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Experimental Study of the Effects of Various Extracts of Albino Rat Sarcoma upon Their Normal Subcutaneous Tissue

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EXPERIMENTAL STUDY OF THE EFFECTS OF VARIOUS EXTRACTS OF ALBINO RAT SARCOMA UPON THEIR NORMAL SUBCUTANEOUS TISSUE.

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OUTLINE

EXPERIMENTAL STUDY OF THE EFFECTS OF VARIOUS EXTRACTS OF ALBINO RAT SARCOMA UPON THEIR NORMAL SUBCUTANEOUS TISSUE.

dioxide that contain the activating agent. In the study of the chemical nature of this substance, they (3,4) found that it was split products of protein or proteose which showed such abundant and prolonged multiplication of fibroblasts. This type of proteose was also obtained from embryo juice, Witte's peptone rabbit brain, egg white, commercial fibrin and some purified proteins by peptic digestion.

Beside the study of normal cellular proliferation, numerable investigations were carried on neoplastic type of growth. In this train of thought, workers with fowl tumors have produced remarkable facts. Cell free extracts (5) were prepared which were just as potent in producing tumor as living tissue. In attempts to isolate the active principle from these preparations a considerable knowledge of the nature of this stimulating agent was obtained. Murphy and his co-workers (6,7,8), found that this activating agent was associated with a protein fraction. This protein fraction was purified in various ways, (7,8,9,10,11), first by dialysis, second by precipitation with gelatin and third by adsorbing agents such as kaolin, aluminum gel or charcoal. In chemical study of these purified extracts, Claude (12) found that the principle constituents of the active residue were protein and phospholipoids. These workers also demonstrated that the causative principle of the chicken sarcoma can be obtained in purified solutions potentially as

active regarding their powers of inducing tumor as the crude original extract.

Sittenfield and co-workers (13, 14) also working with a fowl sarcoma were interested in the activity of the causative agent in cell-free filtrate under varying pH. They found that by adjusting the filtrate to pH.4.0 a precipitate formed which on analysis was found to be much more active than the original filtrate. Such a precipitate was also obtained by any means which provided adequate acidity. Mineral or organic acids, buffered solutions, removal of cations by means of electrodialysis and bubbling through of carbon dioxide have been used with similar results. Repeated precipitations seem to enhance this substance. The hydrogen ion concentration in which the active agent retained its infectivity was between pH3 and pH9.

III MATERIALS AND METHODS

The tumor studied is a spontaneous neoplasm of the rat uterus kept alive by transplantation.

A. Preparation of the Materials

All of the extracts were prepared from sarcoma of white albino rats and were carried out under the most careful aseptic precautions. All the necrotic tissue was discarded and only the living tissue was used in preparing the extracts.

1. Autolysis

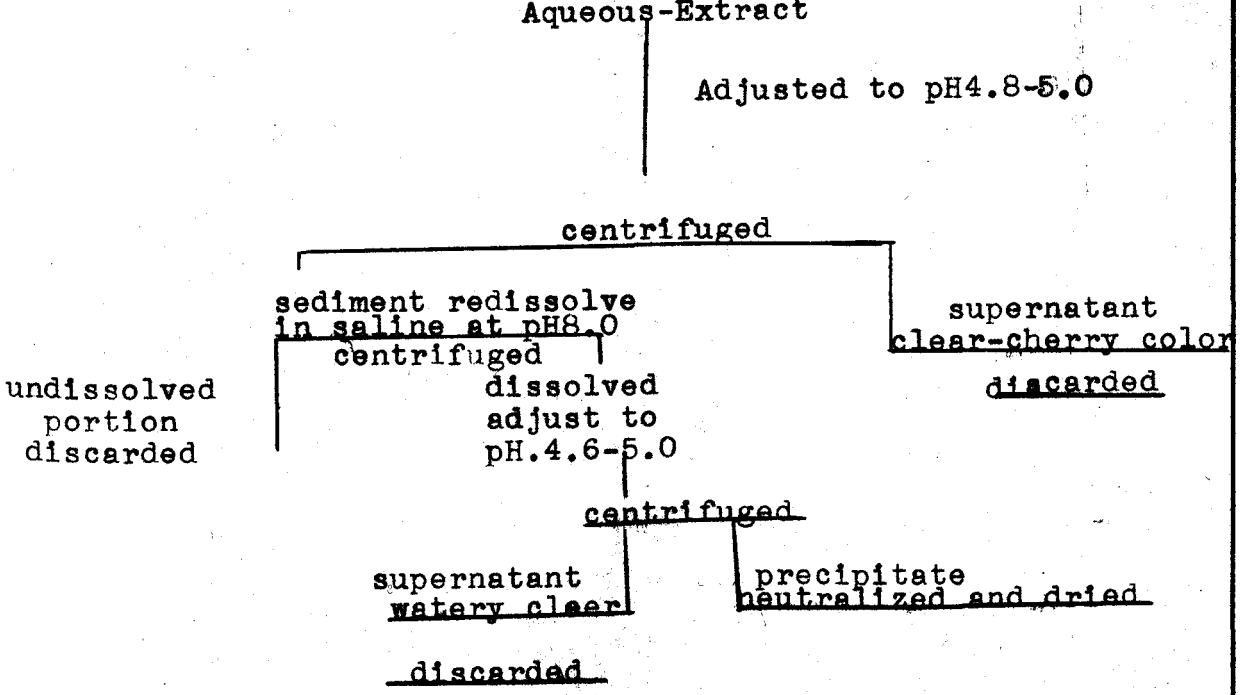
The rats bearing the neoplastic growth were etherized and bled to death. After rapid aseptic removal, the tumor was ground thoroughly in a sterile distilled water at room temperature until a colloidal suspension was obtained. This solution was adjusted to pH-4.5-5.0 by gradually adding drop by drop N/10 hydrochloric acid. The pH was determined by a quinhydrone potentiometer. To this solution toluol was now added to make a ten per cent by volume solution and placed in an incubator at 37° C. to digest.

