and thus altering the results of diagnostic neutralization tests. Certainly further work along these lines, comparing the results obtained with neutralization tests performed in the usual manner and with virus prepared from a 1 hour period of replication is indicated.

Finally, these active, hourly harvests of virus may provide a more delicate means for studying antigenic relationships between members of the arthropod-borne viruses, since animals immunized with active tissue culture virus may produce a more specific antibody than that now obtainable following immunization with virus suspensions containing not only large quantities of animal protein but also varying quantities of inactive virus. These actively replicating HKC cultures may also prove useful as new tools for study of the production of antigens for demonstrating complement-fixing and hemagglutinating activity of the virus.

Summary. Following inoculation of HKC cultures with JBE virus, virus content increases by geometric increment after a brief latent period and yields a maximum titer of virus usually of the order of 10^7 infective units per 0.1 ml of tissue culture fluid 72 to 96 hours following inoculation. Infectivity of the tissue culture fluids then decreases during the next several days as all of the cells of the monolayer are destroyed by the cytopathogenic activity of the virus. Further studies during the 69th to 75th hours following inoculation with JBE virus showed that the infected HKC monolayer is capable of releasing infective virus to a titer of the order of $10^{6.5}$ to $10^{6.8}$ per 0.1 ml at hourly intervals. The practical and theoretical implications of these findings for further studies have been discussed.

1. Kissling, R. E., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 290.

2. Diercks, F. H., Hammon, W. McD., *ibid.*, 1958, v97, 627.

3. Sabin, A. B., Duffy, E. E., Warren, J., Ward, R., Peck, J. L., Jr., Ruchman, I., *J.A.M.A.*, 1943, v122, 477.

4. Warren, J., Hough, R. G., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 109.

5. Schwerdt, C. E., *Spec. Pub. N. Y. Acad. Sci.*, 1957, v5, 157.

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Relation of Myxoma Deoxyribonucleic Acid (DNA) to Fibroma-Myxoma Virus Transformation. (24757)

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Transformation of fibroma into myxoma virus in tissue culture using myxoma virus inactivated by heat as transforming agent (TAM) has been described(1,2). Further findings indicate that TAM consists of myxoma virus particles which have lost infectivity due to denaturation of their outer protein coats. These particles are not affected by deoxyribonuclease (DNase)(3). Continuing investigations have aimed to remove outer coats in an effort to reveal an inner core of DNA. The present communication describes how urea treatment renders TAM susceptible to the destructive action of DNase, while not reducing its ability to transform. These findings suggest that DNA is essential to the Berry-Dedrick phenomenon(4).

Materials and methods. Recent modifications in procedures used in transformation experiments in tissue culture(1,2) may be outlined as follows: *Fibroma virus.* The live virus used was prepared from fibromas made into a 1:10 suspension with the medium used for tissue cultures. After clearing by repeated centrifugations at 3,000-4,000 rpm, the suspensions were stored at –70°C. *Preparation of TAM.* Myxoma virus was prepared as a 1:10 suspension of ground tumors in pH 7.2 phosphate-buffered saline containing 10% calf serum (inactivated) as well as penicillin

and streptomycin. The suspension was cleared with 3 or 4 centrifugations at 3,000 rpm. Virus particles were then concentrated in a pellet by a high speed centrifugation in a Spinco, at 40,000 rpm for 40 minutes, following which they were resuspended in 1/30 their original volume, with use of the same serum-saline medium. Suspensions were then frozen in sealed ampoules at –40°C. These tubes were later thawed and heated 12 minutes when completely submerged in water bath at 65°C. All virus was inactivated in this manner prior to chemical treatment. In preparations 49 through 66 TAM was also twice resedimented and resuspended in buffered saline (no serum) prior to chemical treatment. *Urea treatment.* All preparations of TAM given urea treatment were dialyzed inside a sterile dialysis sac, with gentle rocking, against 10 volumes of 10 M urea in buffered saline for 6 hours at room temperature (23°C). Dialysis was then continued for 2 more hours at 23°, 35°, or 45°C. The outer fluid was then replaced by cold buffered saline and UREA-TAM was dialysed at 4°C against several changes of buffered saline over a period of 4-5 days. *Detergent treatment.* Sodium lauryl sulfate was added to the suspension of TAM to give final solution of 0.1%. The TAM-detergent mixture was exposed to desired temperature for 1 hour, then extensively dialyzed against buffered saline at 4°C. *Enzyme treatment.* Four to 5 μg of crystalline DNase or RNase/ml of TAM were added and the mixture kept at 38°C for 2 hours. The treatment was then repeated before TAM was cooled and stored in frozen state. In every case DNase was tested at time of treatment by adding 4 μg to 1 ml of a sample of DNA in buffered saline. Although conditions were far from optimal due to low concentration of Mg, the viscosity of DNA was destroyed within 5 minutes at 25°C. Stock solution of RNase was assayed by methods of Kunitz(5) and in every case was highly active. *Tissue cultures* consisted of trypsinized rabbit kidney cells maintained in 2-ounce prescription bottles with 3 changes/week of Melnick-Hanks medium containing 10% calf serum and penicillin and streptomycin. The bottles were inoculated within 7 to 9 days of their preparation. *Performance of tissue culture experiments.* TAM and fibroma virus, both in amounts of 1 ml, were mixed in tube with 0.6 ml of inactivated calf serum which had been heated an additional 3-5 minutes at 65°C just prior to use. Fluids were now poured from 2 prescription bottles and their cell sheets were washed by 3 successive changes of nutrient medium, prior to inoculation with equal amounts of TAM, virus, serum mixture. These inocula were allowed to overlie cell sheets for ½ hour at 36°C, then 6 ml of medium were added. Supernatant fluids were harvested at 2-3 and at 5 days. Experiments were terminated at 7 days when cell sheets were thin and the cells rounded. These final harvests were made after bottles had been frozen and thawed. *Rabbit inoculations.* Harvests from individual experiments were inoculated into rabbits intradermally in amounts of 2 ml. Animals developing myxomatosis did so within 5-7 days.

