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Properties of Myxoma Virus Transforming Agent. (24153)

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Investigations of the phenomenon described by Berry and Dedrick(1) have developed currently in several stages, the first having been to transform fibroma into myxoma virus in a predictable fashion. This has been accomplished in tissue culture as previously described(2,3). The present report concerns the nature of the heat-inactivated transforming agent (TAM) used in above experiments and of conditions under which it operates in tissue culture. The effect of enzymes, organic solvents, and the photodynamic action of light on this preparation were studied (Table I). Information gained by such means has proved of value in continuing studies to determine the relation of the nucleic acids of myxoma virus to the Berry-Dedrick phenomenon. The results are presented here.

Materials and methods. Transforming agent myxoma (TAM) was prepared by concentrating myxoma virus in an ultracentrifuge at 30,000 to 40,000 rpm in a No. 40 rotor, then heating the resuspended sediment at 65°C for 40 minutes. TAM mixed with live fibroma virus was inoculated into cultures of trypsinized rabbit kidney cells. Supernatant fluids harvested from these cultures 3 times a week, were tested for presence or absence of transformation by intracutaneous inoculation of rabbits. Since fibroma is a benign and myxoma a fatal disease of rabbits, these tests had a sharp end point. More complete details are given in previous publications(2,3). Additional methods are presented under various headings below.

Results. Effect of organic solvents. Infectivity of myxoma virus was destroyed when

TABLE I. Effect of Various Treatments on Myxoma Virus and on TAM (Transforming Agent Myxoma).

Treatment	Activity of TAM	Infectivity of myxoma virus
Heat 65°C (3 to 45 min.)	Retained	Destroyed
Anesthetic ether	"	"
Chloroform	Destroyed	"
DNAse	Retained	ND*
Trypsin	"	ND
Visible light + toluidine blue	Destroyed	Not totally destroyed
Ultraviolet light	"	*Idem*

* ND = Not done.

1:5 suspensions were extracted with equal volumes of anaesthetic ether for 30 minutes to 2½ hours at room temperature. This was shown by separation and testing of the aqueous layer. No live virus was recovered from 7 lots of myxoma treated in this manner, and tested by intracutaneous inoculation of rabbits, as well as by passage in tissue culture. Two lots, 18 and 19, were further tested in rabbit skin in dilutions of 10^{-1} to 10^{-8}. No live virus was revealed. The 7 lots of myxoma were now designated as ETHER-TAMS and when mixed with live fibroma virus, were tested for transforming activity in a series of tissue culture experiments. Table II shows that 4 of these ETHER-TAMS (Lots 18, 19, 28, and 39) were active in transformation, a finding indicated by appearance of live myxoma virus in 16 of 21 experiments. Aliquots from 2 of these ETHER-TAMS, were heated at 65° for 12 to 40 minutes. These exposures were well beyond those needed to inactivate live myxoma, as elsewhere described(3). The ETHER-TAMS subjected to both treatments in this manner appeared as effective as when ether was the only method used to inactivate.

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TABLE II. Numbers of Positive Transformation Experiments Carried Out with Ether-Prepared TAMS, Shown in Relation to (1) Total Number of Experiments Performed, (2) Use of Heat in Addition to Ether Inactivation and (3) Possible Effect of Serum in Suspending Medium.

Lot No.	Time at 65°C	Serum in preparation medium, %	Transformation in tissue culture, No. pos./total tested
18 a	12 min.	10	5/5
18 b	none	"	3/3
19	12 to 40 min.	"	3/4
39	none	2	2/3
28	"	none	3/6
29	"	"	0/6
30	"	"	0/5
31	"	"	0/2

With ETHER-TAM 18, for example, 3 of 3 experiments were positive (*i.e.* showed transformation) with unheated material, and 5 of 5 other experiments were positive when the same material was heated for 12 minutes. Of 18 negative transformation experiments, 16 fell in a group of 4 ETHER-TAMS (28, 29, 30, 31, Table II) which had been prepared somewhat differently. In these lots, the original myxoma tumors had been suspended in buffered saline (pH 7.2) and no serum was used in subsequent processing. The most effective ETHER-TAMS (18 and 19, Table II) were suspended throughout preparation in Hanks' balanced salt solution containing 10% inactivated horse serum. Suspensions of myxoma virus exposed to chloroform, under conditions similar to those used for above ether extractions in which serum was present, showed no infectivity and no transforming activity in 15 tissue culture experiments.

