ON THE BEHAVIOR OF THE ROUS TUMOR VIRUS TO FREEZING

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In the twenty-three years that have elapsed since the announcement by Rous of his discovery of a fowl sarcoma transmissible by a presumably cell-free filtrate, the exact nature of the causative agent is still undetermined. Of the three principal possibilities, that of filter-passing tumor cells would seem to be excluded rather definitely. But whether we are dealing with a living agent, or with a product of metabolism apparently of enzymatic character capable of inciting the changes which in turn produce it, is still open to question. The production by Fischer of a tumor of this type, by the prolonged treatment of cultures of fowl fibroblasts with weak solutions of arsenic oxide, constitutes strong evidence in favor of the latter view. The principal evidence of contrary character lies in the work of Nakahara, who found that repeated freezing and thawing of the tumor cells greatly impaired the transmissive effect of the agent. His work has been questioned, however, on the ground of neglect to guard against oxidation, to which the agent is peculiarly susceptible.

Partly for the purpose of checking this work of Nakahara, and partly to determine the limits of the possibly lethal effect on the agent of repeated extreme temperature changes, the work reported here was instituted. In it the agent, as well as suspensions of tumor cells, were subjected to repeated freezing by means of carbon dioxide ice, and to rapid thawing, both in practically complete absence of oxygen.

The procedure was relatively simple. The virus, after filtration in physiological salt solution through a sterile "W" Berkefeld filter, was placed in glass ampoules with slender, drawn-out necks. The air was then exhausted by means of a strong water pump, and replaced by pure hydrogen. This procedure was repeated ten times, and the ampoules were finally sealed in the exhausted condition. They were then frozen a variable number of times by means of "dry ice" kept in a large Dewar jar, and after freezing were rapidly thawed by immersion in boiling water exactly to the point of liquefaction of the virus. For comparison, various bacterial suspensions were similarly treated, except as to filtration, at the same time.

The tumor material was obtained from Plymouth Rock chickens originally inoculated with tumor desiccate, and this was transplanted by cell and filtrate inoculation into successive fowls until it was ascertained that the tumor was in the state of cell-free transmissibility. The tumors so obtained were ground in a meat-grinder, and then by hand with the addition of powdered carborundum. The suspension, diluted ten times with physiological salt solution, was centrifuged for
thirty minutes at 2000 revolutions per minute, and the supernatant fluid filtered by pressure through the Berkefeld filter. Subsequent treatment has already been described. Along with the filtrate, which was used uniformly in 1 c.c. doses, sterile kieselguhr was injected. Control injections consisted of fresh tumor cells and of unfrozen filtrate with kieselguhr. All fowls used were of Plymouth Rock breed.

In the first series of injections, 6 fowls were given filtrate frozen and thawed five times. Only one tumor resulted, a 40 gm. mass which grew inwardly, and which was detected only when the fowls were killed six months after injection. Of two fowls injected with tumor cells, both yielded tumors, of 8 and 11 gm. respectively, in one month. None was obtained in two animals injected with unfrozen filtrate.

In the second series, filtrate frozen and thawed twenty times was used. One of the 4 injected fowls developed a tumor, which, 110 days after injection, weighed 16 gm. One of two fowls injected with fresh cells showed a 10 gm. tumor after thirty-three days, the other remaining negative after six months. No tumors were found in the two fowls which received untreated fresh filtrate, after six months.

For the third series filtrate obtained from the positive case of series 1, frozen and thawed twenty times, was used. Three of 7 fowls developed tumors, one of which weighed 15 gm. in forty-five days and another 25 gm. after seventy-four days. The third fowl died four months after injection, and autopsy revealed multiple tumor nodules in the liver, while the site of injection showed no involvement. Both fowls which received fresh cells developed tumors, one in six weeks and the other after four and a half months. Four fowls injected with fresh, untreated filtrate developed tumors: one a 25 gm. mass after seventy-nine days, the others weighing 10, 19, and 27 gm. after four and
a half months. Two animals injected with frozen and thawed suspensions of tumor cells, treated as were the filtrates, gave no tumors in six months.

For the fourth series, filtrate was used from one of the tumors obtained from filtrate frozen and thawed twenty times. It was similarly treated sixty times, and of 3 fowls injected with this which survived six months, all had small tumors at the site of injection, the total mass of which was insufficient to give material for further injections. Of two fowls injected with fresh cells, one had a 12 gm. tumor after three months. One positive result was likewise obtained with unfrozen filtrate, a 5 gm. tumor after three months.

![Image](Fig. 2. Rous Sarcoma Produced by a Filtrate Frozen Sixty Times, Showing Invasion of Pectoral Muscle. Low-power Magnification)

Inspection of the results of fresh cell injections shows little change in the growth rates of the several series of injections; if anything, there was a slight impairment of growth rate in the successive series. To the extent that potency of the untreated filtrate can be estimated from the few control fowls injected, this was at its height in the third series. No inference can be drawn as to the potency of the frozen filtrate, except that there was a very considerable diminution after sixty freezings and thawings. To this the possible lessened potency of the untreated agent, as evidenced by the unfrozen filtrate controls, may have contributed. The fact remains that, even with filtrate subjected to this treatment, positive results were obtained.

The morphology of the resultant tumors was, except in the last series, that of the typical Rous tumor (Fig. 1). Tumors obtained with the filtrate after sixty freezings and thawings showed marked alteration from this (Fig. 2). These tumors showed cells principally of round form, with less regularity of size and shape. There was also a conspicuous lack of encapsulation, more pronounced than with the
other tumors, with diffuse infiltrative growth into the muscle, accompanied by the formation of numerous giant cells. The general appearance was that of enhanced malignancy, which was not supported by the clinical findings.

The bacterial suspensions, frozen and thawed like the filtrate, were tested for viability by inoculation on agar slants. Twenty freezings and thawings were found sufficient to kill *Staphylococcus aureus* and *B. coli*. Sixty caused the complete destruction of spores of *B. subtilis*.

**Summary and Conclusions**

The filtrate of the Rous tumor was found to maintain its tumor-producing power after rapid freezing and thawing for sixty times, when these were so conducted as to avoid accompanying oxidation. There was no apparent change in its action, as determined by rate of tumor growth or by tumor morphology, when this procedure was repeated twenty times. With sixty freezings, there were delay in tumor development and marked change in tumor morphology.

The filtrable agent of the Rous tumor displays a resistance to freezing and thawing greater than that exhibited by known living agents, as bacteria or other cellular organisms. To the extent that this throws light on its nature, it would suggest an unorganized character. However, the possibility that the filtrate may contain organized bodies so minute as to escape the effect of sudden and repeated changes of volume cannot be absolutely precluded.

**References**


NAKAIHARA, W., AND YAOI, H.: Non-enzymatic nature of the entity transmitting chicken sarcoma, Gann 24: 318, 1930.

RIVERS, T. M. (Ed.): Filterable Viruses, Baltimore, Williams and Wilkins, 1928.


1 For comparison in this respect with the disease-producing viruses, the findings of Rivers may be cited, although they are not strictly comparable. Using liquid air and tap water, he found that there was considerable variation in the resistance of different viruses. Vaccine virus, the most resistant, was inactivated by thirty-five freezings and thawings, but only when in high dilution.