Results. The major aim of chemical treatments was to alter TAM particles to expose their inner core of DNA without causing loss of transforming activity. The agents used were urea, which had been used for disruption of bacteriophage(6), and the detergent, sodium lauryl sulfate, which had been used to disrupt tobacco mosaic virus(7). No lots of TAM mentioned below proved infectious when tested in rabbits and tissue cultures by methods previously described(1,2). Lack of infectivity was true of TAMS prepared with detergent and urea as well as by heat alone.

Use of detergents. An initial attempt with detergent was successful from the above point of view. TAM 49 retained its transforming potency after treatment with detergent at 60°C as indicated by positive results in 7 of 7 transformation experiments, all of which led to an appearance of live myxoma virus. A portion of the same material exposed to DNase gave a different result. The enzyme destroyed transforming ability of TAM 49 as indicated by negative results in 5 of 6 experiments. The sixth experiment was barely positive since the rabbits used for testing tissue culture fluids developed only fibro-myxoma which, as previously described(3), represents a minimum of myxoma in presence of large

amount of fibroma virus. These results encouraged an idea that an inner core of DNA could be exposed without destruction of transforming activity. Further efforts in use of detergents, however, met with no success. Eight additional lots of TAM submitted to similar manipulations at temperatures from 39 to 60° lost their transforming ability. Two additional preparations, 67 and 68, exposed to detergent at 58°C (in presence of serum) retained activity but were not destroyed by DNase. Reasons for the inconsistent results obtained with detergent are not yet understood.

Urea treatment. Four successive lots of TAM, 69 to 72, were treated with urea at room temperature and aliquots of each were then exposed to DNase. Twenty-four of 25 experiments performed with these UREA-TAMS were positive. Twenty-six other experiments, performed at same time and under same conditions with aliquots exposed to DNase, were all negative. These results are presented in Table I. It is evident that urea

TABLE I. Transforming Activity of TAM after Treatment with Urea at Different Temperatures Followed by Exposure to DNase.

TAM	Temp. urea R_x, °C	Transformation experiments with UREA-TAM: No enzyme Pos.*	No enzyme Neg.	DNase Pos.	DNase Neg.
4 lots (69-72)	23	24	1	0	26
2 lots (70 and 71)	35-45	11	9	3	20

* Positive = transformation of fibroma to myxoma virus took place in tissue culture. Negative = no change in tissue culture.

treatment at room temperature led to no apparent reduction in transforming ability of heat-prepared lots of TAM but that DNase destroys the transforming activity after such urea treatment. Results were similar but somewhat less constant when urea treatment was carried out at higher temperatures (Table I). Table II presents results obtained for individual lots of UREA-TAM.

Specificity of DNase action. It was reported earlier(3) that TAM is not inactivated by exposure to DNase. This finding has been confirmed and extended by use of longer exposures of the TAM to 2 separate aliquots of DNase at higher temperature of 38°C. One of the lots, TAM 72, had been carried through the dialysis procedure employed in preparation of UREA-TAM, except that urea was omitted. Results in Table III also compare effects of DNase and RNase on the same 2 lots of UREA-TAM. In every case DNase destroyed the transforming ability, whereas RNase had no effect. Thus, destructive action of DNase on UREA-TAM appears to be specific.

TABLE II. Transforming Activity of 4 Lots of TAM after Treatment with Urea at Different Temperatures Followed by Exposure to DNase.

