Table 3. Animals and tissue cultures used in attempts at transmission of kuru.

Primates
Chimpanzee
Gibbon
Black
Golden
Macaques
Rhesus*
Cynomolgus*
Barbary ape
Stump tail
African green monkey
Patas monkey
Spider monkey
Black
Brown
Squirrel monkey
Capuchin monkey
Woolly monkey
Marmoset, white-lipped
Tree shrew
Slow loris
Other mammals
Sheep
Cheviot
Suffolk
Goats
mixed breeds
Pigs
Chester whites
Dorchester
Mice*, 17 inbred breeds
Rats*
Rabbits
Guinea pigs
Hamsters*
Golden Syrian
Avian
Chickens, white leghorns
Embryonated eggs
Day-old chicks
Turkeys
Noryork
Ducks
Long Island
Geese
Tissue cultures
Human embryo kidney
Fetal human embryo brain
BSC-1
WI-26
WI-38
Detroit-6
Hep-2
Primary green monkey kidney

* Newborn as well as immature young inoculated.

brain tissue from other neurological diseases, the close similarity of the disease in both its clinical and pathological features to kuru in man, and the appearance of the disease in seven of eight chimpanzees inoculated with kuru brain material after similarly long incubation periods lead us to believe that we have transmitted kuru to the chimpanzee. The appearance of essentially the same clinical syndrome in two of three chimpanzees inoculated with brain tissue from a killed, "first-passage" animal, and the appearance of the same neuropathological lesions in the third lost from intercurrent infection lead us to believe that passage of the disease from chimpanzee to chimpanzee has succeeded, with a significant reduction in the incubation period.

If the transmissibility and serial transmission in chimpanzees can be confirmed and the filterability of the agent demonstrated, kuru will be the first chronic neurological degenerative disorder of man of demonstrated virus etiology, and the first such disease transmitted to a laboratory animal. The importance of this to the study of neurological degenerative disease and equally to the elucidation of the concept of slow virus infection, needs no further emphasis.

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Malignant Transformation in vitro by Carcinogenic Hydrocarbons

Abstract. *Pieces of ventral prostate from adult C3H mice were cultivated in organ culture for 3 weeks. One group served as a control; another was treated for 1 week with methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene and for 2 additional weeks in normal medium. The pieces were pooled and dispersed with pronase into individual cells that were plated as cell cultures. The control cultures invariably died. The treated cells formed permanent lines that, on subcutaneous injection of from 1 to 2 × 10^6 cells into adult, unconditioned, male C3H mice, produced progressively growing, transplantable tumors. The tumors were mostly sarcomas, but included two anaplastic carcinomas. Malignant transformation in vitro has thus been achieved with carcinogenic hydrocarbons in this system.*

This laboratory has long been concerned with the biologic and molecular mechanisms whereby carcinogenic hydrocarbons initiate malignancy (see *1*). Studied primarily were the interactions of labeled hydrocarbons with constituents of mouse skin in vivo. It appeared, however, that production of carcinogenesis with hydrocarbons in vitro was required for answering a number of critical questions.

Transformation in vitro by oncogenic viruses is a well-established phenomenon (*2*); the "spontaneous" transformation of embryonic mouse fibroblasts in long-term cultures has been reviewed (*3*). Berwald and Sachs (*4*) have reported the malignant transformation in vitro, with carcinogenic hydrocarbons, of mixed embryonic cells from mice and golden hamsters; Borek and Sachs have obtained the same results by x-irradiation (*5*). Transformation and chromosomal abnormalities in a highly selected, permanent, hamster cell line have been produced with carcinogenic hydrocarbons (*6*).

In order to correlate our research in vivo with the system in vitro, it was necessary to use the mouse, although mouse-embryo fibroblasts undergo a high frequency of spontaneous transformation on prolonged cultivation (*3*). It then seemed that cells derived from adult tissues might have a lower frequency of spontaneous transformation, and this fact has now been demonstrated (*7*). We chose Lasnitzki's system (see *8*), in which small pieces of young-adult-mouse prostate are maintained in organ culture for 2 to 3 weeks. Pieces cultivated in normal medium remained histologically differentiated, but pieces maintained in medium containing hydrocarbon exhibited in their epithelial cells massive hyperplasia, squamous metaplasia, and anaplasia, including pleomorphism, multipolar mitoses, and occasional invasion through the basement membrane.

