INDUCTION OF TUMORS IN DOMESTIC RABBITS WITH NUCLEIC ACID PREPARATIONS FROM PARTIALLY PURIFIED SHOPE PAPILLOMA VIRUS AND FROM EXTRACTS OF THE PAPILLOMAS OF DOMESTIC AND COTTONTAIL RABBITS*

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(Received for publication, May 19, 1961)

The virus-induced papillomatosis (1) of domestic rabbits has at least three desirable features which commend it for studies of the mechanism of viral tumorigenesis. (*a*) The tumor is readily induced at the site of inoculation on the skin of the animal, (*b*) continued growth of the tumor may occur in the apparent absence of persistent virus (2), and (*c*) development from a benign growth to malignant tumor can be observed at the primary site (3).

Recently the newer techniques for isolating biologically active nucleic acids (4) were successfully applied to SE polyoma virus (5). Utilizing a similar approach, we have demonstrated that nucleic acid–containing extracts of glycerinated cottontail papilloma tissue have a tumor-inducing capacity (6).

The present paper deals with the further characterization of the tumor-producing factor which now clearly seems to be DNA in nature. In addition, the extraction of a similar active factor from partially purified virus has been accomplished and tumors have been induced with the nucleic acid–containing extracts of papillomatous tissue of domestic animals which fail to yield evidence of viruses when tested by ordinary procedures.

Materials and Methods

Experimental Animals.—New Zealand white rabbits of mixed sexes were used in these experiments. They were obtained from local breeding farms. At the time of inoculation the weight of most animals ranged from 1.5 to 2.5 kg. They were housed individually or in pairs in metal cages and were maintained on a diet of Purina rabbit pellets and water.

Virus.—The source of viral material was 4 pools of warts of the wild cottontail rabbit

* The study was supported in part by grants from the National Institutes of Health (USPHS C-2668, CRT-5040) and by an Institutional Grant of the American Cancer Society, Inc.

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(Earl Johnson Farm, Rago, Kansas) preserved in 50 per cent buffered glycerin at 4°C for 12 to 18 months. These glycerinated tissues served as the starting material for extraction of nucleic acid from cottontail tissue and for purification of Shope papilloma virus. Stock virus was a 10 per cent suspension of washed tumor tissue ground with alundum (crystalline alumina, Norton Company, Worcester, Massachusetts) diluted in 0.14 M NaCl solution, and stored at −42°C. The virus preparations were infective for domestic rabbits at dilutions of 10^{-4} to 10^{-5}.

Virus Assay.—Tests for the presence of intact virus and of virus titer were carried out by inoculating the test suspension into the clipped and shaved skin of domestic rabbits by the scarification method or by the injection and puncture method. The details of these techniques will be referred to later.

Preparation of Partially Purified Virus.—A slight modification of the fractionation procedure of differential centrifugation developed by Beard *et al.* (7) was employed. Ten gm of cottontail papilloma tissue was ground with alundum and mixed with 10 volumes of phosphate-buffered saline free of magnesium and calcium (PBS) (8). Extraction was carried out overnight at 4°C. in a magnetic mixer. Tissue debris was sedimented by centrifugation at 10,000 *g* for 10 minutes and virus in the supernatant was then removed by sedimentation at 75,000 *g* for 1 hour. The pellet, resuspended in 30 ml of PBS was subjected to a second cycle of centrifugation at 10,000 *g* and 75,000 *g*. The final pellet was taken up in 10 ml of PBS and stored at −42°C.

Extraction of Nucleic Acid from Cottontail Papilloma.—Two basic procedures were employed. They will be referred to herein as the "cold phenol" (4, 9–11) and the "hot phenol" (12) method. The details of the two procedures have been described previously (6).

Extraction of Nucleic Acid from Partially Purified Virus.—The cold phenol and hot phenol methods were used on 10 ml of the partially purified virus suspension which originated from 10 gm of the papillomatous tissue. The extraction procedure was the same as in our previous study except that the time of the first extraction in the cold phenol method was shortened to 1 hour.

Extraction of Nucleic Acid from Domestic Papilloma Tissue.—Many extraction methods were tried in attempts to obtain infectious nucleic acid from domestic rabbit papillomas. None of these gave as consistent results as were obtained with cottontail papilloma tissue. The procedure which seemed to be the most effective and which was tentatively chosen as a standard technique is the following:

Twenty gm of fresh domestic papilloma tissue and 20 ml of PBS were homogenized in a Waring blendor at 4°C for 2 minutes. Then 40 ml of 88 per cent phenol and 2 ml of ethylenediamine-sodium-tetracetate (EDTA) (5.6×10^{-4} M solution in PBS) were added quickly, and the mixer was run for another 2 minutes. The homogenate was then transferred to a flask with 10 gm of alundum placed on a magnetic mixer and ground at 4°C for 1 hour. The homogenate was then placed in a 56°C waterbath for 5 minutes and, after immediate cooling, grinding and extraction were continued at 4°C for 30 minutes. After centrifugation at low speed, the unseparated milky suspension was separated from the tissue debris and was extracted with an equal volume of 80 per cent phenol at 4°C for 30 minutes. The same procedure was repeated on the withdrawn aqueous layer and interphase material. The final aqueous phase was extracted with ether, and the residual ether was evaporated by bubbling nitrogen through the suspension.

Concentration of the tumor-producing factor in several extracts was attempted by ethanol precipitation. Two volumes of cold ethanol were gradually added to the final aqueous phase with continuous stirring. The resultant fibrous precipitate was removed with a glass rod and placed in $\frac{1}{5}$ to $\frac{1}{10}$ of the original volume of PBS. According to the well established method (13) of extracting DNA from tumor and other mammalian tissues, PBS with 1 M and 1.6 M NaCl was also tried in place of the usual 0.14 M.