TAM	Temp. urea R_x, °C	Transformation experiments with UREA-TAM: No enzyme Pos.*	No enzyme Neg.	DNase Pos.	DNase Neg.
69	23	10	0	0	9
70 A	23	3	1	0	6
B	35	2	6	0	5
C	45	4	2	0	6
71 A	23	6	0	0	6
B	35	5	1	3	3
72 A	23	5	0	0	5

* Positive = transformation of fibroma to myxoma virus took place in tissue culture. Negative = no change in tissue culture.

DNase activity of rabbit kidney tissue cultures. A consideration of importance was whether UREA-TAMS might not be destroyed by enzymes present in the tissue cultures, particularly DNase. Assays were made, therefore, of fluids from cultures of rabbit kidney. These fluids had remained on the tissue culture for 2 days prior to harvesting them for the determinations. Viscosimetric procedure and units are described elsewhere(8). Results showed that rabbit

TABLE III. Comparison of Effect of RNase and DNase on Transforming Activity of UREA-TAM.

TAM lot numbers	Treatment	Enzyme used on TAM	Transformation exp. Pos.†	Neg.
58, 72, 74	Heat* alone	DNase	4	0
73, 74	" * + urea	"	0	6
73, 74	*Idem*	RNase	7	0

* 65°C, 12 min.

† Positive = transformation of fibroma to myxoma virus took place in tissue culture. Negative = no change in tissue culture.

kidney cells released a considerable amount of DNase I, 2.8-3.0 units/ml, and a small amount of DNase II, .15 unit/ml, into their nutrient media. The amounts of this enzyme remained fairly constant over a 2-week period in which the cultures increased in age from 1 to 3 weeks. Fresh, uninoculated tissue culture media had only .15 unit of DNase I/ml and no detectable DNase II. Neither DNase I nor DNase II activity was detectable when tissue culture cells alone were frozen and thawed into a volume of saline about 1/5 that of the tissue culture fluid removed. It is probable that the quantity of cells in the sample was so low that the methods employed would not detect the intracellular enzyme. It is clear, however, that most of the DNase I produced by kidney cells is released into the medium. Results of the above experiments led to a change in technics used. As described above, tissue cultures were washed in 3 changes of nutrient fluid to get rid of all possible DNase activity, prior to their inoculation with TAM plus fibroma virus.

Discussion. Earlier studies(1) have indicated that TAM is essentially a myxoma virus particle which has lost infectivity due to denaturation of its outer protein coat. The present problem has been to remove this coat and expose a postulated inner core of DNA. Two agents have been used in this attack. The first of them, a detergent, did not prove to be of use, in spite of one early success. Possibly conditions for its action are too critical. The action of a second agent, urea, was consistent and definite, for UREA-TAMS have proved susceptible to destructive action of DNase, in contrast to TAMS prepared with heat alone or heat followed by detergent and tested under same conditions. This effect of DNase appears to be specific

It is not yet possible to decide whether the particles of UREA-TAM are without a protein coat or whether the DNase sensitivity results from formation of crevices which permit approach of the enzyme to the nucleic acid. At least one consideration suggests that the final product is not completely free DNA. This is that activity of UREA-TAM is comparable to that of TAM itself, whereas the viral activity of nucleic acids derived from viruses is generally very low, particularly with animal viruses as reported by Schramm(9). It is hoped that the nature of UREA-TAM may be elucidated by continued chemical, physical, and biological studies.

Summary. (1) TAM (Transforming Agent Myxoma) became susceptible to destructive action of DNase when treated with urea. (2) DNase used under same conditions had no effect on TAM prepared by heat alone. (3) RNase did not destroy UREA-TAM. (4) Potency of UREA-TAM was comparable to that of heated TAM from which it was prepared. (5) Evidence accumulated indicated that myxoma DNA is intimately concerned with transformation of fibroma into myxoma virus in tissue culture.

1. Kilham, L., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 59.

2. ———, *J. Nat. Cancer Inst.*, 1958, v20, 729.

3. Kilham, L., Lerner, E., Hiatt, C., Shack, J., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 689.

4. Berry, G. P., Dedrick, H. M., *J. Bact.*, 1936, v31, 50.

5. Kunitz, M., *J. Biol. Chem.*, 1946, v164, 563.

6. Cohen, S. S., *Cold Spring Harbor Symp., Quant. Biol.*, 1947, v12, 35.

7. Hart, R. G., *Proc. Nat. Acad. Sci.*, 1955, v41, 261.

8. Shack, J., *J. Biol. Chem.*, 1957, v226, 573.

9. Schramm, G., *Ann. Rev. Biochem.*, 1958, v27, 101.

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