Enzyme treatment. TAMS prepared by heating retained transforming activity after treatment both with *desoxyribonuclease* (DNase) and with trypsin. The TAMS were originally centrifuged and heated in media containing 10% calf serum, as described for previous experiments(3), but were given 2 additional centrifugations at 40,000 rpm to wash and resuspend them in buffered saline, pH 7.2. The pH was that of the nutrient tissue culture fluids. These TAMS were now exposed to 4-5 μg/ml of crystalline bovine pancreatic DNase (Worthington) for 1 to 2 hours at room temperature. Three different lots of TAM, including one previously treated with trypsin and one suspended in the original media with 10% calf serum, retained activity in the presence of DNase, as revealed by positive results in 9 experiments. Tests on aliquots of TAM suspensions showed that added DNA was rapidly hydrolyzed in the presence of the enzyme. Crystalline trypsin (Worthington), was allowed to act on one lot of TAM for 20 hours, in amounts of 0.8 mg/ml. The mixture was afterward heated at 65°C for an hour to destroy the enzyme. Six positive transformation experiments indicated that the activity of this single lot of TAM was unaffected by trypsin.

Inactivation by photodynamic action. A number of animal viruses have been shown to lose infectivity when illuminated with visible light in the presence of dissolved oxygen and a photodynamic dye(5,6). The reaction is a photosensitized oxidation of portions of the virus to which the dye is chemically bound. The mechanism of this reaction has not been reported in detail, but it is supposed that the viral nucleic acids provide the critical reaction sites. With these considerations in mind, 2 TAM preparations were irradiated with intense visible light after addition of toluidine blue O to a final concentration of 0.61 mg%. These preparations were found to have lost their ability to induce transformations, when they were tested subsequently in 6 tissue culture experiments. A control sample containing the dye and maintained in the dark, remained active as shown in 2 experiments.

Inactivation by ultraviolet light. Two experiments indicated that TAM exposed to ultraviolet light lost its activity under conditions which did not completely destroy the infectivity of live myxoma virus. The samples were irradiated for 10 minutes in a layer thickness of 2 mm with an incident intensity of 36 microwatts/cm^2 at 2537 Å.

Sedimentation of TAM particles. TAM prepared by heat-inactivation presumably consists of particles which are essentially of the same size as those of unheated myxoma virus, a finding suggested by the following experiment. Two lots of TAM were centrifuged

in a Spinco at 40,000 rpm for 1 hour. This centrifugation was the same as that used in the original preparation and transforming activity was recovered from the sediments, but not from the supernatant fluids.

Fate of TAM in tissue cultures. Simultaneous additions of TAM and live fibroma virus to rabbit kidney cultures led to transformations in nearly all experiments whereas addition of TAM alone, under similar circumstances, never resulted in appearance of any infectious virus(2,3). A number of questions arose from these experiments. First, how long might TAM survive in tissue cultures and second, would it attach to or enter cells in an absence of fibroma virus? Titrations of TAM were not attempted in experiments designed to answer these questions. A dosage of 0.45 ml of TAM, used for each 2 ounce bottle of tissue culture, possibly represented at least 3 times the amount required for transformation. In initial experiments, supernatant fluids were poured from 3 pairs of tissue culture bottles. TAM was then allowed to overlay the cell sheets directly for varying periods, ranging 5 to 30 minutes, after which an effort was made to wash it away with 3 changes of nutrient fluid. Fibroma virus was then added to the cultures. Transformations subsequently took place in the 15- and 30-minute experiments. No transformation took place after the 5-minute interval. The first of the wash fluids used in these 3 experiments were combined with live fibroma virus as a basis for 3 duplicate experiments, all of which were positive. There was thus evidence that TAM had been used originally in amounts exceeding those needed for transformation. The experiments were now repeated at intervals of 1 and of 24 hours before the addition of fibroma virus. Results were the same as at intervals of 15 and 30 minutes with the exception that wash fluids were not tested. It was concluded from this set of experiments that TAM could become bound to rabbit kidney cells by itself, and that it was not readily destroyed when incubated in such cultures at 36°C. A further question was whether TAM had to be added at the same time or before fibroma virus, or whether it could be added afterward. Two experiments were carried out for this purpose. TAM was added 24 hours and 48 hours respectively after fibroma virus, with transformations taking place within a week in both experiments. It has become evident that TAM can be effective whether added before, or as much as 48 hours after fibroma virus.