Infectivity Assay of Nucleic Acid Preparations.—The infectivity and tumor-inducing potency of the extracted nucleic acid preparations were determined by their capacity to induce macroscopic tumors in the skin of domestic rabbits. The standard inoculum was 0.1 ml for the cottontail preparations and 0.1 or 0.2 ml for domestic rabbit extracts. In early experiments three different methods, referred to herein as (*a*) scarification, (*b*) injection, and (*c*) injection and puncture, were employed. The scarification method was the standard procedure employed in our laboratory for inoculating Shope papilloma virus. The injection method was an intradermal injection made with a 27-gauge needle on a tuberculin syringe. The most effective technique and the one used as routine after the early experiments has been referred to as injection and puncture method and consisted of multiple punctures of the small bleb formed by intradermal injection of the extracts. Usually the tip of a scalpel and sometimes fine needles held together with forceps were used as tools. Fifty to eighty punctures were made in one bleb. Care was taken to avoid too deep punctures which led to undesirable bleeding at the inoculated sites. Deoxyribonuclease (DNAase) activity has been demonstrated in normal rabbit serum (14). Therefore, bleeding and exudation might result in the inactivation of the tumor-producing factor in the extracts. Excess mechanical damage also might kill the epithelial cells and reduce the chances of successful infection by the extracts. Usually 4 to 8 sites were utilized on the back of each rabbit and two to four inoculations of 0.1 or 0.2 ml of the nucleic acid preparation were made in each site.

Positive "takes" were determined by the detection of definite macroscopic growth of a papilloma, which persisted for at least a week. In most cases the papillomas persisted indefinitely. The animals were checked and the findings were recorded every day until the first appearance of the tumor or for at least 8 weeks if the results were negative. After positive growth was established, the rabbits were checked two to three times a week for 60 days.

Antisera.—Two different kinds of antiviral antisera were employed. One was a pooled serum collected from rabbits which once had fairsized tumors that subsequently regressed. The serum had a 100 per cent endpoint of 1:40 by neutralization test. The other pool was sera from domestic rabbits given 3 injections of stock virus mixed with Freund's (complete) adjuvant. The titer measured as above was 1:80. The pools of sera were frozen and stored at −42°C. In order to determine the effect of the antisera on the test substances, 0.5 ml of the sera (1:1 or 1:10 dilution) were mixed with equal volumes of nucleic acid preparation or with Shope papilloma virus suspension and were held at 25°C for 20 minutes.

Enzymes.—All enzyme preparations used in the present experiments were dissolved in PBS and 0.5 ml of test substance and enzyme were mixed to give the desired final concentration. The mixtures were incubated at 25°C for 10 to 20 minutes. The enzymes used were DNAase 1 times crystallized and RNAase crystalline (Nutritional Biochemicals Corporation, Cleveland) and trypsin[1] 2 times crystallized (Worthington Biochemical Co.) and α-chymotrypsin[1] prepared by the method of Northrop and Kunitz.[2] The DNAase was applied in presence of 0.005 M $MgSO_4$. Trypsin was dissolved to a concentration of 7.8 mg/ml in 10^{-3} M HCl and kept frozen till the time of use. Alpha chymotrypsin was recrystallized 2 times and kept in a dry state under refrigeration. Fresh enzyme solutions were made up each time before use.

Ultraviolet Absorption Measurements.—Absorption curves were obtained with a Beckman spectrophotometer model DK-2. The nucleic acid-containing extracts were diluted 10 to 30 times with PBS (pH 7.5) prior to the measurement.

Electron Microscopic Examination.—Conventional electron microscopy was carried out on

[1] The two proteolytic enzymes were kindly supplied by Dr. M. Yamazaki of the Department of Biochemistry, University of Washington.

[2] Northrop, J. H., Crystalline Enzymes, New York, Columbia University Press, 2nd edition, 1948.

partially purified virus preparations by Dr. V. C. Chambers of our Department. Virus specimens were mounted on a carbon-coated specimen grid, air-dried, and shadowed with palladium. Electron micrographs were taken on an RCA EMU-2C electron microscope.

TABLE I

*Development of Tumors in Domestic Rabbits Inoculated with Nucleic Acid Preparations from Cottontail Papilloma Tissue**

Extraction method	Nucleic acid preparation	No. of rabbits used	Site of inoculation	Mode of inoculation	No. tumors per No. inoculation sites‡	Incubation period
						days
Cold phenol extraction	Undiluted	9	Back	Scarification	0/2, 2/3, 2/2, 0/2, 4/4, 3/4, 4/4, 4/4, 1/2	—, 43, 16, —, 22, 29, 31, 28, 27
	"	4	"	Injection	0/3, 2/8, 0/8, 2/4	—, 20, —, 30
	"	11	"	Injection and puncture	1/1, 1/1, 3/4, 4/4, 2/2, 2/2, 2/2, 2/2, 2/2, 4/4, 4/4	21, 18, 16, 16, 12, 16, 20, 14, 19, 12, 19
	Diluted§ (1:2)	6	"	" "	2/2, 2/2, 4/4, 4/4, 3/4, 4/4	16, 17, 11, 12, 24, 20
	" (1:5)	3	"	Scarification	1/2, 2/2, 1/2	36, 36, 47
	Undiluted	3	Ear	Injection and puncture	2/2, 0/2, 2/2	16, —, 22
	Precipitated‖	6	Back	" "	2/2, 2/2, 2/2, 2/2, 2/2, 2/2	22, 19, 15, 19, 14, 16
Hot phenol extraction	Undiluted	3	Back	Scarification	1/2, 2/2, 2/2	24, 19, 21
	"	1	"	Injection	5/10	22
	"	2	"	Injection and puncture	3/4, 3/4	16, 18
	"	2	Ear	Scarification	2/2, 2/2	25, 22
	Diluted (1:2)	2	"	"	4/4, 4/4	28, 26
	"	2	"	Injection and puncture	2/4, 2/4	29, 26

* A summary of the results of 28 extractions carried out independently.
‡ Each fraction represents data from one rabbit.
§ Diluted with phosphate-buffered saline.
‖ Precipitated with 2 times volume of ethanol and resuspended in the same volume of phosphate-buffered saline.