Thus the histological changes suggested that carcinogenesis may have occurred in this system in vitro. Nevertheless, for 3½ years no tumors were produced when hydrocarbon-treated organ cultures from C3H mice were

implanted into hundreds of C3H mice under various conditions (*9*), so the cells of these treated prostates did not meet the essential biologic criterion of malignancy—production of tumors on implantation into inbred mice of the same strain. It appeared that this failure may have been caused by the inoculation of too-few malignant cells (if, indeed, any were present in the organ-culture pieces). It was therefore decided to disperse the pieces of prostate after maintenance in organ culture and to grow the resulting cells in culture in the hope of selection of the more–rapidly growing transformed cells. The following experiment is typical of many.

Ventral prostates from C3H mice, 6 to 8 weeks old, were carefully dissected and cut into four 1-mm^3 pieces. Four pieces from a random pool were cultivated at 37°C in each dish (Falcon plastic, 35-mm) on a rayon strip placed on a stainless-steel grid so that the medium soaked the bottom of the pieces (*9*). The gas phase was a mixture of air, oxygen, and carbon dioxide (50 : 45 : 5) saturated with water, and Eagle minimal essential medium (*10*), plus 10 percent horse serum, penicillin, and streptomycin, was used. Eight dishes served as controls, and eight were cultured in the same medium with the addition of 5 μg/ml of methylcholanthrene. The methylcholanthrene was dissolved in acetone (0.2 mg/ml) and added rapidly to the medium, and the acetone was removed by a current of air. The protein in the medium stabilizes the solution of the hydrocarbon. The media were changed twice weekly. After 1 week, the hydrocarbon-treated cultures were washed repeatedly with and transferred to normal medium. After 3 weeks of organ culture, the pieces from one dish of the control and one dish of the treated prostates were fixed for histologic examination.

The remaining pieces from each group were carefully removed from the rayon strips, rinsed in Hanks balanced salt solution, combined, and incubated for 15 minutes at 37°C in a shaking water bath in Hanks solution with 1 mg/ml of pronase (grade B; CalBiochem). The cells in the pieces were dispersed by pipetting 20 times with a 2-ml Pasteur pipette, an equal volume of Eagle basal medium (*10*), plus 20 percent fetal-calf serum, was added, and the resultant suspension was mixed and passed through a sieve of 200-mesh bolting silk and centrifuged; the pellet was suspended in the above-mentioned medium after an additional centrifugation and washing. The resultant cells were suspended, and counted in a hemocytometer, and 2 × 10^5 cells were plated in 2 ml in each 35-mm plastic dish; three dishes were obtained from each group. The cells were incubated at 37°C in a humidified incubator, the gas phase being a mixture of air and carbon dioxide (95 : 5).

Table 1. Tumors induced in mice by subcutaneous inoculation of from 1 to 2 × 10^6 cells from cultures transformed in vitro by one of two hydrocarbons. The tumors generally appeared within 3 to 5 weeks and grew progressively. Numbers of tumors appear in parentheses.

Days in culture	Tumors
Methylcholanthrene	
94–222	Fibrosarcomas (8)
122, 143	Anaplastic carcinomas (2)
143	Reticulum cell sarcoma (1)
115	Fibroliposarcoma (1)
122	Malignant histiocytoma (1)
94, 115	Atypical fibroplasias (2)
9, 10-Dimethyl-1, 2-benzanthracene	
137–148	Fibrosarcomas (4)
137	Rhabdomyosarcoma (1)

After 1 day there were much debris and many dead cells in both groups, and the media were replaced with Eagle basal medium (made with Earle salt solution; GIBCO) plus 20 percent fetal-calf serum. The medium was changed every 2 days, and the dishes were examined with an inverted phase-contrast microscope (Zeiss). At first the cells from the controls were attached better than those from the treated cultures. Eight days after plating, foci of small refractile cells were seen in the cultures treated with methylcholanthrene, but usually not in the controls. The foci grew slowly into colonies, while the other cells detached, and at 5 weeks a subculture was made by combining the cells from the three dishes of the primary culture into a single dish, although a confluent monolayer had not yet been obtained; the medium was Eagle basal plus 10 percent fetal-calf serum.

On the 1st day after this initial subculture, groups of attached cells were observed growing progressively to form a monolayer. The doubling time of the first subculture was 6 days; of the second subculture, 4 days; of the third, 3½ days; and of the fourth, 3 days. On the fifth subculture, a larger inoculum of cells was used to attain a doubling time of 2 days, which time later decreased to about 1 day; this line has grown continuously for more than 10 months. Results were comparable in two additional successive experiments with methylcholanthrene (1 μg/ml in 0.5-percent dimethyl sulfoxide) and in three experiments in which 9,10-dimethyl-1,2-benzanthracene (DMBA) was added during the first week of organ culture at concentrations of 0.01 and 0.05 μg/ml in 0.5-percent dimethyl sulfoxide. Thus, permanent cell lines have been obtained, in all of six attempts, by dispersing organ cultures of mouse prostate that had been treated for the 1st week with methylcholanthrene or DMBA.

