17157. Effect of Testis Extract on Growth of a Transplanted Lymphosarcoma in AKm Mice.*

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(Introduced by C. P. Rhoads).

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Mammalian testis extract, as well as many bacteria, contains a factor which enhances the *in vivo* spread of dyes, infective organisms and toxins. This substance is accepted to be identical with the enzyme hyaluronidase.[1] Since the first description of the spreading factor by Duran-Reynals,[2] a number of workers have considered the possible relationship between this factor and the local and distant spread of malignant growths. Such investigations have sought to determine, 1) the actual content of spreading factor in tumors, as demonstrated by *in vivo* and *in vitro* methods, and 2) the behaviour of tumor transplants under the influence of the factor. Duran-Reynals and Stewart[3] demonstrated the spreading factor in some human carcinomas, but not in sarcomas. McCutcheon and Coman[4] also offered evidence of the spreading factor in human carcinomas. In a series of animal tumors Boyland and McClean[5] found a great variation in spreading activity, with the highest values in mouse and fowl sarcomas. The concentration of spreading factor in tumors never approached that of normal testis tissue. Hakanson and Glick[6] reported an elevation in the anti-hyaluronidase titer in sera from patients with malignant tumors. This increase was more marked in cases of metastatic than of non-metastatic tumors. Coman, McCutcheon and Zeidman[7] gave several injections of hyaluronidase in or around transplanted mouse Sarcoma 241, and Shope Papilloma, but observed no effect on the growth of the tumors.

When the malignant transplants were combined with testis extract prior to inoculation, Duran-Reynals[8] found that the growth of the Brown-Pearce carcinoma was retarded or prevented. In his discussion he favors the explanation that this phenomenon is due to the increased cellular and tissue permeability brought about by the spreading factor. He states, however, that this unitarian interpretation of the effects of testis extract is not positively established. Using a similar technic, Prime and Haagensen[9] could not demonstrate any effect on a transplanted rat sarcoma, a rat carcinoma or a guniea pig sarcoma. Hoffman, Parker and Walker[10] were able to show that rabbit testis extract enhanced the spread of the Chicken Tumor I, while Tanzer[11] found that testis extract inhibited the growth of mouse Sarcoma 180, but was without effect on Sarcoma 37 and the Bashford Adenocarcinoma. Tanzer suggests that the testis extract may either injure the cells of the tumor, or increase the resistance of the inoculated animal, and that the Duran-Reynals spreading factor may or may not, be identical with the factor acting upon tumors.

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1 Meyer, Karl, *Physiol. Rev.*, 1947, **27**, 335.

2 Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327.

3 Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15**, 2790.

4 McCutcheon, M., and Coman, D. R., *Cancer Res.*, 1947, **7**, 379.

5 Boyland, E., and McClean, D., *J. Path. and Bact.*, 1935, **41**, 553.

6 Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, 1948, **9**, 129.

7 Coman, D. R., McCutcheon, M., and Zeidman, I., *Cancer Res.*, 1947, **7**, 383.

8 Duran-Reynals, F., *J. Exp. Med.*, 1931, **54**, 493.

9 Prime, F., and Haagensen, C. D., *Am. J. Cancer*, 1934, **20**, 630.

10 Hoffman, D. C., Parker, F., and Walker, T., *Am. J. Path.*, 1931, **7**, 523.

11 Tanzer, R. C., *J. Exp. Med.*, 1932, **55**, 455.

It is apparent that the experimental data are not consistent, and do not permit general conclusions as to the significance of the spreading factor in the growth mechanism of malignant tumors. In the present work we have investigated the effect of various testis extracts on the growth of a transplanted mouse lymphosarcoma. We have also considered the relationship of the principle affecting the growth of tumors to the Duran-Reynals spreading factor, identified with the enzyme hyaluronidase.

Material and methods. The experimental work has been done with mice of the AKm stock. The tumor used was derived from the 9417 strain of lymphatic leukemia, described by Burchenal, Lester, Riley and Rhoads.[12] This leukemia is of spontaneous origin. According to Willis[13] we have used the term "lymphosarcoma" for the localized growth of the disease. The tumor was propagated by subcutaneous implants into the right side of the abdomen of the mice. A piece of tumor was minced with scissors in physiological saline solution, filtered through cotton, and the cell suspension diluted to contain 1 million cells in 0.1 ml.