EXPERIMENTAL

Properties of Nucleic Acid Extracts of Cottontail Papilloma Tissue

Induction of Tumors in Domestic Rabbits.—The results of tests on the infectivity and tumorigenic activity of the preparations obtained from 28 independent extractions are summarized in Table I. In the initial experiments the incubation periods of the tumors fluctuated widely even when undiluted nucleic acid extracts were used. Later, after the standardization of the extraction and inoculation techniques, a fairly consistent incubation period of 2 to 3 weeks was observed and the rate of positive "takes" rose to almost 100 per cent.

The gross and microscopic similarity of these tumors induced by the nucleic acid–containing extracts to the ordinary virus-induced papillomas was reported in a preceding paper (6).

In extracting the active factor from the papillomatous tissue, both the cold phenol and the hot phenol methods were effective. Because of its seemingly less drastic nature, the cold phenol extraction was preferred. Inoculations by injection and puncture were effective and convenient for quantitation and the method was employed throughout the remainder of the study. The "precipitated" preparations also gave good results. The undiluted aqueous preparation of the cottontail papilloma extract in PBS retains its biological activity for at least 4 months under storage at −42°C. (Table II).

Fate of Nucleic Acid–Induced Tumors.—Some of the tumors induced by the nucleic acid preparations regressed after a certain period of growth (Table III).

TABLE II

Stability of Cottontail Papilloma Nucleic Acid Preparation Stored at −42°C*

Experiment No.	Days stored at −42° C						
	0‡	1	12	27	35	72	129
1	2/2§ (19)		2/2 (17)‖				
2	2/2 (17)¶	4/4 (14)			2/2 (22)		
3	2/2 (13)					2/2 (20)	
4	2/2 (15)						2/2 (20)‖
5	4/4 (15)			2/2 (16)‖			
6	4/4 (15)	2/2 (14)	2/2 (<15)				

* Undiluted nucleic acid extracts; preserved in phosphate-buffered saline (pH 7.5).

‡ Inoculated immediately after the extraction without any storage at −42°C.

§ Number of tumors over number of inoculation sites. In parentheses average incubation period in days.

‖ Precipitated once with ethanol prior to inoculation.

¶ Diluted 2 times prior to inoculation.

This regression was considered similar to those we have seen in ordinary virus-induced papillomas for the following reasons: the regression was systemic; *i.e.*, multiple tumors on the back of one animal regressed together. In rabbits with both nucleic acid–induced and virus–induced tumor, all regressed simultaneously, regardless of their origin. Regression took place approximately 5 to 6 weeks after inoculation but no precise study was carried out on regression rate.

Among those papillomas which persisted, some showed unusually rapid growth resulting in a huge tumor similar to that seen in Fig. 1. Seven to 10 months after inoculation about half of these tumors developed malignant growth which was morphologically indistinguishable from the carcinomas derived from the virus-induced papillomas. Detailed studies on these tumors will be reported later.

Some Other Properties of the Extract.—The undiluted nucleic acid extract ob-

TABLE III

Regression of Multiple Tumors Induced by the Nucleic Acid Preparations in Domestic Rabbits

Rabbit No.	Preparation*	No. of tumors‡	Regression	
			Beginning§	Completion‖
			days after inoculation	
N-738	CP-NA¶	14	—	—
N-739	"	14	—	—
N-742	CP-NA (&V)**	10 (4)	28 (28)	34 (30)
N-743	" (")	14 (4)	— (—)	— (—)
N-746	CP-NA	26	—	—
N-747	"	26	—	—
N-748	CP-NA	18	34	45
N-749	"	16	—	—
N-755	PPV-NA‡‡	13	33	46
N-756	"	13	—	—
N-761	CP-NA (&V)	12 (2)	... §§	...
N-762	" (")	12 (4)	31 (31)	38 (38)
N-767	PPV-NA	14	30	... ‖‖
N-768	"	13	27	37
N-772	CP-NA	4	33	44
N-773	"	4	—	—
N-778	CP-NA	14	—	—
N-779	"	14	35	42

—, no regression observed.

* 0.1 ml of undiluted extracts were employed as an inoculum for each inoculation site.

‡ Number of tumors counted at the time of maximal growth.

§ First sign of regression in any of the tumors.

‖ Complete disappearance of all of the tumors.

¶ Cottontail papilloma nucleic acid preparation.

** In parentheses; V = Shope papilloma virus preparation, figures in parentheses refer to tumors produced at the site of virus inoculation and their data on regression.

‡‡ Nucleic acid preparation extracted from partially purified virus.

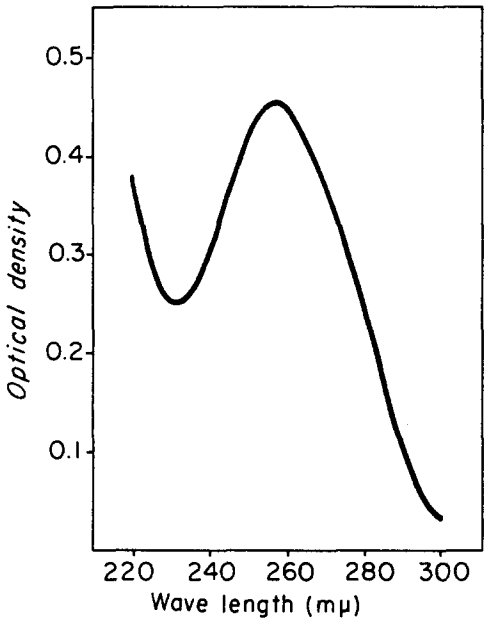
§§ Died on 27th day, no sign of regression noticed.

