DEMONSTRATION OF PAPOVAVIRUS IN HUMAN WART TISSUE BY ELECTROPHORESIS

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
EDITH E. STEWARD

1968

This is to certify that the

thesis entitled

Demonstration of Papovavirus
in Human Wart Tissue by Electrophoresis

presented by

Edith E. Steward

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

W. N. Imack.
Major professor

Date June 5, 1968

O-169

ABSTRACT

DEMONSTRATION OF PAPOVAVIRUS
IN HUMAN WART TISSUE
BY ELECTROPHORESIS

by Edith E. Steward

Although the wart virus can be observed by electron microscopy, the agent has not been easily demonstrated by other conventional means.

In this study, macerated wart tissues were examined by agar-gel electrophoresis to detect the wart virus. A series of callus and normal skin tissues were included as controls.

Of 31 wart tissues tested, 27 (87.1%) yielded protein zones. A 20 mm protein zone occurred with greatest frequency (38.7%). This protein zone was not observed in electrophoretic patterns from either normal skin or callus tissue.

Concentrates of wart tissue pools produced a single 20 mm protein zone.

Wart viral particles were demonstrated in concentrates by electron microscopy. The wart virus was also observed in eluates of the 20 mm protein zone.

By special staining technics, the 20 mm zone produced by the wart virus was shown to consist of nucleoprotein.

The migration of the wart virus in an electrical field was shown to be independent of a protein carrier.

The wart virus was shown, by Ouchterlony double diffusion technics, to be serologically related to sera from subjects with histories of warts.

DEMONSTRATION OF PAPOVAVIRUS IN HUMAN WART TISSUE BY ELECTROPHORESIS

By

Edith E. Steward

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1968

This work is respectfully

dedicated to my mother,

Venice E. Steward

whose patience, understanding,

and encouragement made the total

endeavor possible

ACKNOWLEDGEMENTS

The author wishes to express her sincere appreciation to Dr. Walter N. Mack, Professor of Microbiology and Public Health, for his assistance, encouragement and direction throughout this study.

Appreciation is also extended to Dr. Robert B. Foy, Technical Director, Clinical Laboratories, Edward W. Sparrow Hospital, for his suggestions and advice which were incorporated in the development of technics used throughout this study.

Indebtedness is recognized to Edward W. Sparrow Hospital and Dr. John F. Dunkel, Pathologist, for facilities used in the study. Gratitude is also expressed to Dr. Dunkel for his continued interest, encouragement and support given generously through this entire academic endeavor.

Thanks are also given to Drs. A. P. Ulbrich, C. L. Lewis, and J. E. Snyder, for provision of wart, normal skin and callus tissue specimens.

Electron micrographs used in this study were produced by the Electron Microscope Laboratory, Michigan State University.

Sincere thanks are expressed to Mrs. Radene Winterton for technical assistance given at various periods during the study.

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INTRODUCTION

The tumor-producing viruses have been accepted as members of a group denoted Papovaviruses (42). These agents include: Shope papilloma virus of rabbits; polyoma virus of mice; vacuolating agent of monkeys and the human wart virus. These agents range from 40-50 millimicrons in diameter, are icosahedral in shape, and are DNA composed viruses (45).

The infectious nature of the common human wart, verruca vulgaris, was first reported by Jadassohn in 1896 (16). Following the injection of macerated wart tissue into the skin of this investigator and his colleagues, 33 reproductions of warts were accomplished.

During the next quarter of a century, the transmissibility of wart tissue filtrates was demonstrated. In 1907, Ciuffo produced warts by the injection of Berkefield N filtrates of extracted wart tissue (8). The infectiousness of wart tissue filtrates was verified by Serra in 1908 (32), and Wile and Kingery in 1919 (43). In 1921, Kingery produced a second generation wart tumor mass by injecting filtrates of an experimentally produced wart (18).

Demonstration of virus particles in sections of wart tissue was made possible by electron microscopy. Williams et al in 1961 (44), as well as a number of other investigators (3, 24, 35, 36, 37) have employed electron microscopic methods to study the wart virus in tissue.