Lack of immunizing capacity of TAM. TAMS were tested routinely in rabbits and no sign of infection ever resulted, when a maximum of 2 ml of concentrated material was given by intracutaneous and intraperitoneal routes of inoculation(3). One wondered, however, whether such inoculations might not protect test animals against subsequent challenge with live myxoma virus. Seven rabbits were challenged accordingly, 12 to 14 days after inoculation. Of these animals, 3 different lots of ETHER-TAM had been tested in 3 of them and 4 different lots of heat-prepared TAM in 4. All of the animals challenged developed myxomatosis, in a minimum incubation time of 5 days. There was thus no evidence that TAM had any protective effect.

Discussion. The structure of myxoma virus and alterations resulting from TAM preparation are of interest to an analysis of the Berry-Dedrick phenomenon. Electron microscope studies indicate that myxoma has a relatively large protein coat surrounding an inner core of DNA, a structure which, as Farrant and Fenner(4) have shown, resembles that described for the related vaccinia virus (7). From another point of view, there is a basic structural difference between these 2 pox viruses. Andrewes and Horstman(8) found that fibroma and myxoma viruses were ether-sensitive while vaccinia was ether-resistant. The former viruses may have a soluble outer layer, possibly lipid in nature. One may speculate that heat and ether methods of preparing TAM both denature the protein coat, and that ether may also remove this outer lipid layer. Several things appear to be evident concerning the structure of the TAM particle. First, TAM particles sediment as readily in an ultracentrifuge as those of myxoma virus, indicating that their size has not

altered significantly. Second, it is probable that the central core of DNA remains intact. This core, lying within a coat of denatured protein, is relatively stable to storage and to moderate heating, and it is unaffected by exposure to DNase. It is, however, readily destroyed by visible light in the presence of toluidine blue and by ultraviolet irradiation.

TAM is seemingly inert when studied alone *in vivo* and *in vitro*. Inoculated rabbits not only fail to develop any signs of infection, but also fail to develop immunity to a subsequent challenge with live myxoma virus. A question of interest is what happens to TAM in the presence of live fibroma virus. A first thought was that the live virus might function in promoting entry of TAM into tissue culture cells. Once inside, TAM particles would lose their outer coats and the central DNA would replicate itself. The action of fibroma virus, however, is probably more specific. Several experiments indicated that TAM particles can attach to and possibly enter cells alone and even a related virus, vaccinia, will not lead to transformation when given simultaneously. Fibroma virus sets up special conditions after infecting a susceptible cell. Febvre *et al.*(9) have shown that all recognizable fibroma virus disappears shortly after entry and an area of viroplasm, the inclusion body, appears within the next 5 hours. Virus particles begin to emerge from this area about 8 hours after infection. One wonders whether TAM particles coming to this matrix area might not lose their outer coats and then replicate themselves from the central cores of DNA. Investigations are being continued to better characterize the transformation process and the interactions involved.

Summary. The transforming agent prepared from myxoma virus (TAM) is resistant to heat, to ether extraction, and to mild enzymatic digestion but is inactivated by the photodynamic action of toluidine blue. TAM particles can be sedimented by ultracentrifugation (Spinco). TAM becomes attached to rabbit kidney cells in 15 minutes, when inoculated by itself, is not destroyed by incubation in tissue cultures for 48 hours at 36°C, and can be added before or after live fibroma virus in transformation experiments. The transforming agent is not immunogenic against myxoma virus in rabbits.

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On the Nature of Oxytocin and Vasopressin from Human Pituitary.* (24154)

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Comparison of pressor-antidiuretic hormones isolated in this laboratory from posterior pituitary glands of beef and hog has shown them to be cyclic octapeptide amides differing in one amino acid residue, the beef vasopressin containing arginine(1) and vasopressin from hog glands, lysine(2). The structure of arginine vasopressin, the cyclic disulfide of L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-arginylglycinamide, may be repre-

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