‖‖ Died on 41st day with approximately 80 per cent regression completed.

tained from cottontail papilloma tissue was highly viscous (in PBS) and had a slightly yellowish color in a diffuse light. On addition of ethanol, a fine flocculent precipitate formed. This precipitate dissolved slowly in PBS to give a colorless suspension.

The ultraviolet absorption curves of the undiluted nucleic acid extract obtained from cottontail papilloma tissue were rather inconsistent. After precipitation with ethanol, however, the spectrum was characteristic of a nucleic acid–containing solution, with a maximum around 256 mμ and a minimum at 232 mμ, as shown in Text-fig. 1.

The Dische reaction (15) for deoxyribonucleic acid (DNA) was positive in either "undiluted" or "precipitated" preparations. The orcinol reaction (Bial, 16) was positive in both cases.



TEXT-FIG. 1. Ultraviolet absorption spectrum of a nucleic acid preparation obtained by phenolic deproteinization (3 times) and ethanol precipitation (1 times) of cottontail papilloma tissue. Diluted (10 times) in phosphate-buffered saline (pH 7.5).

Differentiation between the Tumor-Inducing Capacity of Cottontail Papilloma Nucleic Acid Preparations and of Intact Shope Papilloma Virus

Effect of DNAase on Tumorigenic Activity of Cottontail Nucleic Acid Preparation.—Since the Shope papilloma virus seems to be definitely a DNA virus (18, 19), it was predictable that the tumorigenic factor of the extract would be sensitive to DNAase provided virus DNA were involved. In Table IV this is shown to be the case. When the undiluted nucleic acid extract was used, the minimum effective dose of DNAase blocking the tumorigenic activity of the factor was 2 μg/ml. However, in the precipitated preparation as little as 0.02 μg/ml was effective.

Effect of RNAase on Tumorigenic Activity of Cottontail Nucleic Acid Preparation.—Despite the sensitivity of the tumor-producing factor to DNAase, it was decided to study the effect of RNAase on the active factor. The results of the assay are shown in Table V. Relatively high concentrations of the enzyme, 100 μg/ml and 20 μg/ml, exhibited an inhibitory effect when held with the extracts

TABLE IV

Effect of DNAase on the Tumor-Inducing Capacity of Cottontail Papilloma Nucleic Acid Preparation in Domestic Rabbits

Experiment No.	CP-NA preparation	Concentration of DNAase*	No. of tumors per number inoculation sites	Average incubation
		μg/ml		days
1	Undiluted	0	4/4	25
	"	100	0/4	
2	Undiluted	0	4/4	16
	"	20	0/4	
	"	2	0/4	
3‡	Undiluted	0	4/4	20
	"	20	0/4	
	"	2	0/4	
4	Undiluted	0	8/8	12
	"	0.2	8/8	12
	"	0.02	8/8	12
	"	0.002	6/6	11
5	Precipitated	0	4/4	14
	"	0.2	0/4	
	"	0.02	0/4	
	"	0.002	4/4	18
	"	0.0002	4/4	13

CP-NA, cottontail papilloma nucleic acid preparation.

* Extracts were exposed to DNAase for 20 minutes at 25°C at a pH of 7.5.

‡ Extracts were exposed to DNAase for 10 minutes at 25°C at a pH of 7.5.

for 20 minutes at 25°C. However, 20 μg/ml for 10 minutes or 2 μg/ml for 20 minutes at the same temperature had no effect on the tumorigenic activity of the extract.

Effect of DNAase and RNAase on the Tumorigenic Activity of Intact Shope Papilloma Virus.—For comparison, the effect of the two nucleases on the Shope papilloma virus preparation was examined. A relatively massive dose of 100 μg/ml of both DNAase and RNAase had no effect on a 10^{-1} and 10^{-2} dilution of the virus under conditions of exposure and inoculation identical with those used with the nucleic acid preparation.

Effect of Antiviral Antisera on Tumorigenic Activity of Cottontail Nucleic Acid Preparation.—Three experiments were conducted employing four different lots of nucleic acid preparations (two undiluted and two precipitated) with combinations of three dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of virus. The results were clear-cut (Table VI). In no case was the nucleic acid preparation inactivated by either of the two pools of antisera used, whereas all of the virus preparations were completely neutralized. Both antisera were employed in dilutions of 1:1 and 1:10 for 20 minutes at 25°C.

TABLE V

Effect of RNAase on the Tumor-Inducing Capacity of Cottontail Papilloma Nucleic Acid Preparation

Experiment No.	CP-NA preparation	Concentration of RNAase*	No. of tumors per number inoculation sites	Average incubation
				days
1	Undiluted	0	4/4	25
	"	100	0/8	
2	Undiluted	0	4/4	15
	"	20	0/4	
	"	2	4/4	22
3‡	Undiluted	0	4/4	20
	"	20	4/4	21

CP-NA, cottontail papilloma nucleic acid preparation.

* Extracts were exposed to RNAase for 20 minutes at 25°C at a pH of 7.5.

‡ Extracts were exposed to RNAase for 10 minutes at 25°C at a pH of 7.5.

Effect of Trypsin and Chymotrypsin on Tumorigenic Activity of Cottontail Papilloma Nucleic Acid Preparation and on Intact Shope Virus.—As the nucleic acid preparations probably contained an appreciable amount of protein, it was essential to test for the effect of proteolytic enzymes. There was also a possibility that the tumorigenic factor in this system resembled the phage-derived "protoplast-infecting agent" which is sensitive to trypsin (17). Two proteolytic enzymes, trypsin and chymotrypsin, were employed, and the results are listed in Table VII. Neither the cottontail nucleic acid preparation nor the intact Shope virus was affected by exposure to a relatively large concentration of 100 μg/ml of these two enzymes.