Procedures used to gain evidence of viruses have proved not to be easily adapted to the study of the wart virus. Prior to 1960, viral isolation attempts by the usual cultural methods were unsuccessful. The chick chorioallantoic membrane was used in an unsuccessful attempt to culture the wart virus by Felsher in 1947 (11). A filterable agent from a wart tissue was isolated on the chorioallantoic membrane by Bivins in 1953 (6) but this was subsequently shown to be a contaminating strain of avian pox virus (34). Attempts to culture the infectious agent of verruca vulgaris and condyloma acuminatum in tissue culture were unsuccessful when conducted by Siegel and Novy in 1955 (33). Primary isolation of the virus in monkey kidney tissue cells was reported by Mendelson and Kligman in 1961 (22) but their results have not been repeated either by themselves or other investigators.

While infected wart tissue cells have produced sharp and distinct cellular degeneration when inoculated on a

particular cell line (15), no agent has been recovered. Serial passage was accomplished only by transfer of infected cells.

In our laboratory, unsuccessful attempts to demonstrate the virus in cell-free fluids from tissue cultures prevented the application of conventional serological procedures to a study of the virus.

Agglutination of the virus in the presence of specific antibody has been shown to occur (4) but only after differential centrifugation and electron microscopic procedures were utilized. The laborious and expensive nature of these technics does not make them readily usable for extensive or routine study of wart virus serology.

The present study demonstrates that the wart virus can be detected in wart tissue by electrophoresis, a readily available and applicable tool of research. This is not the first attempt to apply the electrophoretic process to tissue analysis. In 1952, Demling, upon examining rat and human liver homogenates by electrophoresis, demonstrated that species specific patterns were produced (9). Kessel in 1959 applied the procedure to kidney as well as liver tissues (17). Other investigators have showed that distinctly different electrophoretic patterns

were produced by such divergent tissues as liver, kidney, spleen, mucous membranes and various types of muscle (31). Each type of tissue was distinctive in the number, amount and mobility of proteins produced.

Electrophoretic analysis of malignant soft tissues have shown not only abnormal patterns when compared to similar normal tissues but distinctions between various pathologic states (2).

Similarly, healthy and diseased epidermal tissues have been analyzed (12, 13).

Cultivated viruses also have been successfully submitted to electrophoretic analysis (19, 29, 38). However, in contrast to the present study, free or density-gradient electrophoretic procedures were employed. This is the first instance in which electrophoresis has been used to demonstrate a viral agent in human tissues.

MATERIALS AND METHODS

Tissue processing.

Wart tissues were obtained from individuals by total enucleation (39). Normal skin fragments were obtained at the time of various surgical procedures. Callus specimens were collected by a local podiatrist.

Tissue segments measuring 3 x 5 x 2 mm and weighing 100-200 mg were used. Specimens were stored at -20°C or in 50% phosphate buffered glycerin at 4°C.

Prior to use, each tissue was ground in 1 ml of 0.7 M urea in a borate buffer (0.77 g boric acid, 1 g ethylene-diamine tetra-acetic acid, and 10.1 g Tris dissolved in distilled water and brought to 1 liter volume). Urea was incorporated into the suspending medium to enhance protein release from tissue cells. This substance has been used by other investigators to enhance solubility of epidermal proteins (30). Tissue grinding was accomplished in a glass tissue homogenizer (Corning #7725) either manually or by the use of a motor-driven pestle (Model #77-717, Eberbach K and L Scientific Co.). An ice bath surrounded the receptacle holding the tissues during the automatic grinding. The majority of wart tissues and all of the

normal skin and callus segments were mechanically ground by three 4 minute grindings at approximately 200 rpm. A few wart tissues were mechanically ground by three 2 minute grindings at approximately 400 rpm. The suspensions were allowed to settle for 18 hours at 22°C. Supernatant fluids were used for electrophoretic studies.

Influence of glycerol storage medium on protein detection from tissue samples.

Prior to use, wart tissues used in this study were stored either at 4°C in 50% buffered glycerol or at -20°C without any suspending medium. The effect of the storage medium was evaluated.

Two wart tissues were used, one having been stored in glycerol while the other had not. Before use, any residual glycerol was carefully blotted from the refrigerated tissue.

