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Isolation of Toxohormone from Tumor Tissues of Germfree Mice.* (31419)

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Investigators in cancer research are generally careful about the state of the tumor tissues they use for chemical studies. Adherent normal tissues and necrotic areas in the tumor are carefully trimmed away, and it is tacitly understood that any grossly infected tumors are not to be used at all. On the other hand there is always a possibility that some microbial contamination could escape notice, and so introduce extraneous substances into tumor tissue components. A confusing development became apparent in studies on toxohormone, the tumor cell product which is responsible for the depression of liver catalase in tumor-bearing animals(1), when Kampschmidt(2) claimed that tumor toxohormone comes not from the tumor tissue itself but from contaminating bacteria. The tumor used by Kampschmidt was very heavily infected with *Salmonella typhimurium,* and *Salmonella* endotoxin was found to be highly active in depressing liver catalase activity when injected into normal animals.

In making this sweeping statement Kampschmidt ignored all the relevant experimental data to the contrary(1), and our recent study on the relation of bacterial contamination to tumor toxohormone showed anew that Kampschmidt's allegation is unfounded(3). The experiment presented in this paper should clear away any possible doubt in the matter. We have found that a tumor, produced and maintained in germfree mice, is as productive of toxohormone as other tumors derived from conventional, non-germfree mice.

Material. Germfree tumor tissue was collected from 7 germfree Lobund C3H mice carrying transplants of fibrosarcoma tissue which was originally induced by injections of a sterile solution of 20-methylcholanthrene in olive oil(4). The germfree mice and their tumors were free of bacterial flora, as determined by test procedures described by Wagner(5). Individual tumors weighed 5 to 10 g, wet weight. A pool of 41.4 g of tumor tissue was stored in the freezer until dried at 70°C in the vacuum oven. The desiccated tumor tissue was shipped from Notre Dame to Tokyo for further examination.

Methods. Procedures for extraction of toxohormone and its bioassay were carried out at the National Cancer Center Research Institute. 7.04 g of desiccated tumor was reconstituted in 200 ml of water, and homogenized with a Waring blendor. The homogenate was centrifuged and the supernatant fluid was boiled for 2 hours in a water bath. It was condensed to about 1/10 original volume, and centrifuged again to remove precipitated material. Two volumes of absolute ethanol were then added to the clear supernatant fluid. The precipitate thereby formed was collected by centrifugation, washed with ethanol and then with ether. This is the original toxohormone fraction of Nakahara and Fukuoka (1). The yield of the toxohormone fraction was 650 mg (9.25% of the original dried material), which is approximately the yield to be expected from any other tumor tissue.

The toxohormone activity of the preparation from germfree tumor tissues was tested by injecting it intraperitoneally into adult mice of ddN strain, about 20 g in body weight, and determining the liver catalase activity of the mice at 24 hours after the

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injection. The mice were killed by decapitation and exsanguination. The liver was removed at once and weighed. The whole liver was homogenized with 99 volumes of ice cold deionized water in a Waring blendor, and 1 ml of the homogenate, diluted with 4 ml of deionized water (equivalent to 0.2% liver homogenate), was used as the enzyme solution. Substrate was prepared by diluting 5.4 ml of 3% H_2O_2 to 1 liter of deionized water with enough $Na_2HPO_4 \cdot 2H_2O$ and KH_2PO_4 to adjust the reaction to pH 7.0. Titanyl sulfate solution was prepared by diluting a saturated solution of it in 2 N Na_2SO_4 to 5 times its volume with the same solvent.

In the determination of catalase activity, 0.5 ml of the enzyme solution was added to 24.5 ml of the H_2O_2 solution, the mixture was stirred vigorously in an ice bath for 1 minute, and then 1 ml of the reaction mixture was pipetted out and mixed immediately and thoroughly with 4 ml of the titanyl sulfate solution. After 30 minutes to 1 hour the yellowish color which developed was measured with a Leitz photometer at 415 mμ. For blank control 0.5 ml of deionized water was used in place of the enzyme solution under otherwise identical test procedures.

The reaction constant k of the catalase activity was calculated from the equation,

$$k = \frac{1}{t} \log \frac{a}{a-x}$$

where t, a, and x represent time, the initial concentration of substrate, and the amount of the substrate decrease per unit time, respectively.

TABLE I. Effect of Germfree Tumor Toxohormone on Liver Catalase Activity of Normal Mice.

Toxohormone injected (mg/mouse)	No. of mice	Catalase activity (k/100 mg N) Lowest	Highest	Mean
0	10	154	219	172 ± 19
34	5	133	145	140 ± 5
80	6	66	122	98 ± 22

The results of the assay are expressed as catalase activity in the term of k/100 mg N (Table I).

Correct method for the isolation of toxohormone is essential, although any of the usual methods can be safely used for catalase determination.

Summary. We have given a detailed description of the methods for extraction and bioassay of toxohormone. Tumor tissues from conventional and from germfree mice produce toxohormone in abundance.

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