Induction of Tumors with Extracts from Partially Purified Shope Papilloma Virus

Although the preceding evidence suggests that the biologically active nucleic acid obtained from the cottontail papilloma tissue is likely to be of viral origin, it was felt that more direct proof could be obtained by extracting the virus

TABLE VI

Effect of the Antiviral Antisera on the Tumor-Inducing Capacity of Cottontail Papilloma Nucleic Acid Preparation and of Shope Papilloma Virus Preparation

Experiment No.	CP-NA* or SPV	Anti-serum*	Dilution of serum added	Tumor-inducing capacity	
				No. tumors per No. inoculation sites	Average incubation
					days
1	CP-NA (undiluted)	I	1:1	2/2	21
		"	1:10	2/2	13
		"	0‡	2/2	13
	SPV (10^{-3})	I	1:1	0/2	
		"	1:10	0/2	
		"	0	2/2	20
2	CP-NA (undiluted)	II	1:1	4/4	18
		"	1:10	4/4	16
		"	0	4/4	17
	SPV (10^{-1})	II	1:1	0/4	
		"	1:10	0/4	
		"	0	4/4	14
	SPV (10^{-3})	II	1:1	0/4	
		"	1:10	0/4	
		"	0	4/4	25
3	CP-NA§ (precipitated)	II	1:1	4/4	18
		"	1:10	4/4	19
		"	0	4/4	17
	CP-NA‖ (precipitated)	II	1:1	4/4	21
		"	1:10	4/4	18
		"	0	4/4	20
	SPV (10^{-2})	II	1:1	0/4	
		"	1:10	0/4	
		"	0	4/4	20

CP-NA, cottontail papilloma nucleic acid preparation.

SPV, Shope papilloma virus preparation.

Antiserum I, pooled serum from regressed rabbits (see text).

Antiserum II, pooled serum from rabbits hyperimmunized with Shope papilloma virus and Freund's adjuvant.

* The test mixture was held at 25°C for 20 minutes.

‡ Control; same amount of PBS was added instead of antiserum.

§ Stored at −42°C for 12 days.

‖ Stored at −42°C for 129 days.

itself, using the same procedure. As seen in Fig. 4, the electron micrographs of the specimens drawn from the final suspension of the partially purified virus showed an appreciable homogeneity. Three lots of virus preparations were extracted by both the cold phenol and the hot phenol method. As shown in Table VIII, all gave positive results regardless of the extraction method used. Antiviral antiserum also showed no effect on tumorigenic activity of the extract.

TABLE VII

Effect of Trypsin and Chymotrypsin on the Tumor-Inducing Capacity of Cottontail Nucleic Acid Preparation and of Shope Papilloma Virus Preparation

Experiment No.	CP-NA or SPV	Enzyme*	Concentration	No. tumors per No. inoculation sites	Average incubation
			μg/ml		*days*
1	CP-NA	Trypsin	0	4/4	12
		"	100	8/8	11
		"	20	8/8	12
2	CP-NA	Chymotrypsin	0	4/4	12
		"	100	4/4	12
3	SPV	Trypsin	0	2/2	10
		"	100	8/8	12
		Chymotrpysin	0	2/2	14
		"	100	8/8	12

CP-NA, cottontail papilloma nucleic acid preparation (undiluted).

SPV, Shope papilloma virus preparation (10^{-1}).

* The extracts of viruses were exposed to the enzymes for 20 minutes at 25°C minutes at 25°C at a pH of 7.5.

The gross appearance (Fig. 2) and the microscopic feature of the tumors were identical with those induced by the cottontail nucleic acid preparation and by intact Shope papilloma virus.

Attempts to Induce Tumor with the Extracts from Domestic Rabbit Papilloma Tissue

It is well known that the papillomatous tissue of domestic rabbits ordinarily contains little or no demonstrable virus (2), although Shope had limited success in serial transmission by utilizing extracts of domestic tumor produced with selected strains of virus (22). It was interesting to determine, therefore, whether a tumor-producing factor is demonstrable in domestic papilloma extracts that are non-pathogenic. Sixteen extractions by a variety of methods were carried out. The starting material included papillomas induced both by virus and by nucleic acid–containing extracts. The age of the tumor varied from 8 to 27 weeks

after inoculation. Some nucleic acid extracts were from a single source, and others were pooled material from several hosts. Eight specimens were tested for the presence of intact virus in the starting material, and ordinary infectivity tests were carried out utilizing a 10 per cent tissue suspension in PBS. The results of these experiments are summarized in Table IX. Among 16 extracts, 12 induced papillomatous growth in some animals and only 4 extracts yielded completely negative results. The rate of positive "takes" per number of inoculation sites, however, was approximately 21 per cent. The incubation period ranged from 25 to 46 days. The tumors that appeared were typical papillomas

TABLE VIII

Tumor-Inducing Capacity of Nucleic Acid Preparations Extracted from Partially Purified Shope Papilloma Virus

Virus preparation	Extraction		Nucleic acid preparation	Tumor-inducing capacity	
	No.	Method		No. tumors per No. inoculation sites	Average incubation
					days
Lot A	1	Cold phenol	Undiluted	8/8	20
	2	Hot "	"	6/8	18
Lot B	3	Cold phenol	Undiluted	6/7	16
	4	Hot "	"	6/6	18
Lot C	5	Cold phenol	Undiluted	4/4	14
	"	" "	Diluted*	7/8	16
	"	" "	" ‡	8/8	20

* Mixed 1:1 with phosphate-buffered saline and held at 25°C for 20 minutes.