By contrast, 25 successive experiments, in which the organ cultures from the control groups that had not been treated with carcinogenic hydrocarbons were dispersed and plated for cell culture, yielded no permanent line of cells. In the primary cell cultures, the cells detached over a period of 2 to 4 weeks, and usually one could not make a subculture. In some of the experiments the usual Eagle basal medium, plus 20 percent fetal-calf serum, was supplemented with 2 percent chick-embryo extract. The fact that none of the 25 control cultures formed permanent cell lines demonstrates that under these conditions no spontaneous transformation occurred. However, the fact that permanent cell lines were established from all the carcinogen-treated cultures demonstrates that the hydrocarbons produced a transformation.

In the early stages of the cell cultures derived from the carcinogen-treated organ cultures, the cells exhibited epithelial-like morphology (Fig. 1A). However, these cell lines were not cloned; some of the lines later became fibroblast-like in appearance, while others retained their epithelial morphology.

It is generally considered that normal cells in culture remain as monolayers, whereas transformed cells lose contact inhibition and form multilayered cultures. This piling up of cells, with random orientation, was the main criterion of malignancy used by Sachs and his group (*4*, *5*). When the dishes were allowed to become crowded, our cells from the carcinogen-treated cultures also piled up and formed a multilayered, criss-crossed appearance (Fig. 1B).

It is now generally agreed that normal rodent cells grown in culture are very susceptible to the toxicity of carcinogenic hydrocarbons, whereas transformed cells are resistant (*11*, *12*). The

growth rate of our transformed cells was not significantly inhibited by methylcholanthrene or DMBA at 5 μg/ml, a concentration that kills almost all normal cells (see *12*). These cells are also rich in acid phosphatase, as determined histochemically (*13*), a condition characteristic of prostate epithelial cells (*14*). Multipolar mitoses, which are frequently considered to be characteristic of malignancy, are often seen in our cultures.

In order to determine whether these transformed cells were malignant, 1 to 2×10^6 cells from cultures were injected subcutaneously into unconditioned, adult, male C3H mice. A number of progressively growing tumors were produced; those obtained with three of the cell lines are listed in Table 1. Malignant tumors were obtained in 90 percent of the mice inoculated; five were transplanted into male C3H mice and all grew. Cell cultures, established from the first two tumors that were tried, grew continuously and gave tumors when mice were inoculated; some of the mice died from their tumors. Of the first 20 tumors, 18 were sarcomas (Fig. 1C) and two were anaplastic carcinomas (Fig. 1D).