In the experimental animals 0.1 ml (1 million cells) of the tumor suspension was mixed with 0.1 ml of testis extract and inoculated at once into the mice. The control animals were given 0.1 ml of the suspension of malignant cells mixed with 0.1 ml of physiological saline solution. In the control animals a tumor developed at the site of injection after 8-12 days. Successively it attained the size of 3-4 cm in diameter, infiltrated the abdominal musculature and peritoneum, became necrotic, hemorrhagic and finally ulcerated. The homolateral inguinal and axillary lymph nodes were aways involved. The disease usually spread to the internal organs before the animals died on an average of 30 days following the inoculation.

We have used 3 preparations of testis extract from AKm mice designated "crude extract", "granule fraction", and "supernate fraction". All of these represented a concentration of 50% in relation to the amount of testis tissue employed. The "crude extract" was prepared by grinding the testis tissue with equal amounts of distilled water and centrifuging once for 20 minutes at 600 x g. The resulting supernate constituted the "crude extract." The "granule fractions" were prepared by grinding testis tissue with equal amounts of either distilled water or 30% sucrose solution. The homogenates were centrifuged 3 times for 20 minutes at 600 x g and the sediments discarded. The supernates were then centrifuged 30 min. at 20,000 x g, and the sediments consisting of granules staining with Janus Green B, washed once or twice in either distilled water or 30% sucrose solution. After the final centrifugation at 20,000 x g, the sediments were taken up in distilled water, and the fractions designated "granule, water" and "granule, sucrose". Because of the observation of Hogeboom, Schneider and Pallade[14] that the large granules of the cytoplasm (mitochondria) were preserved best in 30% sucrose solution, we found it necessary to compare the activity of the "granules" prepared in distilled water with those prepared in sucrose solution. Since it was not considered desirable to use hypertonic sucrose solution for injection purposes, we used only distilled water for the final suspension. The "supernate fraction" represented the supernate from the first high speed centrifugation of a "granule fraction" prepared with distilled water. This supernate was purified by one or two more centrifugations at high speed.

In preliminary experiments we have investigated the content in the testis extracts of the factor, supposed to be hyaluronidase,

TABLE I.
Showing the Effects of Various Testis Extracts on the Intradermal Spread of India Ink.

Extracts	Spreading coefficient
Crude extract	21
Supernate fraction	21
Granule, water	3
Granule, sucrose	3
Control	1

[12] Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P., *Cancer*, 1948, **1**, 399.

[13] Willis, R. A., *Pathology of Tumours*, Mosby, 1948.

[14] Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.

TABLE II.
Showing the Growth of the Lymphosarcoma as Influenced by Testis Extract.

Inoculum	Total No.	No. with tumor	No. without tumor
Lymphosarcoma + testis extr.	99	55 (55.5%)	44 (44.5%)
Lymphosarcoma + physiol. saline	43	39 (91%)	4 (9%)

TABLE III.
Showing the Relative Effect of Different Testis Fractions on Growth of the Lymphosarcoma.

Inoculated with lymphosarcoma +	Total No.	No. with tumor	No. without tumor
Crude extr.	39	18 (46%)	21 (54%)
Granule fraction	32	19 (59%)	13 (41%)
Supernate "	28	18 (64%)	10 (36%)
Physiological saline	43	39 (91%)	4 (9%)

which affects the spread of India Ink injected intradermally. Testis extracts were combined with equal amounts of India Ink, diluted 1:5 with distilled water, and 0.4 ml of the mixtures injected intradermally on the shaved back of albino rabbits. India ink plus distilled water served as control. After 24 hours the longitudinal (D) and latitudinal (d) extent of the spread were measured and the area of spreading estimated according to the formula $\pi \frac{D \times d}{4}$.[15] The figure representing the spread of the extract, divided by that of the control, was designated the spreading coefficient.

Results. Table I shows the spread of the India Ink as influenced by the various fractions of testis extract.

As shown in Table I both the "crude extract" and the "supernate fraction" gave very marked spreading effects. On the other hand the "granule fraction" produced a spread of India Ink which was small and hardly significant. It is emphasized that the "granules" gave the same spread, whether they were prepared with distilled water or with 30% sucrose solution.

