FRACTIONATION OF CHICKEN TUMOR EXTRACTS BY HIGH SPEED CENTRIFUGATION [1]

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Recent studies of filtrable chicken tumors have shown that rapid separation of the causative agents could be accomplished by means of high-speed centrifugation (1, 2). This method has been applied to the purification of the agent causing chicken tumor I.

TABLE I: *Fractionation of Chicken Tumor Filtrate by High-Speed Centrifugation: Precipitation of the Washed Sediment at pH 4.2*

Fractions tested *	Inoculation tests		Tyndall effect
	Number of tumors (per cent)	Average size of tumors (cm.)	
Washed sediment	100	1.7 × 1.5	+++++
pH 4.2 Supernatant fluid	50	0.9 × 0.8	++
pH 4.2 Precipitate (redissolved in Tyrode's)	100	1.8 × 1.2	++++±

* Solutions made up to the same volume and brought to pH 7.1 before injection.

Berkefeld filtrates of the tumor were submitted to a centrifugal force of 14,000 times gravity for one to three hours, according to the viscosity of the fluid. The collected sediment was suspended in Tyrode's solution and the coarse particles discarded by low-speed centrifugation. The material remaining in the solution was deposited again by high-speed centrifugation, and taken up once more in Tyrode's solution. Coarse particles were removed by low-speed centrifugation and the volume of the fluid adjusted to that of the original filtrate. Methods and discussion of the results are given in another paper (3). Inoculation tests showed that the effect of high-speed centrifugation was to reduce markedly the tumor-producing activity of the filtrate. Concentration of the agent occurred during centrifugation, as shown by the increased activity of that part of the filtrate at the bottom of the tube. In spite of the successive manipulations to which the sediment was submitted, and loss of some active material in the process, the washed sediment exhibited a tumor-producing power even greater than that of the control filtrate. The enhanced activity of the sediment can be explained by the fact that the tumor extract contains inhibiting elements which are eliminated by repeated high-speed centrifugation. From analysis of the results, it was estimated that about 90 per cent of the tumor-producing power of the original filtrate remained associated with the washed sediment.

Preparations, obtained by taking up the washed sediment in Tyrode's or buffered solutions of pH 7.2, are always more or less opalescent. Such material appears to contain a great number of particles which can be seen in the dark field microscope, but whose size is below the power of resolution of an ordinary microscope. Amies (4), who has confirmed the original observation

[1] Read before the American Association for Cancer Research, Chicago, Ill., March 24, 1937.

of Ledingham and Gye and those of McIntosh, is inclined to consider at least part of these bodies as representing the causative agent of the tumors.

In an attempt to study further the properties of the material separated by high-speed centrifugation, the effect of acid solutions on the washed sediment was tested. The material, suspended in Tyrode's solution, was brought to pH 4.2 by means of buffered solution. The precipitate which formed was separated by low-speed centrifugation and redissolved in Tyrode's solution, the volume being adjusted to equal that of the neutralized supernatant fluid. The relative opalescence of the solutions was determined in the dark room against focused light. The results are shown in Table I.

As shown in Table I, most of the tumor-producing activity of the solution was carried along with the acid precipitate. The opalescence of the solution was found to parallel more or less the tumor-producing powers of the solutions.[1] As a preliminary to a more detailed study of the properties of the tumor agent, methods for the preparation of larger quantities of active material were next investigated.

Activity tests carried out with a plain tumor extract, an extract purified by adsorption on colloidal aluminum hydroxide (5), or the Berkefeld filtrate of the crude extract showed that the process of filtration through a Berkefeld candle would deprive the extract of about 90 per cent of its original tumor-producing power. Therefore, plain water extracts were used in the next experiments in place of the usual Berkefeld filtrates.