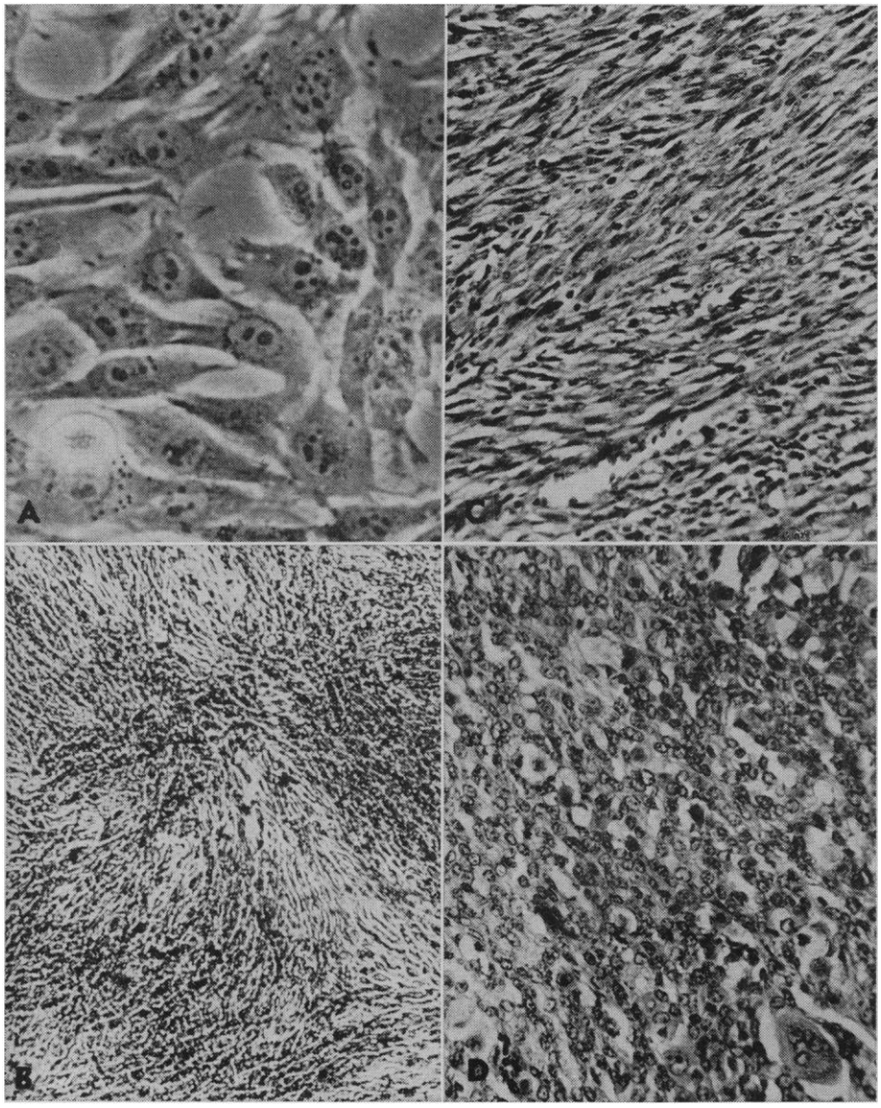

Fig. 1. (A) Epithelial-like cells from organ culture treated with methylcholanthrene, after 153 days in cell culture (phase contrast; × 620). (B) Culture treated with methylcholanthrene, after 113 days in cell culture (fixed in methanol and stained with Giemsa; × 240). (C) Fibrosarcoma induced in a male C3H mouse by implantation of 1×10^6 cells transformed in vitro with methylcholanthrene (fixed in formalin and stained with hematoxylin and eosin; × 210). (D) Anaplastic carcinoma induced in a male C3H mouse by implantation of 1×10^6 cells transformed in vitro with methylcholanthrene (fixed in formalin and stained with hematoxylin and eosin; × 210).

The following criteria establish that malignant transformation has been produced in vitro from primary cultures of mouse prostate by carcinogenic hydrocarbons in this system: (i) the carcinogen-treated cultures all form permanent lines, whereas cells from the control cultures eventually die; it is considered (*15*) that normal diploid cells can undergo only a limited number of cell divisions; (ii) the treated cells pile up and grow as randomly oriented multilayers; (iii) the treated cells resist the toxicity of carcinogenic hydrocarbons; (iv) aneuploid cells and multipolar mitoses are seen in the cell cultures; (v) the fact that the treated cells are rich in acid phosphatase confirms their prostatic origin; and (vi) inoculation of the cells into adult, unconditioned mice produces malignant, transplantable tumors.

In order to investigate the possibility that in this system the carcinogens activated a latent oncogenic virus, cell-free extracts were made from the treated organ and cell cultures. These extracts produced no cytopathic effect when added to cultures of mouse-embryo cells, HeLa cells, or adult-mouse prostate cells. Moreover, the serums of normal C3H mice and of C3H mice bearing tumors induced by the cultured cells were examined for polyoma, Rauscher, and Maloney virus antibodies and were found to be negative.

Interpretation of the results of our experiments remains highly speculative. It seems clear that we have demonstrated malignant transformation of cultures of mouse prostate cells in vitro with carcinogenic hydrocarbons. The question of whether this is a single transformation process, or whether the initial transformation that produced a permanent line of cells (which has never occurred in the controls) merely allowed a second and spontaneous transformation to malignancy, is now of particular concern. The facts that adult cells were used (*7*) and that fetal-calf serum was present in the medium suggest that the latter alternative may not have occurred during these experiments, since it has been demonstrated that fetal-calf serum prevents or delays spontaneous transformation of mouse-embryo cells in culture.

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Tryptophan Hydroxylation: Measurement in Pineal Gland, Brainstem, and Carcinoid Tumor

Abstract. *Development of a rapid and sensitive radioassay has permitted study of the conversion of tryptophan to 5-hydroxytryptophan in mammalian tissues. Of normal tissues examined, beef and rat pineal gland contained the highest activity. This is the first direct demonstration of tryptophan hydroxylase in this hydroxyindole-rich tissue. Rat and rabbit brainstem and human carcinoid tumor also had quantities of enzyme that could be measured easily. The reaction requires a reduced pteridine and oxygen and is inhibited by para-chlorophenylalanine.*

It is generally accepted that hydroxylation of tryptophan at the five position is the first and presumably rate-limiting step in 5-hydroxytryptamine (serotonin) biosynthesis. Although there have been reports (*1–4*) on the detection of tryptophan hydroxylase activity in mammalian tissues in vitro, study of this enzyme has been elusive, owing to low activity and the lack of a sensitive and specific assay. We reported (*5*) that a malignant mast cell tumor of the mouse is rich in tryptophan hydroxylating activity, and this enzyme source has been used to develop a radioassay. The assay, together with the finding that enzyme activity is enhanced in the presence of the reducing agent, 2-mercaptoethanol (*6*), has permitted detection of tryptophan hydroxylation in several mammalian tissues and assay of enzyme activity in certain serotonin-rich tissues including pineal gland, brainstem, and carcinoid tumor.