‡ Mixed 1:1 with pooled serum (undiluted) from rabbits hyperimmunized with Shope papilloma virus and Freund's complete adjuvant and held at 25°C for 20 minutes.

in gross (Fig. 3) and microscopic appearance. The tests for infective virus in the tissue homogenates before phenol extraction were still negative after 60 days of observation.

The low rate of "take" and the prolonged incubation period seemed to suggest a relatively small amount of the tumorigenic factor in the domestic rabbit extracts. It may also be possible that in some cases, though not all, the "regression mechanism" begins to operate before the tumor becomes detectable macroscopically, because the incubation period described above falls within the period when the regression of the tumor is known to take place in this breed of animals.

PBS made hypertonic with NaCl (1 and 1.6 M final concentration) was used

in three extractions, and five attempts to concentrate the factor by ethanol precipitation were also made. Extraction with the hypertonic salt solutions yielded a more viscous aqueous solution and gave a fine fibrous precipitate

TABLE IX

Induction of Tumors in Domestic Rabbits with Phenolic Extracts of Domestic Rabbit Papillomas

Extraction No.	Source* (CP-NA- or SPV-induced)	Extracted in PBS of	Nucleic acid preparation‡	No. tumors per No. inoculation sites	Average incubation
					days
1	SPV	0.14 M§	Undiluted	2/16	24
2	"	"	"	3/8	30
3‖	CP-NA	"	"	0/4	
4	SPV	"	"	0/4	
5	"	"	"	1/8	46
6‖	CP-NA	"	"	1/4	34
7	SPV (pooled)	"	"	5/12	30
8	" "	"	"	1/9	36
9	" "	"	"	2/9	27
10‖	SPV	1.0 M	"	2/12	26
10‖	"	"	Precipitated¶	2/12	32
11	"	"	" ¶	1/6	39
12‖	SPV (pooled)	1.6 M	"	1/6	25
13‖	SPV	0.14 M	"	0/6	
14‖	"	"	"	0/4	
14‖	"	"	Undiluted	1/4	32
15‖	SPV (pooled)	"	"	6/8	34
16‖	" "	"	"	1/4	29
16‖	" "	"	Diluted**	1/8	27

CP-NA, cottontail papilloma nucleic acid preparation.

SPV, Shope papilloma virus preparation.

PBS, phosphate-buffered saline.

* "Source" indicates material used to induce the domestic tumor employed as the source of tissue to be extracted. Age of the tumor used varied from 8 to 27 weeks.

‡ 0.2 ml was inoculated by the injection and puncture method.

§ Molarity according to NaCl in PBS (see text).

‖ Tests for intact virus in the starting material were carried out. No positive results were obtained in all of the cases after 60 days' observation.

¶ 0.1 ml. was inoculated by the injection and puncture method.

** Diluted (1:2) preparation.

typical of highly polymerized DNA after addition of ethanol. However, no improvement of infectivity has been seen.

DISCUSSION

The experimental evidence indicates that the tumorigenic factor in the nucleic acid preparation from cottontail papillomas is not intact virus surviving

phenolic extraction. This has been clearly demonstrated by comparison of the effect of DNAase, RNAase, and antiviral antisera on the tumor-inducing capacity of the nucleic acid preparation and of the intact Shope papilloma virus. Crystalline DNAase showed a blocking effect on the tumorigenic activity of the nucleic acid preparation; however, the antiviral antisera had no effect on it. Even the 1:1 dilution of the antisera did not block the appearance of the tumor. On the other hand, the intact virus in a 10^{-1} tissue suspension was completely neutralized by sera of 1:10 dilution. The tumor-inducing capacity of the intact virus was unaffected by either DNAase or RNAase. When the cottontail nucleic acid preparations were exposed to relatively large concentrations of RNAase, a suppressive effect on the induction of the tumor was observed. One might speculate that this is the result of a non-specific binding of the basic enzyme protein to the nucleic acid.

The relative ineffectiveness (minimum effective dose 2 μg/ml) of the DNAase action on the undiluted nucleic acid extract may have been due to an effect of the residual EDTA, which would bind essential metal ions. The procedure of precipitating the nucleic acid with ethanol and resuspending it in PBS probably removed most of the EDTA. At any rate, the minimum effective concentration (0.02 μg/ml) of DNAase was 10^{-3}-fold less than that of the RNAase. It has been reported recently by Watson and Littlefield (19) in their studies on the nucleic acid of purified Shope papilloma virus that the constituent nucleic acid is a double helical DNA. This confirms the earlier concept that the Shope papilloma virus is a DNA virus (18). Although further characterization of the tumorigenic extracts from the partially purified virus is desirable, it seems very likely that the active factor in the extracts of both cottontail papilloma tissue and of virus itself is DNA.

Two proteolytic enzymes, trypsin and chymotrypsin, failed to inactivate the tumor-producing capacity of the nucleic acid preparations. This is evidence against the possibility that the factor is protein.

The state of affairs responsible for the failure to recover Shope papilloma virus from the growths induced therewith in domestic rabbits has long been referred to as "masking" (2). Recently, it has been shown by fluorescent antibody studies (21) that domestic papilloma tissue actually contains so small an amount of demonstrable viral antigen as to yield only a minute fluorescence limited to the keratinizing cells. The stimulating question remains: Is the apparent absence of virus in the domestic papilloma merely a manifestation of a quantitative difference (20), or is the virus present in an altered, non-infective, "masked" form (2) ? Noyes and Mellors (21) have suggested that the "masked" virus may lack the antigenic protein coat and exist as the nucleic acid moiety alone. The fact that a nucleic acid–containing extract with tumorigenic potency was obtained from domestic tumor tissue which showed no evidence of demonstrable virus by the ordinary tests falls in with this assumption.