1. Effect of maceration speeds.

Triplicate 3 x 25 x 2 mm segments were obtained from each tissue. One representative segment from each tissue was ground 4 minutes at 200 rpm, while another segment from each tissue was ground for 4 minutes at 800 rpm. The remaining fragment from each sample was ground for 4 minutes at 1200 rpm. The grinding protocol was repeated

three times during a 4 hour period. Each tissue fragment was ground in 1 ml of 0.7M urea in borate buffer (pH 8.2). Following maceration, each suspension was kept at 22°C for 18 hours. Electrophoretic evaluation was conducted on each supernatant fluid.

2. Effect of storage-time and pulp contact.

Supernatant fluids from the glycerol-preserved tissue ground at approximately 200 and 1200 rpm and those from the non-glycerol preserved tissue ground at approximately 200 and 800 rpm were divided into two portions. One aliquot from each pair was stored for one week at 4°C in sealed, small tubes. The remaining aliquot of each pair was returned to the pulp from which it was derived. These mixtures were stored for one week at 4°C in sealed, small tubes. All suspensions were gently shaken by hand for 5 seconds each day of storage. At storage termination, the mixtures were allowed to settle and the supernatant fluids used for electrophoretic evaluation.

Electrophoresis.

A Spinco modified Durrum electrophoretic cell (Beckman Instrument Co.) was adapted for use with agar-gel strips. A borate buffer (18.5 g boric acid and 2.5 g sodium hydroxide dissolved in distilled water and brought to 1 liter volume), pH 8.2, was used within the cell. Eight agar-gel strips were prepared for each test. To prepare strips, 5 ml of molten 0.6% agarose (SEAKEM - Bausch and Lomb) solution in borate buffer were applied to 3.5 x 15 cm film leader strips (DuPont P40B) and the agar allowed to solidify. A 25 x 3 mm paper strip (Spinco #319328) was pressed into the agar creating a sample well 25 x 3 x 2 mm deep, equidistant from each end of the strip. The prepared strips were placed in the cell. Prior to sample application, excess moisture was removed from the sample well by blotting with filter paper wicks (Whatman #1) for a few seconds. Twenty microliters of sample were applied to each strip. Following sample application, the cover of the cell was put in place, tightly closed and migration begun.

Migration of proteins was allowed to continue at a constant voltage of 145 volts. Power to the cell was provided by a Spinco Duostat. Between 18 and 23 milliamps

were maintained. Reproducible protein zone migration patterns were obtained by using a polychrome dye marker (Gelman Instrument Co.). The migration time was approximately 70 minutes. After migration, the agar strips were fixed in 90% methanol for 10 minutes. Following fixation, the strips were washed in distilled water and dried at 100-105^{o}C for 30 minutes.

A 0.4% solution of Buffalo black dye (Allied Chemical Co.) in acidified 50% methanol was used to demonstrate the presence of protein. The strips were rinsed in 2% aqueous acetic acid to reduce background color and then air dried.

Wart tissue ultracentrifugation.

Pools of wart material which had been stored at -20°C or glycerinated and stored at 4°C were concentrated by ultracentrifugation. Each pool of 2.89 g was ground by hand with a mortar and pestle for one hour in 75 ml of Hanks' balanced salt solution (14). Suspensions were centrifuged at 2000 rpm for 10 minutes to remove gross particles. The resulting supernatant fluid was placed in rotor 30 of a preparatory ultracentrifuge and spun at 78,480 x g for one hour. The supernatant fluid was removed and clarified at 15,000 rpm for 10 minutes in a multi-speed centrifuge. The sediment was discarded. The supernatant fluid was placed in rotor 50 and subjected to 54,333 x g for one hour. Seven tenths molar urea in borate buffer was used to resuspend the pellet. Amounts of buffer used to resuspend pellets ranged from 0.5 ml to 3.0 ml. Portions of the samples were submitted to electrophoresis as well as other studies.

Ultra-violet absorption studies.

A pool of 2.89 g of wart tissue, stored at 4°C in 50% glycerin, was concentrated by two ultracentrifugation cycles as previously described. A concentrated preparation, consisting of the final pellet resuspended in 0.5 ml of distilled water, was prepared. A 1:20 dilution was made by diluting 0.15 ml of the concentrate to a 3.0 ml volume with 0.7M urea borate buffer. A 1:100 dilution was obtained by further dilution of the 1:20 suspension.