The fresh tumor tissue was extracted with sterile, redistilled water, or with Tyrode's solution of pH 7.2, the volume of solvent being equivalent to fifteen times the weight of the pulp. The extract was centrifuged for twenty minutes under a centrifugal force of 2400 times gravity and the supernatant fluid filtered through sterile gauze. In some instances this extract was purified by treatment with an equal volume of alumina gel (5). The extract was then centrifuged at high speed, the centrifugal force at the center of the tube being equal to 17,000 times gravity. High-speed centrifugation was continued for two hours to two hours and forty-five minutes, according to the viscosity of the extracts, which varied from 2.6 to 3.8 times that of water, as measured in the viscosimeter of Ostwald.

The supernatant fluid from high-speed centrifugation was discarded and the deposit suspended in Tyrode's solution. This crude suspension was centrifuged at high speed for a short run of two minutes, not counting the two minutes necessary for deceleration of the centrifuge. The supernatant fluid was saved. The deposit was resuspended in a small volume of Tyrode's solution and the material centrifuged again at high speed for a short run of two minutes. The last sediment was discarded and the washings were combined with the main supernatant fluid. The material in suspension was deposited again by one hour's centrifugation at high speed and the entire process (high-speed centrifugation, suspension of the sediment in Tyrode's solution, short run at high speed and washing of the sediment) was repeated three times. During centrifugation the temperature of the fluid did not exceed 18° C. Between runs the material was kept on ice when possible.

When taken up in Tyrode's or phosphate buffer solutions of pH 7.2, the material isolated by the foregoing method gives a colloidal solution which is transparent to transmitted light and presents a marked bluish opalescence under reflected light. In the dark field microscope this material appears to be composed of a great quantity of granules, comparatively small, similar in size and agitated by the Brownian movement. Large particles and clusters, usually abundant in the sediment prepared by the first method from Berkefeld filtrates, were absent. When concentrated by high-speed centrifugation the

[1] The active material separated by high-speed centrifugation from the filtrate of a chicken fibrosarcoma (chicken tumor 10) was also precipitated at pH 4.2 and the acid precipitate was found to carry with it most of the tumor-producing activity.

material deposited is amber in color and completely transparent. This may suggest that the major part of the sediment exists in the form of a gel.

A concentrated solution of this purified fraction (0.4 mg. substance per c.c.) will precipitate in acid solutions at a wide range between pH 2.0 and pH 5.0. Beyond these two points the substance becomes again soluble. There is indication of an isoelectric point around pH 3.5. The difference in the degree of precipitation was emphasized when diluted solutions were used for the test. In this case a precipitate separated at pH 3.8, but no flocculation could be detected at pH 4.0. The results are shown in Table II.