The major steps and principles of the assay are as follows. (i) L-Tryptophan-2-^{14}C is incubated with enzyme, a monoamine oxidase inhibitor, and necessary components of the reaction mixture in the presence of a known amount of 5-hydroxytryptophan (5HTP). The latter serves as an inhibitor of tryptophan decarboxylation (*7*) as well as a trap for the newly formed 5HTP. (ii) After the reaction has proceeded, a portion of the 5HTP is enzymatically decarboxylated with partially purified aromatic L-amino acid decarboxylase (*8*). (iii) The serotonin formed is isolated and its specific radioactivity determined. The amount of tryptophan converted to 5HTP can then be calculated.

Rat, rabbit, and guinea pig tissues were removed immediately after decapitation; beef pineal glands were obtained at a slaughter house and removed within 10 minutes of exsanguination; carcinoid tumor was a liver metastasis obtained at surgery. All tissues were iced immediately and homogenized in two volumes of ice-cold 0.05*M* tris-acetate buffer, *p*H 7.4.

A typical assay is done as follows (*9*): 0.05 to 0.2 ml of a 33 percent tissue homogenate is incubated in the presence of 0.25*M* tris-acetate buffer (*p*H 7.5), 1.8 m*M* DL-5HTP, 0.4 m*M* pargyline hydrochloride, 1 m*M* 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$), 0.05*M* 2-mercaptoethanol, 0.1 m*M* ferrous ammonium sulfate, and 0.12 m*M* L-tryptophan-2-^{14}C (16.7 μc/μmole) in a total volume of 0.25 to 0.50 ml. After the desired period of incubation in air at 37°C, 0.5 ml of 1*M* tris-acetate buffer, *p*H 9.0, is added to stop the hydroxylation reaction. The mixture is diluted to 2 ml, and 250 units of the decarboxylase enzyme (*10*) and an additional 1.2 μmole of DL-5HTP are added. This mixture is incubated for an additional 20 minutes at 37°C. At the conclusion of the second incubation the sample is diluted to 20 ml and applied to a 0.5 by 3 cm Permutit column (*10*). The column is washed with 20 ml of boiling H_2O. Tryptophan and 5HTP do not adhere to the column and are discarded in the wash. Serotonin is eluted from the column with 4 ml of 2*N* NH_4OH. Two milliliters are placed in a counting vial; 0.3 ml of glacial acetic acid is immediately added to the remaining 2.0 ml of eluate to stabilize the serotonin. A 0.4-ml portion of this latter fraction is diluted to 2.0 ml with H_2O, 0.6 ml of concentrated HCl is added, and the serotonin content is determined by fluorescence assay (*11*). To the portion of the Permutit eluate in the counting vial, 10 ml of fluorophore solution (*12*) are added, and radioactivity is determined in a Packard Tricarb liquid scintillation counter.

The validity of the assay was first established with the use of the mouse mast cell tryptophan-hydroxylating enzyme which is normally assayed by a colorimetric procedure (*5*). Comparison of the results of radioassay and

Table 1. Comparison of tryptophan hydroxylation as measured by radioassay and colorimetric assay. Enzyme preparations used in these experiments were ammonium sulfate fractions of the mouse mast cell tumor (*5*). The enzyme solution contained 20.0, 8.1, and 5.5 mg/ml of protein for experiments 1, 2, and 3, respectively. One milliliter of enzyme solution was used for the colorimetric assay (*5*) and 0.1 ml for the radioassay. Incubations were for 1 hour, as described in the text. Results for radioassay in this and subsequent experiments were calculated with the following formula: Tryptophan hydroxylated = (specific activity of serotonin × total L-5HTP added)/specific activity of substrate. Tryptophan and L-5HTP are expressed in micromoles; specific activity of serotonin and the substrate in counts per minute per micromole.

Exp. No.	Activity (mμmole/mg protein per hour)	
	Radioassay	Colorimetric assay
1	14.3	17.5
2	16.3	17.3
3	12.3	14.9