SUMMARY

A deoxyribonucleic acid preparation which showed infectivity and tumorigenic activity in domestic rabbits was isolated from the papillomatous tissue of wild cottontail rabbits by phenolic deproteinization procedure. The activity of the preparation could be completely abolished by its exposure to a minute amount (0.02 μg/ml) of DNAase. Antisera against Shope papilloma virus did not block the tumorigenic activity of the preparation, and trypsin and chymotrypsin had no effect on it. The extraction with phenol of a partially purified virus preparation also yielded extracts with tumorigenic potency.

Extracts obtained from the domestic rabbit papilloma and submitted to phenolic deproteinization also proved infective and tumorigenic in rabbits of this sort, although the level of "tumorigenicity" was much lower than that of the cottontail preparations. Tests for intact virus, carried out with half of the extracts yielded wholly negative findings.

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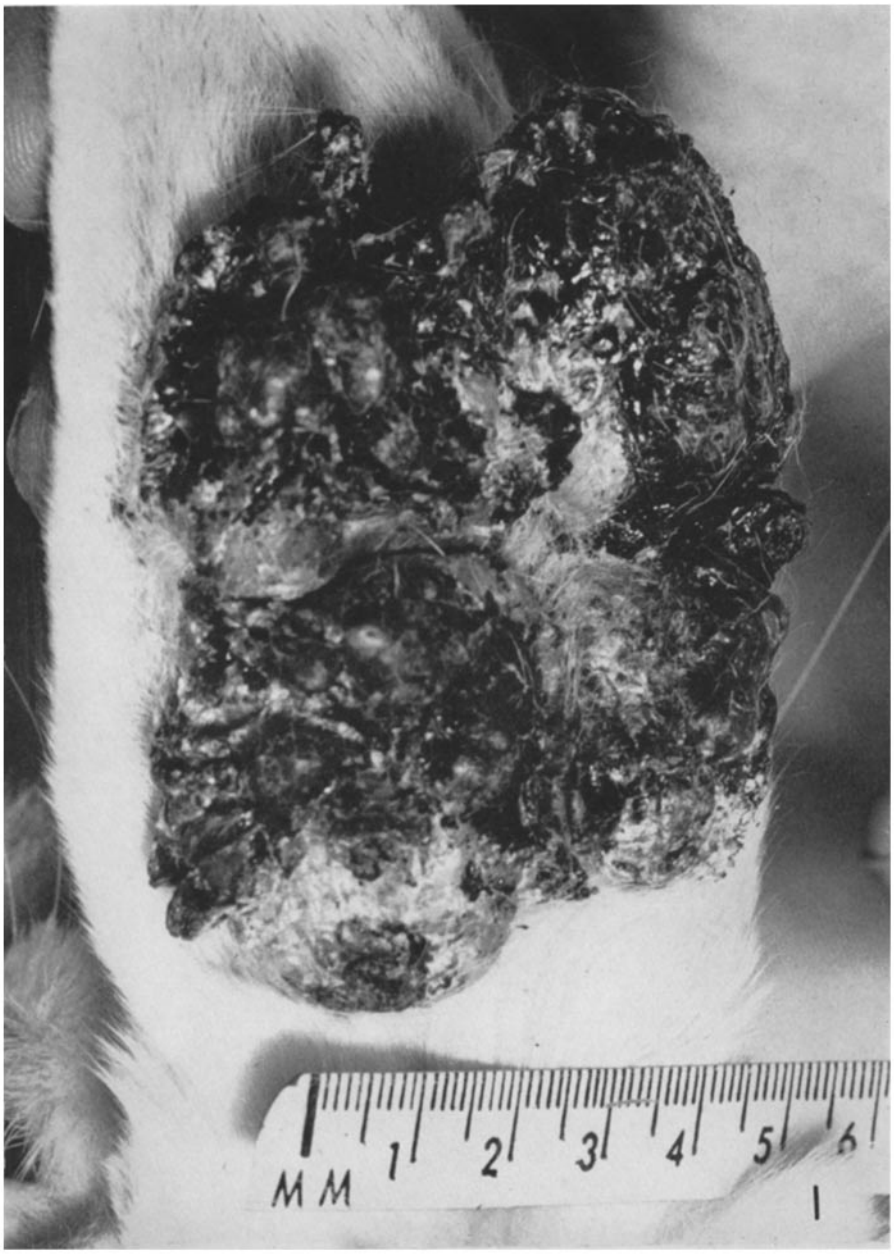
EXPLANATION OF PLATES

PLATE 45

FIG. 1. A huge tumor on an ear of a domestic rabbit at the site of inoculation of nucleic acid preparation derived from cottontail papilloma tissue. Photographed 106 days after inoculation.