After digesting for a definite length of time, it was removed from the incubator and centrifuged. The undigested material or the sediment was discarded while the supernatant was treated as follows: a portion of it was dried and tested while the other portion was subjected to the following treatment: to the solution 95% alcohol concentration was obtained, i.e. to each 1cc. of extract 18cc. of 95% alcohol was added. At this concentration a very fine flocculating precipitate was formed. This was removed by filtration and the precipitate was again redissolved in distilled water in similar manner. This doubly reprecipitated material was fanned dry at room temperature and tested. (Table II)

2. Iso-electric precipitation

All these experiments were performed at low temperatures. The tumor was removed in a similar manner as described above. The aequeous extract was placed in a refrigerator for one hour, then removed and centrifuged. The supernatant was now gradually subjected to increasing acidity until at pH-4.8-5.0 a definite precipitate was formed. This was removed by centrifugalization, redissolved in slightly alkaline solution and again treated in similar manner. The precipitate was neutralized and dried. The whole procedure is explained in the following table:

TABLE I



A. Removal of Lipoids

The lipoids were removed as follows:

The powder was covered with acetone for one hour. After the acetone was decanted, the dried powder was now covered with a mixture of two parts of ether and one part of absolute alcohol for one hour. This was decanted and the powder was fanned dry and tested.

B. Method of Bio-Assay

After an extensive search for a satisfactory procedure in which uniformity and reliability in testing the extracts could be accomplished, the following method was finally developed. The operations were performed with the greatest care in order to avoid as much injury to the normal subcutaneous tissue as possible. As a rule male albino rats weighing from 100-143 grams were used. The animals were etherized, the groin area shaven off and a deep subcutaneous pocket was formed in each groin. A weighed portion of the powdered material was placed in a sterile No. 5 gelatin capsule. After dipping the capsule in a 1/10 India ink solution, it was placed in one of the pockets. Into the other pocket, a control or an empty capsule dipped in 1/10 Indis ink was placed. After the operation the animal was returned to a clean cage with no change in environmental or dietary condition until the autopsy.

The animal was killed and the area of the tissue dyed with the ink was removed and fixed in Zenker's solution for twenty-four hours. Microscopic sections of these tissues which were eight microns in thickness and stained with Hematoxylin and Eosin dyes were studied.

Uniformity and reliability in this method was established by the fact that the tissue was subjected to the same size and about the same amount of material. Therefore all the tissue reactions were limited to an equal area and in these areas the type and the amount of cellular response was studied.

IV RESULTS

Many experiments were performed to determine at which stage the tissue response toward the extract was maximum. The duration of experiment varied from twenty-four hours to ninety-six hours. From these experiments it was concluded that the best cellular response was around the third day.

From Carrel and Baker's work, it is evident that the higher cleavage products of protein including proteoses definitely stimulate fibroblastic growth in vitro. With this fact in mind, a systematic search was employed to obtain a product of similar nature and determine their effect upon tissue growth in vivo. Autolysis of various duration were performed. 3½, 16, 24 and 48 hour autolysate products were prepared and their effects studied.