A portion of the concentrate was purified by two additional ultracentrifugation cycles. The final pellet was resuspended in 0.5 ml of 0.7M urea borate buffer. A 1:100 dilution was prepared by diluting 0.03 ml of the concentrate to a 3 ml volume with 0.7M urea borate buffer.

UV absorbance measurements of the 1:20 and 1:100 dilutions of the 2 cycle concentrate and the 1:100 dilution of the purified concentrate obtained following 4 ultracentrifugation cycles were made in a Beckman DU Spectrophotometer. Absorbance readings were obtained at 5 nM increments from 246 to 290 nM.

Preparations for electron microscopy.

A pool of glycerin-stored wart tissues was ultracentrifuged as above but with two additional cycles to

remove debris as well as any traces of the buffer suspending medium. The sediment was resuspended in small amounts of distilled water. Minute amounts of the resuspended sediment were placed upon carbon prepared screens. Equal volumes of a 2% phosphotungstic acid solution were placed on each screen (26). Immediately the solution was permitted to flow onto filter paper by touching the edge of the filter paper to the edge of the electron microscopic screen. After drying, in a dust-free atmosphere, the screen was observed in the electron microscope.

An electrophoretic procedure, using the glycerinated wart tissue concentrate, was employed to obtain a preparation for electron microscopy. Two of eight agar strips were stained following electrophoresis to locate protein zones. Using the stained patterns as guides, the agar of the six remaining strips was cut so that the protein zone was midway in a 15 mm agar segment. Corresponding 15 mm agar segments from each of these six strips were removed from the supporting medium and pooled in 1 ml of distilled water. Similar eluates were obtained from 15 mm agar segments preceding and following this protein-containing segment. The origin of sample application was included in the agar segments which preceded the protein-containing

segment. Eluates of these three segment pools were separately submitted to electron microscopy.

A similar procedure was conducted to evaluate agar segments which did not include the origin of sample application. Agar segments of 15 mm width were cut to encompass the detected protein zone. Corresponding 15 mm agar segments from six strips were pooled and eluted in 1 ml of distilled water. A similar eluate was derived from 10 mm agar segments preceding this protein-containing segment. This eluate did not contain the origin of application.

Special stains of concentrated wart virus preparations.

Concentrated wart virus preparations were derived by ultracentrifugation as previously described. Twenty microliter quantities of concentrate were submitted to electrophoresis in the usual manner.

At the conclusion of electrophoresis, agar-gel strips were fixed for 3 hours in either a 2% acetic acid in 50% ethanol or a 2% aqueous acetic acid solution (40). Subsequently, agar-gel strips were washed and dried in the manner previously outlined.

Agar-gel strips were immersed for one hour in a 2% aqueous solution of Pyronine Y (Hartman-Leddon Co., Inc.) for the detection of nucleoprotein (40). Excess stain was removed by a sodium acetate-acetic acid buffer wash (pH 4.7, 0.2M).

A glycoprotein stain, based on the Schiff's reaction and recommended by Uriel in 1964 (40), was used with the following modifications. A "cold Schiff's" reagent (20) was employed and a sulfurous acid solution prepared in the manner recommended by McManus in 1960 (21) was used as a final rinse.

A staining procedure for lipoprotein material, used by Uriel in 1964 (40), was employed with the following modifications. The stain used contained a 4:1 mixture of Oil Red O (Hartman-Leddon Co., Inc.) and fat Red 7B (Ciba Products Co.) dyes. The staining solution was prepared by saturating warm absolute methanol with the dye mixture. Following dissolution, a 70% methanol solution was prepared by the addition of sufficient distilled water. Agar-gel strips were stained for one hour in this staining mixture.

Electrophoresis of E. coli T_3 bacteriophage.

A 24 hour nutrient broth culture of E. coli type B was inoculated on nutrient agar plates. The organisms were distributed over the surface with a glass spreader. Inoculated plates were incubated at 37°C for 18-24 hours. At the end of the incubation period, three drops of T_3 bacteriophage were inoculated onto each plate and uniform dispersal of the bacteriophage was accomplished by a glass spreader. The plates were reincubated at 37°C for 18-24 hours.