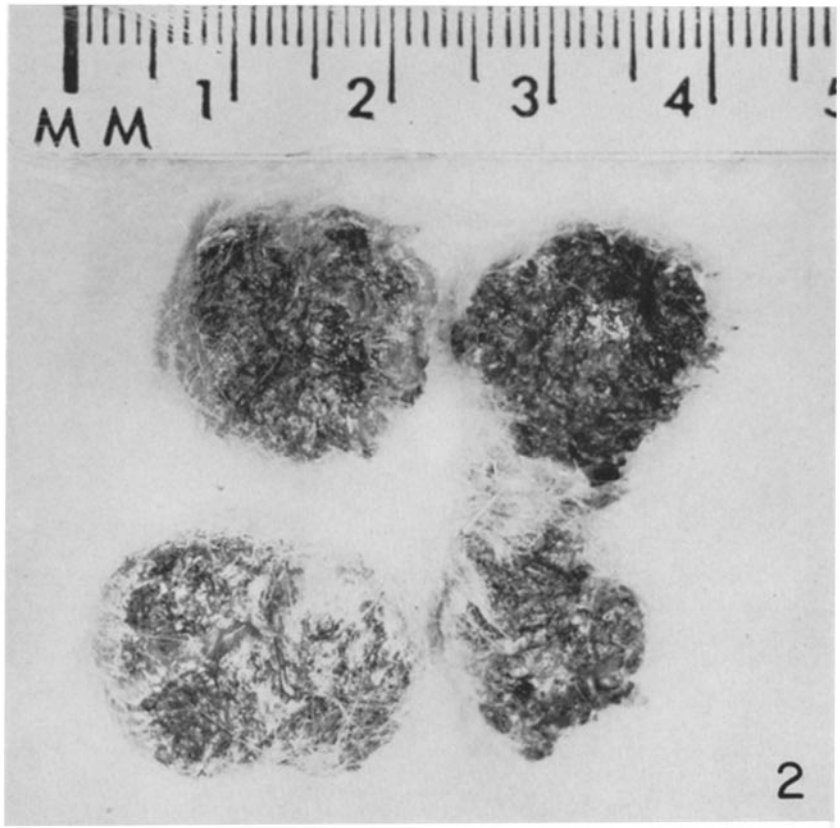


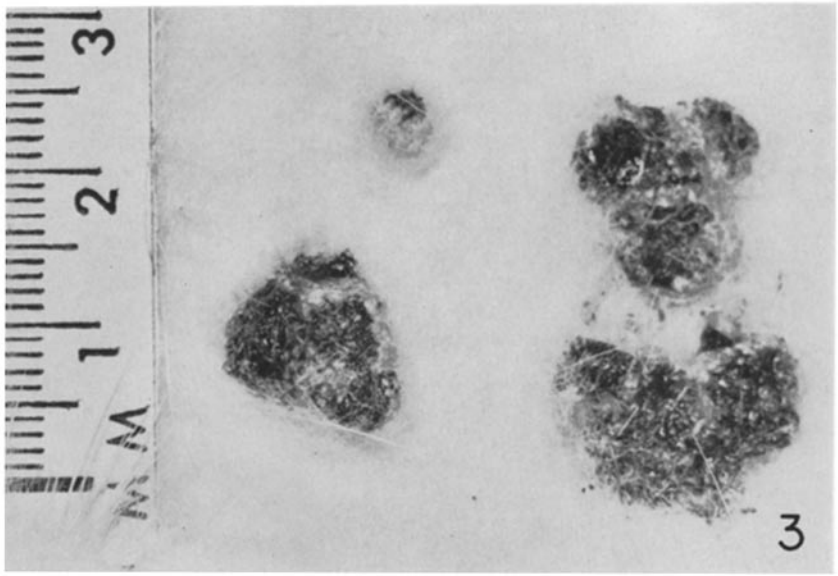
(Ito and Evans: Tumor induction with nucleic acid)

PLATE 46

FIG. 2. Domestic rabbit papillomas growing from 4 adjacent sites inoculated with nucleic acid preparation extracted from partially purified Shope papilloma virus preparation. 68 days after inoculation.

FIG. 3. Domestic rabbit papillomas induced by phenolic extracts of the papillomatous tissues from rabbits of the same breed. 62 days after inoculation.

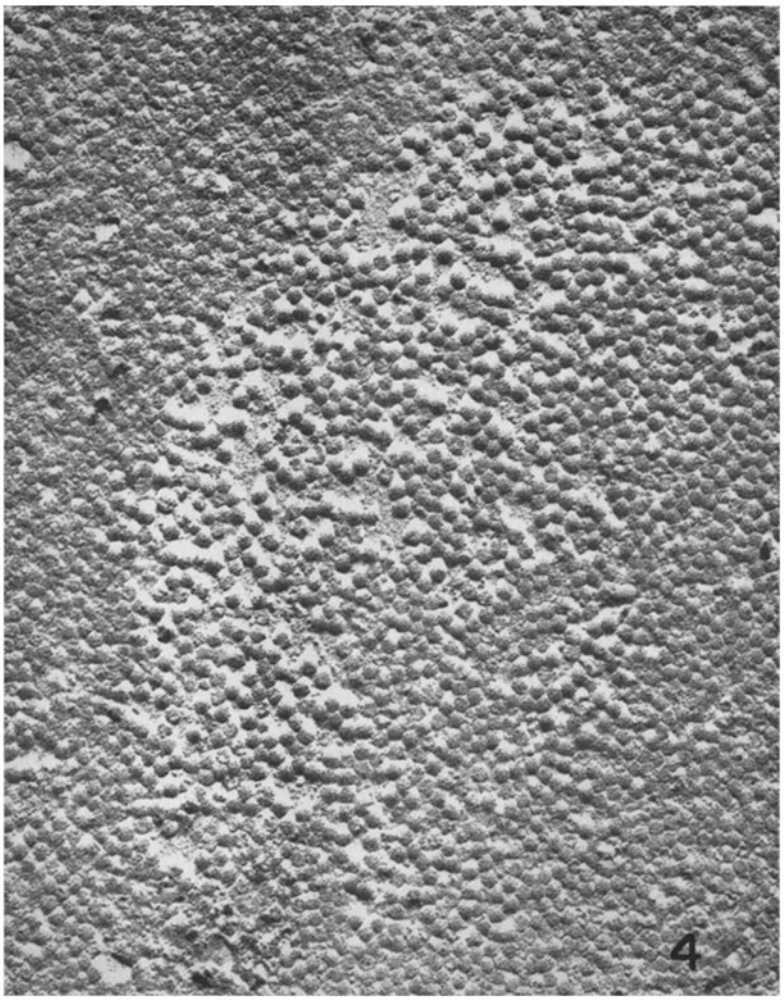




(Ito and Evans: Tumor induction with nucleic acid)

PLATE 47

FIG. 4. Electron micrograph of partially purified Shope papilloma virus preparation, shadowed with palladium. × 37,000.



(Ito and Evans: Tumor induction with nucleic acid)