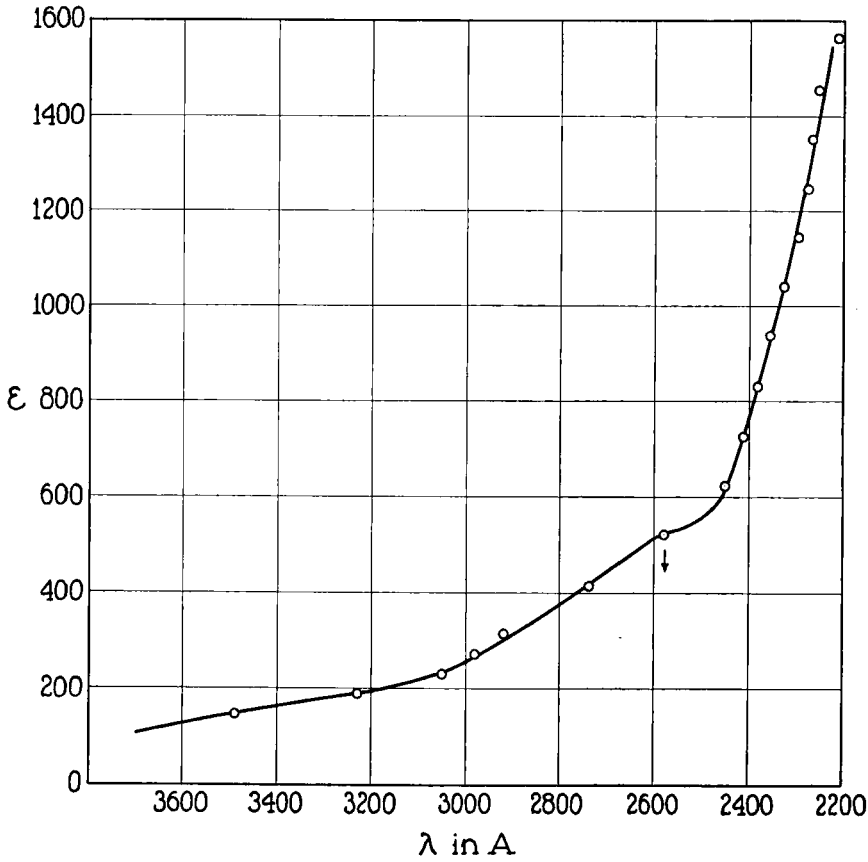

FIG. 1

The observation of a maximum precipitation of the substance around pH 3.8 is of interest when correlated with the fact that pH 3.5–3.8 is also the range of inactivation of the tumor agent, as shown by Lewis and Michaelis (6). That an ordinary tumor protein, carried along with the sediment, is responsible for the acid precipitation has not been completely excluded, although as far as can be judged, the material isolated appears homogeneous.

On the alkaline side the solution retains its opalescence up to pH 11.5 where a sudden change seems to take place. At that point the solution becomes more transparent, indicating a dissociation of the particles into smaller units, but never loses a certain opalescence even in the presence of concentrated sodium

hydroxide. In our experiments the agent retained its full activity in alkaline solutions of pH 11.4 for about one hour, but was immediately and completely inactivated at pH 12.

The ultraviolet absorbing power of the material isolated by high-speed centrifugation was determined by Dr. A. Rothen. The absorbing power of the solution for ultraviolet light was found to increase rapidly between λ 3000 and λ 2200, with indication of a maximum at $\lambda \backsimeq 2575$. The absorption curve, shown in Fig. 1, is very similar in shape to that previously obtained with active fractions prepared from chicken tumor I extract by a different

TABLE II: *Properties of a Fraction Prepared from Chicken Tumor Extract by High-Speed Centrifugation: Range of Precipitation in Acid Buffers*

pH	1.5	2.6	3.8	3.9	4.0	4.4	5.0	7.0
Diluted solution	−	+	+	±	−	−	−	−
Concentrated solution (0.04 per cent)	±	++++	++++	+++	++	++	+	−

method (7). Whether in both cases the same substance is responsible for the characteristic absorption curve is as yet undetermined. The material isolated by high-speed centrifugation shows an absorbing power for ultraviolet light much greater than that exhibited by the fraction prepared by adsorption and dialysis (7), suggesting that centrifugation was more effective in concentrating the absorbing elements.

For activity tests the purified material was taken up in a volume of Tyrode's solution equal to the volume of the original tumor extract. Increasing dilutions of both fractions were tested by inoculating 0.4 c.c. doses into adult Plymouth Rock hens. Results showed that the tumor-producing power of the material isolated by high-speed centrifugation was about 20 per cent that of the untreated extract. The absolute loss in tumor-producing activity may be partly accounted for by loss of substance during the preparation of the material. The tumor agent deteriorates rapidly *in vitro* and it is probable that part of the loss in tumor-producing power resulted from spontaneous inactivation. In terms of weight of solids in solution the tumor-producing power of the purified material was about ten times greater than that of the original extract.

Information regarding the nature of the substance separated must await results of the chemical analysis which is now under way. With the present method of preparation the amount of material separated from tumor extracts by high-speed centrifugation was 0.6 mg. per gm. of fresh tumor tissue.

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