Following incubation, three ml of sterile distilled water were pipetted onto each plate, and the crude lysate suspended with a glass spreader. The lysate was centrifuged at 9,2000 rpm for 30 minutes in a multispeed centrifuge to remove debris. The supernatant fluid was then filtered through a Millipore HA filter. The filtrate was stored at 4°C until 600 ml of lysate were obtained. Plaque assay was done by the method of Adams (1).

The T_3 virus was concentrated at 29,000 rpm in a rotor 30. The pellet was resuspended in 3 ml of the supernatant fluid, and clarified by centrifugation at 9,200 rpm for 20 minutes in a multispeed centrifuge. The resulting supernatant fluid was reconcentrated at 31,000

rpm for 1 hour in a rotor 50. The final pellet, resuspended in minimal quantities of sterile, distilled water, was submitted to electron microscopy and electrophoresis.

In the electrophoretic procedure, a 20 microliter sample was applied to a 3 x 25 mm filter strip as was done with other samples. In a second electrophoretic procedure, a 5 microliter sample was applied to a narrow 25 mm slit cut in the agar in place of the filter strip.

Immuno-electrophoretic technics.

Agar-gel strips were prepared as previously described. A single 3 x 25 mm filter strip or two strips of the same size (2 x 10 mm or 5 mm square) were used for sample application. Filter strips were pressed into the agar at a point equidistant from each end of the agar strip. When two filter strips were used for sample application, they were placed either 8 mm apart for the 2 x 10 mm strips or 16 mm apart for the 5 mm squares. Twenty microliters of sample were applied to the 3 x 25 mm filter strip while 10 microliter quantities were applied to filter strips of smaller dimensions.

Immediately following the migration period, the agar strips were refrigerated for 20 minutes to firmly solidify

the agar, softened in the electrophoretic process. Upon hardening of the agar, the sample applicator strip was removed and a central, longitudinal trough (1 x 70 mm) was cut in the agar parallel to the sides of the strip. Agar was aspirated from the trough by means of a suction apparatus. Upon filling the trough with either the antibody or antigenic material, the agar strips were incubated three days at room temperature in a closed container saturated with water vapor.

Following incubation, the agar strips were washed in 0.9% w/v NaCl for 2 days to remove unreacted proteins. A one hour wash in distilled water was sufficient for salt removal. The strips were then dried and stained for protein in the usual manner.

Simple immuno-diffusion technics.

1. Micro-modified Ouchterlony double diffusion. Hyland "Immuno-Plates", Pattern B 085-072, (Hyland Laboratories) were used. The unit consisted of a polystyrene dish with 1 x 3 inch cavity containing 4 ml of agar-gel. The agar consisted of Difco Special Noble agar (Difco Laboratories) 2%, glycine 7.5%, NaCl 1%, and Sodium azide 0.1%. The pH of the agar ranged from 7.0 to 7.2. Each plate contained three series of pre-cut wells, 3 mm in diameter and 7 mm apart.

After filling the wells with the reactants, the "Immuno-Plate" was incubated at 22°C for 48 hours in sealed, humidified containers. Following incubation, the agar was washed with saline solution for 48 hours to remove nonreactive protein. Subsequent washes with distilled water for at least 24 hours were sufficient for salt removal. The 1 x 3 inch section of agar, dissociated from the supporting polystyrene tray during the washing procedures, was placed on a portion of film leader strip (DuPont P-40B) and dried at 45°C for 18 hours. A stained, permanent record of any reaction was obtained by staining the dried strip with Buffalo black.

2. Double diffusion with cellulose acetate medium. Sepraphore III (Gelman Instrument Co.) cellulose acetate strips were used. A 1 x 3 inch strip was used for each test. Prior to use, cellulose acetate strips were immersed in 0.04 M veronal buffer, pH 8.6, for at least 4 hours. The buffer was prepared by dissolving 1.38 grams of diethyl barbituric acid and 7.7 grams of sodium diethyl barbiturate in 1 liter of distilled water. After soaking, the cellulose acetate strips were drained of excess buffer solution and placed in humidified containers which would be used for the duration of the test. Two by five millimeter and two by ten millimeter filter strips (Spinco #319328) were used for sample application. Two filter strips were placed on the cellulose acetate, parallel to the long axis of the strip and at a 10 mm distance from each other. Excess buffer solution was carefully blotted from the filter strips and the supporting medium immediately prior to sample application. Samples were applied to the filter strips in such a way that immediate overflow was avoided.