TABLE II

Effect of Autolytic Products:

Duration of Digestion	Dose	Duration of Experiment	Mitotic Count		Fibroblastic Proliferation
			Extract	Control	
3½ hrs.	40mg	72 hrs.	1	0	--
3½ hrs.	45mg	72½ hrs.	1	3	--
16 hrs.	40mg	72 hrs.	2	0	--
16 hrs.	35mg	72½ hrs.	1	1	--
16 hrs.	45mg	73 hrs.	0	1	--
16 hrs.	45mg	72 hrs.	1	0	--
24 hrs.	30mg	72½ hrs.	1	1	--
24 hrs.	40mg	73 hrs.	0	0	--
48 hrs.	40mg	71 hrs.	3	5	--
48 hrs.	35mg	72 hrs.	1	0	--

In removing the tissue for microscopic examination grossly, there was no evidence of growth. The groin tissue was stained black with dye. On microscopic examination nothing but normal groin or fat tissue with a few macrophages containing ink particles could be observed. Therefore, in view of the experimental results the higher cleavage products of autolysis have shown no reaction upon normal tissue.

Proteose Fraction:

Proteoses as described above from the autolysates were also studied. Results are tabulated in table three.

TABLE III

Proteose Fraction

Dose	Duration of Experiment	Mitotic Count		Fibroblastic Proliferation
		Extract	Control	
30mg	72½ hrs.	0	2	--
35mg	78 hrs.	0	1	--
40mg	72 hrs.	1	1	--
40mg	72 hrs.	0	0	--
45mg	73 hrs.	2	1	--
45mg	74 hrs.	1	0	--

From the above results it is evident that the proteose Fractions of the autolysates show no response from the groin tissue.

Iso-electric precipitates:

Since the autolysis failed to produce results the problem was now shifted to another angle. By iso-electric precipitation Sittenfield and co-workers (14) were able to separate the so-called causative agent from the original filtrate. This active principle came down with the precipitate formed at pH 4.0. With this fact in mind, a systematic search was instituted to study the effect of various pH values upon the acqeous extract of the rat sarcoma. The tumor extract subjected to increasing acidity with N/10 hydrochloric acid showed gradual development of opalescence and finally a precipitate separated when the reaction of the fluid attained pH.4.8.

After, the precipitate was separated by centrifugalization, it was neutralized and tested. The results are tabulated in table four.

TABLE IV

First Precipitates at pH.4.8-5.0 :

Dose	Duration of Experiment	Mitotic Count		Fibroblastic Proliferation
		Extract	Control	
45mg	72 hrs.	40	0	+ +
40mg	78 hrs.	46	0	+ +
40mg	78 hrs.	46	1	+ +
45mg	7 days	21	0	+(+-)

Grossly, definite growths could be seen which were rather whitish in color, firm in consistency, and irregular. A microscopic examination revealed many fibroblastic cells invadint the area which on closer study showed many mitosis. These types of growths remained in the animal for seven days after which they gradually disappeared.

With these encouraging results an attempt was now made to increase the potency of this preparation. Studies on growth factors by Mc. Junkin and co-workers (15) revealed that a lipoid fraction of tissue extracts inhibited cellular proliferation. Therefore, a lipoid extraction was now performed as described above, and the resulting material tested. Results are tabulated in table five.

TABLE V

Lipoid Free First Precipitates:

Dose	Duration of Experiment	Mitotic Count		Fibroblastic Growth
		Extract	Control	
40mg	72 hrs.	17	0	+
40mg	72 hrs.	16	0	+
45mg	72 hrs.	12	0	+
45mg	6 days	5	0	+

Gross and microscopic examination revealed some activity but in no way compared to the original.

In further attempt to increase the potency, a repre - cipitation was done. The precipitate obtained at pH.4.8. or pH5.0. was dissolved in slightly saline solution and again treated in the similar manner as described above. Results are tabulated in table six.

TABLE VI

Second Precipitate at pH.4.8-5.0

Dose	Duration of Experiment	Mitotic Count Extract	Fibroblastic Growth
45mg	48 hrs.	1	--
20mg	70½ hrs.	1	--
40mg	71 hrs.	4	--
45mg	72 hrs.	25	+ +
30mg	73½ hrs.	2	--
40mg	73 hrs.	2	--
45mg	148 hrs.	2	--

Only in one instance that this showed any sign of stimulation. However, subjecting it to lipoid extraction produced a completely different picture. Results are tabulated in table seven.

TABLE VII

Lipoid Free Second Precipitate at pH.4.8-5.0

Dose	Duration of Experiment	Mitotic Count Extract	Fibroblastic Proliferation
30mg	70½ hrs.	5	++
45mg	71 hrs.	41	+++
25mg	72 hrs.	11	+++
45 mg	72 hrs.	46	+++
45mg	72 hrs.	28	+++
40mg	72 hrs.	42	+++
45mg	72 hrs.	47	+++
40mg	74 hrs.	56 88	+++
40mg	78 hrs.	11	+++
30mg	148 hrs.	46	+++
45mg	7 days	growth	disappeared

Gross examination revealed large growths about the size of a navy bean, grayish in color; irregular and firm. Microscopic examination revealed a very fine fibroblastic reaction. This reaction was the most encouraging and convincing obtained during the course of investigation. The groin tissue was almost completely replaced with fibroblastic cells.