Tables II and III present the effect of testis extract on the transplanted lymphosarcoma in 99 mice. There were 43 controls. The whole group of animals given testis extract is presented without regard to the particular fractions of the extract in Table II, while Table III relates the effects of the inividual fractions.

In the control group 9% of the animals do not develop tumors, whereas, in the group treated with testis extract, 44.5% fail to take the transplant. This difference is statistically significant. Some of the test animals were killed when the controls died. On autopsy slightly enlarged subcutaneous lymph nodes were found on the same side as the injections. The spleens were small, and there were no macroscopical signs of metastasis to other inner organs. Most of the animals, however, were allowed to live 2 months or more after the inoculation, and appeared to remain in good health.

Table III indicates the relative effect of the "granule" and "supernate" fractions of testis extract on the growth of the lymphosarcoma.

The "crude extract" showed slightly greater activity than the more purified fractions. Interestingly the "granule" and "supernate" fractions seemed to have practically the same activity, with a slight difference in favor of the "granule fraction". The "granules" showed the same effect whether they were prepared with 30% sucrose or distilled water.

If the principle affecting the growth of this lymphosarcoma was identical with the spreading factor (hyaluronidase), we would expect to demonstrate a significant difference between the activity of the "granule" and "supernate" fractions, a difference comparable to that demonstrated in the spreading coefficients (Table I). As shown there the spreading coefficient

[15] Madinaveitia, J., *Biochem. J.*, 1938, **32**, 1806.

of the "supernate" is 7 times that of the "granule fraction". In contrast the tumor influencing activity in the "granule fraction" is slightly higher than that of the "supernate fraction". (Table III). These findings indicate that the "lymphosarcoma factor" is not identical with the spreading factor (hyaluronidase).

Summary. A factor has been demonstrated in testis extract which inhibits the growth of a transplanted lymphosarcoma in AKm mice.

This factor is of unknown nature, but appears to be different from the Duran-Reynals spreading factor.

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17158. Effects of Urethane on Living Tissue.*

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Ethyl Carbamate (urethane) has been shown to be a useful agent in the treatment of wound infections.[1,2] When extensive wounds or large areas of intact skin are exposed to the prolonged application of 10% urethane solution[2] about 20 to 30% of the patients treated develop nausea and vomiting. This suggests that the drug is absorbed through open wounds and normal skin surfaces. The nausea and vomiting reported by Howe and Weinstein,[2] and Howe[1] in urethane treated wound infections and by Paterson *et al.* in leukemia patients receiving the drug by mouth[3] appeared to be benign in nature. However, because the treatment of human wound infections requires prolonged exposure to large amounts of this drug, experiments were conducted to investigate its effect on the organs of animals treated with similar doses.

A group of 10 guinea pigs was given lethal amounts of urethane administered intraperitoneally in rapidly ascending doses over a short period of time (Table I, Pigs 1-10). The gross and histological tissue changes were compared with those of a second group of 10 animals receiving daily subcutaneous injections of urethane in dosages simulating those used in the topical treatment of infected wounds in man (Table II, Pigs 14-23). Hypnotic effects ranging from drowsiness and ataxia to terminal anesthesia were noted. Some animals recovered and ate heartily after 8 hours of complete anesthesia. In the treatment of a large infected wound a person is exposed to a maximum of 60 g of urethane per day, although only a fraction of this is absorbed due to loss in dressings. The animals in Group II received 0.1 g per 100 grams of body weight per day for 28 consecutive days which is about twice as long as the average duration of therapy in 39 urethane treated patients.[2] This is comparable to the maximal dosage used in man, and it was given subcutaneously to insure complete absorption. Dosages were corrected weekly for fluctuations in weight. Control animals (Table I, Pigs 11-13; Table II, Pigs 24-26) received corresponding amounts by volume of normal saline. Diet consisted of Purina chow, lettuce and cabbage. All animals in Group II gained weight and at the end of the experiment were healthy and had lustrous silky coats. They were killed on the 29th day.

Examination of the organs was made immediately after death. Sections were fixed in 5% formaldehyde solution, imbedded in paraffin and stained with hematoxylin eosin. Brain, striated muscle, myocardium, lungs,

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1 Howe, C. W., *Surg., Gynec. and Obst.*, 1948, **87**, 425.

2 Howe, C. W., and Weinstein, L., *Surg., Gynec. and Obst.*, 1947, **84**, 913.

3 Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **1**, 677.