Following sample application, the cellulose acetate strips were sealed in the humidified chambers and incubated at 22^{o}C for 24 hours. After incubation, cellulose acetate strips were washed in saline solution

for one hour. A distilled water wash of 30 minutes was conducted for salt removal. Strips were placed on blotter paper and allowed to completely dry. Immuno-precipitates on the cellulose-acetate strips were stained with Buffalo black. For permanent preparations the strips were again dried at 22°C. Clearing of the strips was accomplished by submerging them in microscope immersion oil. Strips were mounted between two glass slides with histologic mounting medium.

RESULTS

In this study, the technic used for viral protein detection in tissue was found satisfactory after preliminary trials with different buffers, agars and varying potentials.

Table 1 illustrates the effects of several environmental conditions upon soluble proteins obtained from triplicate segments of two wart tissues. One wart tissue had been stored in glycerol, while the other had not.

One segment from each tissue was ground at the usual speed of 200 rpm while the remaining segments were ground at 800 and 1200 rpm. To analyze the effect of maceration speeds on protein detection, a portion of the supernatant fluids was tested without delay. Table 1 shows that as the maceration speed increased from 200 to 800 rpm, loss of protein zones occurred regardless of glycerol storage. When the tissue stored without glycerol was ground at the higher speed, a decrease in zone intensity as well as a zone loss was observed. The lower temperature obtained by use of an ice bath around the tissue grinder was not sufficient to prevent protein loss at high speeds.

The remaining portion of two supernatant fluids from each tissue was used to study the effect of storage on

Table 1. Stability of soluble protein fractions detected from wart tissue by electrophoresis.

Effect of maceration speed, storage-time, sediment contact and glycerol storage

Homogenate supernatant fluid-freshly prepared				Homogenate supernatant fluid-stored at 4 C for one week	
				Without pulp contact	With pulp contact
Wart Tissue No.	Storage suspending medium[1]	Grinding speed (rpm)	No. zones/ zone intensity	No. zone/zone intensity	No. zone/zone intensity
1	None	200	5/* * * + +	3/* * +	5/* * * + +
		800	4/+ + + + ±	4/+ + + + ±	4/* * * +
		1200	4/+ + + + ±		
2	Glycerol	200	3/* * *	2/+ +	2/* +
		800	2/* *		
		1200	2/* *	1/+	2/* *

Key: * intense protein zone, + faint protein zone, ± questionable zone

[1]Refers to storage suspending medium prior to any treatment. All tissues were ground in 1 ml quantities of 0.7M urea borate buffer.

suspended proteins. One half of each supernatant fluid was returned to the original sediment. Fluids with and without pulp were stored at 4°C for one week. Table 1 shows that storage of supernatant fluids without pulp resulted in protein loss. The loss of protein was retarded or prevented by storage of supernatant fluids with sediment from which the proteins originated.

Glycerol storage of wart tissues did not influence protein detection. Tissues stored with or without the suspending medium exhibited similar electrophoretic patterns. Storage of supernatant fluids from tissue homogenates resulted in protein loss, while supernatant fluid-pulp mixtures did not do so. In addition, identical electrophoretic patterns were produced by concentrated wart tissue pools regardless of previous storage in glycerol.

Table 2 gives results of wart tissue electrophoretic analysis. It will be observed that of 31 wart tissues processed, 27 (87.1%) exhibited one or more protein zones. At least 2 protein zones were produced from 22 (70.9%) of the 31 wart samples. Over half (54.8%) of the wart tissues yielded at least 3 zones. Although 5 of the 31 wart tissues (16.1%) exhibited only one protein zone, 4

Table 2. Electrophoretic analysis of 31 wart tissues.