In the center of the tissue there were a few polymorphonuclear cells and macrophages containing ink particles but periphery, the whole tissue was just flourishing with fibroblasts. Closer study of these cells showed many mitosis of which a few appeared atypical.

However in allowing this preparation to remain in the animal for longer period it was found that about the seventh or eighth day the growth began to decrease and later disappeared completely.

In attempting to gather more knowledge concerning the nature of the substance, experiments to determine its reaction to heat were performed. The powder was dissolved in saline and placed in boiling water until completely dried. The results are tabulated in table eight.

TABLE VIII

Heated Lipoid Free Second Precipitates:

Dose	Duration of Experiment	Mitotic Count Extract	Fibroblastic Proliferation
45mg	71 hrs.	31	++
45mg	71½ hrs.	23	++
40mg	72 hrs.	35	++
45mg	78 hrs.	3	+-

The growth was smaller than the one obtained with the non-heated extract. On microscopic examination the fibroblastic proliferation was greatly diminished.

Although some activity was still present it did not compare favorably with the non-heated.

V. DISCUSSION OF RESULTS

Study of the fibroblastic proliferation obtained in this investigation did not reveal whether these growths were normal or neoplastic. The growths were carefully scrutinized for atypical mitotic figures. A few of the mitoses suggested abnormalities but however no definite conclusion could be made as to whether these signified a neoplastic property. The experimental results only indicate that there is some substance in these preparations which stimulates tissue reaction.

VI. CONCLUSIONS

Under the concitions of the experiments and within the limits of accuracy of the method, the following conclusions made:

1. Higher autolytic products including the proteose fraction do not produce any response from the subcutaneous tissue.
2. Aqueous extracts of rat sarcoma adjusted to pH.4.6 produce precipitates which temporarily stimulated fibroblastic growth. This stimulation lasted from seven to eight days.
3. Reprecipitation followed by lipoid extraction greatly increases its potency.
4. Heating destroys the activating principle to a slight degree.

VII BIBLIOGRAPHY

1. Carrel, A. and Ebeling, A. H.

On the permanent life of tissues outside of the organisms.

J. Exp. Med. 1612-15:516

2. Baker, L. E. and Carrel, A.

Action on fibroblasts of the Protein fraction of embryonic tissue extract.

J. Exp. Med. 1926-44:387

3. Baker, L. E. and Carrel, A.

Chemical nature of substance required for cell multiplication.

J. Exp. Med. 1926-44:508

4. Baker, L. E. and Carrel, A.

The Effect of Digest of Pure Protein on Cell Proliferation

J. Exp. Med. 1928-47:353

5. Rous, P.

A Sarcoma of the Fowl transmissible by an agent separable from tumor cells.

J. Exp. Med. 1911-13:397

6. Murphy, Jas. B.,Helmer, O. M. and Sturm, E.

Association of the causative agent of a chicken tumor with a protein fraction of the tumor filtrate.

Science 1928-68:18

7. Murphy, Jas. B., Helmer, O. M., Claude, A. and Sturm, E.
Observation concerning the causative agent of a chicken tumor.
Science 1931-72:266

8. Murphy, Jas. B. and Sturm, E.
Further observation on an inhibitor principle associated with the causative agent of a chicken tumor.
Science 1931-74:180

9. Murphy, Jas. B., Claude, A., Sturm, E. and Helmer, O. M.
Properties of the causative agent of chicken tumor.
Three Attempts at the Isolation of the active principle
J. Exp. Med. 1932-56:91

10. Claude, A. and Murphy, Jas. B.
Transmissible Tumors of the Fowl
Physiological Review-1933-13:246

11. Claude, A.
Properties of the Causative Agent of Chicken Tumor
Chemical Properties of the Tumor Filtrate.
J. Exp. Med. 1935-61:27

12. Claude, A.
Properties of the Causative Agent of a Chicken Tumor.
Chemical Composition of Purified Chicken Tunor Extracts
Containing the active principle.
J. Exp. Med. 1935-61:41

13. Sittenfield, M. J. and Johnson, B. A.
Concentration of the Causative Agent in the Filtrate of the Rous Chicken Sarcoma.
Proc. of Soc.
for
Exp. Biology and Med. 1630-31-28:206

14. Sittenfield, M. J., Johnson, B. A. and Jobling, J. W.
Demonstration of inhibitory substances in the filtrate of the Rous Chicken Sarcoma and their separation from the active agent.
Am. J. Cancer 1931-15:2275

15. Mc. Junkin, F. A. and Henry, J. W.
Antigrowth Effect of Lipoid Fractions of Tissue Extracts.
Am. J. Pathology 1935-11:353