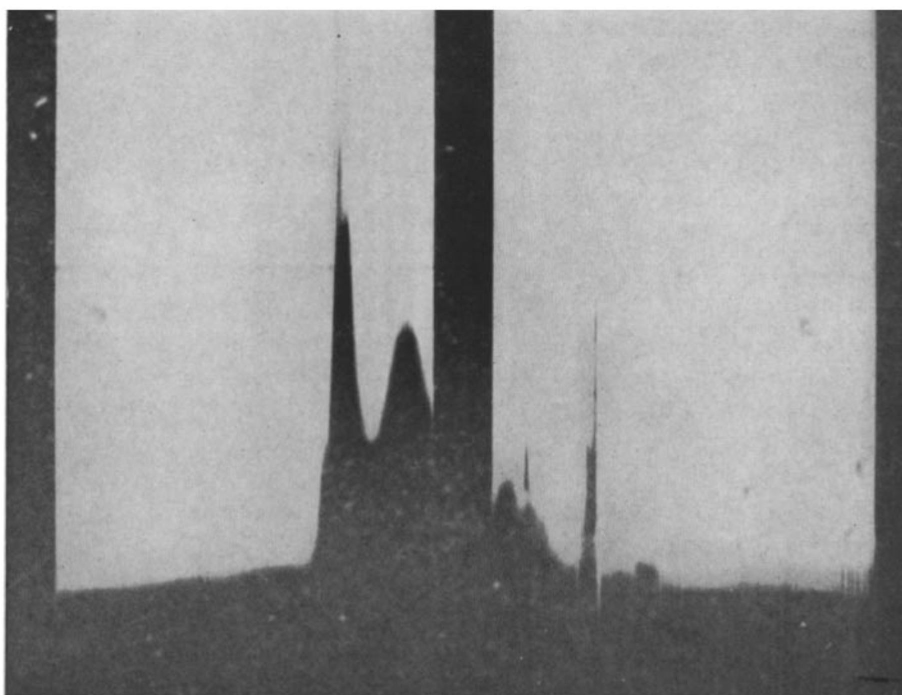

FIG. 1. Electrophoretic pattern of purified papain-agent-complex: papain moving toward cathode on left, components from tumor extract moving toward anode on right.

TABLE VI

Activity of the Fractions Obtained by Splitting Papain-Agent-Complex by Electrophoresis

Dilution	Original papain-agent-complex			Fraction A Anode compartment			Fraction B Middle compartment			Fraction C Cathode compartment		
	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor	N	No. tumors / No. inoculated	Average size tumor
	mg./cc.			*mg./cc.*			*mg./cc.*			*mg./cc.*		
1:10	1.8×10^{-1}	4/4	+++	2.6×10^{-2}	4/4	+	1.4×10^{-1}	4/4	+++	2.2×10^{-2}	0/4	–
1:10²	1.8×10^{-2}	4/4	++	2.6×10^{-3}	4/4	++	1.4×10^{-2}	4/4	+++	2.2×10^{-3}	0/4	–
1:10³	1.8×10^{-3}	4/4	++	2.6×10^{-4}	3/4	++	1.4×10^{-3}	3/4	+++	2.2×10^{-4}	0/4	–
1:10⁴	1.8×10^{-4}	3/4	++	2.6×10^{-5}	0/4	–	1.4×10^{-4}	2/4	++	2.2×10^{-5}	0/4	–
1:10⁵	1.8×10^{-5}	0/4	–	2.6×10^{-6}	0/4	–	1.4×10^{-5}	0/4	–	2.2×10^{-6}	0/4	–
1:10⁶	1.8×10^{-6}	0/4	–	2.6×10^{-7}	0/4	–	1.4×10^{-6}	0/4	–	2.2×10^{-7}	0/4	–

the precipitate centrifuged off and washed. The activities of the filtrate, supernatant fluid, and the suspension of the histone-agent-complex were compared.

Though the basic protein again precipitated all of the active agent, the

histone-agent-complex was slightly less active than the original filtrate. This may have been due to the extreme insolubility of the histone-agent-complex.

5. Splitting of Papain-Agent-Complex and Separation of Components in Tiselius Electrophoresis Cell.—A salt solution of purified papain-agent-complex was dialyzed overnight against 0.2 M phosphate buffer pH 7.3 to 7.5 containing 3 per cent NaCl and then placed in the Tiselius electrophoresis cell. The papain moved toward the cathode while the components originally coming from the tumor extract moved toward the anode (Fig. 1). After 7 to 11 hours, three fractions were separated, fraction A, the components moving toward the anode, fraction B, the material in the middle cells which contained each of the original components, and fraction C, the components moving toward the cathode. The activity of each fraction was tested and compared with that of the original papain-agent-complex. One can see from Table VI, that fraction A was just as active as fraction B and as the original papain-agent-complex, while the material moving toward the cathode was completely inactive. The latter was identified as papain by its direction and mobility.

DISCUSSION

From the data presented, it is evident that basic proteins can be used to precipitate the active agent of the Rous sarcoma, and the papain-agent-complex containing as little as 1×10^{-6} mg. of N gave rise to tumors. It is reasonable to expect that basic proteins would form a salt linkage with the tumor agent at pH 7, since the former are positively charged while the latter is negatively charged. However, the material containing the active agent can be subsequently recovered free from papain, as they migrate in opposite directions in the Tiselius electrophoresis apparatus.

The papain-agent-complex, at this stage of the investigation, contains other material beside the basic protein and the active agent. This is readily ascertained from the electrophoresis picture, Fig. 1, in which several components moving toward the anode are apparent, indicating that other acid substances are precipitated along with the active agent. A few chemical analyses of the complex support this view. On analyzing several different preparations for purines by the method of Graff and Maculla (14) it was found that the purine nitrogen comprised from 4 to 8 per cent of the total nitrogen. The amount of purine in the complex is rather high if one takes into account that the papain, which is free of nucleic acid, composed as much as 50 to 70 per cent of the complex. This was estimated

by comparing the areas of the components moving toward the anode with those of the papain moving toward the cathode. This high value of purines in the complex may be due to precipitation of free nucleic acids along with nucleoproteins in the extract.

Varying small amounts of lipids were also found in the complex. The presence of these may be due either to the precipitation of free phospholipids by basic proteins as shown by Chargaff (15), to their being carried down mechanically by the precipitate, or because they form an integral part of the tumor agent. Attempt is being made to purify the complex further.

SUMMARY

The viscosity of Rous sarcoma extracts can be reduced with a polysaccharide enzyme isolated from pneumococcus or testis without destruction of the active agent and thus more concentrated active filtrates can be obtained.

The active agent can be precipitated with basic proteins.

The basic protein can be separated from the components originally coming from the tumor filtrate by electrophoresis.

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