No. of samples	No. of zones produced per sample	% of total tissues studied
4	0	12.9
5	1 only	16.1
27	1 or more	87.1
22	2 or more	70.9
17	3 or more	54.8
7	4 or more	22.6
4	5	12.9

tissues (12.9%) produced as many as 5 zones. An example of the multiple protein zone pattern produced by the majority of wart tissues is shown in Fig. 1b.

To summarize the different zone patterns produced from wart tissue electrophoresis, the protein migration distances along with the number of zones/sample were tabulated (Table 3). An evaluation of the zone migration distribution is illustrated in Fig. 2. The migration distance, in millimeters from origin to zone, was plotted against the number of subjects whose wart tissues yielded similar protein zones.

An overall inspection of Fig. 2 indicates that the majority of protein zones occurred in the 15 to 25 mm or narrower 20 to 25 mm area. One protein zone was noted to occur with greater frequency (38.7%) than any other. This zone occurred 20-21 mm from the origin and was demonstrated in 12 of the 31 wart tissues. Studies were undertaken to identify this zone.

For control, normal skin and callus tissue were also submitted to electrophoresis.

Table 4 illustrates the variety of sources from which skin samples were collected. It can be seen from Table 5 that although 24 normal skin samples were electrophoretically

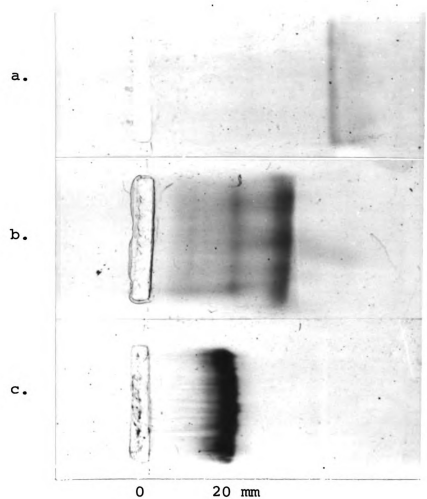


Fig. 1. a. Typical normal skin electrophoretic pattern demonstrating one rapidly migrating protein zone.

b. Migration pattern of a wart tissue fragment exhibiting multiple zones including one 20 mm from the origin.

c. Electrophoresis of concentrated viral particles yielded an intensely stained zone 20 mm from the origin.

Table 3. Protein migration patterns from 31 wart tissues examined electrophoretically.

Sample No.	No. of protein zones*	Protein zone location (mm from origin)
1	3	6-20-33
2	3	7-20-35
3	3	10-20-40
4	1	52
5	5	10-20-29-32-35
6	3	44-49-53
7	1	34
8	1	48
9	3	10-20-32
10	3	41-45-48
11	3	38-52-54
12	3	15-27-39
13	5	12-25-35-40-45
14	2	14-38
15	4	12-35-37-41
16	4	12-25-38-41
17	5	13-25-37-45-51
18	3	10-20-33
19	2	20-34
20	2	20-35
21	2	21-34
22	1	53
23	3	10-20-31
24	5	10-20-24-30-32
25	4	11-20-28-30
26	1	25
27	2	10-25

*4 of the samples exhibited no protein zones.

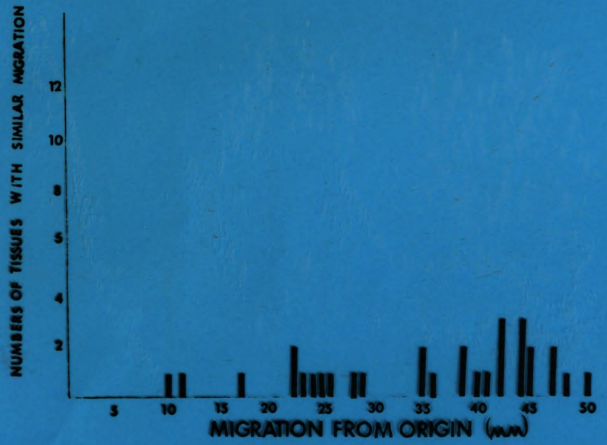


Fig 3. Migration Distribution of Proteins from callus skin tissues

Fig 3. Migration Distribution of Proteins from Collagen Strip Tissues

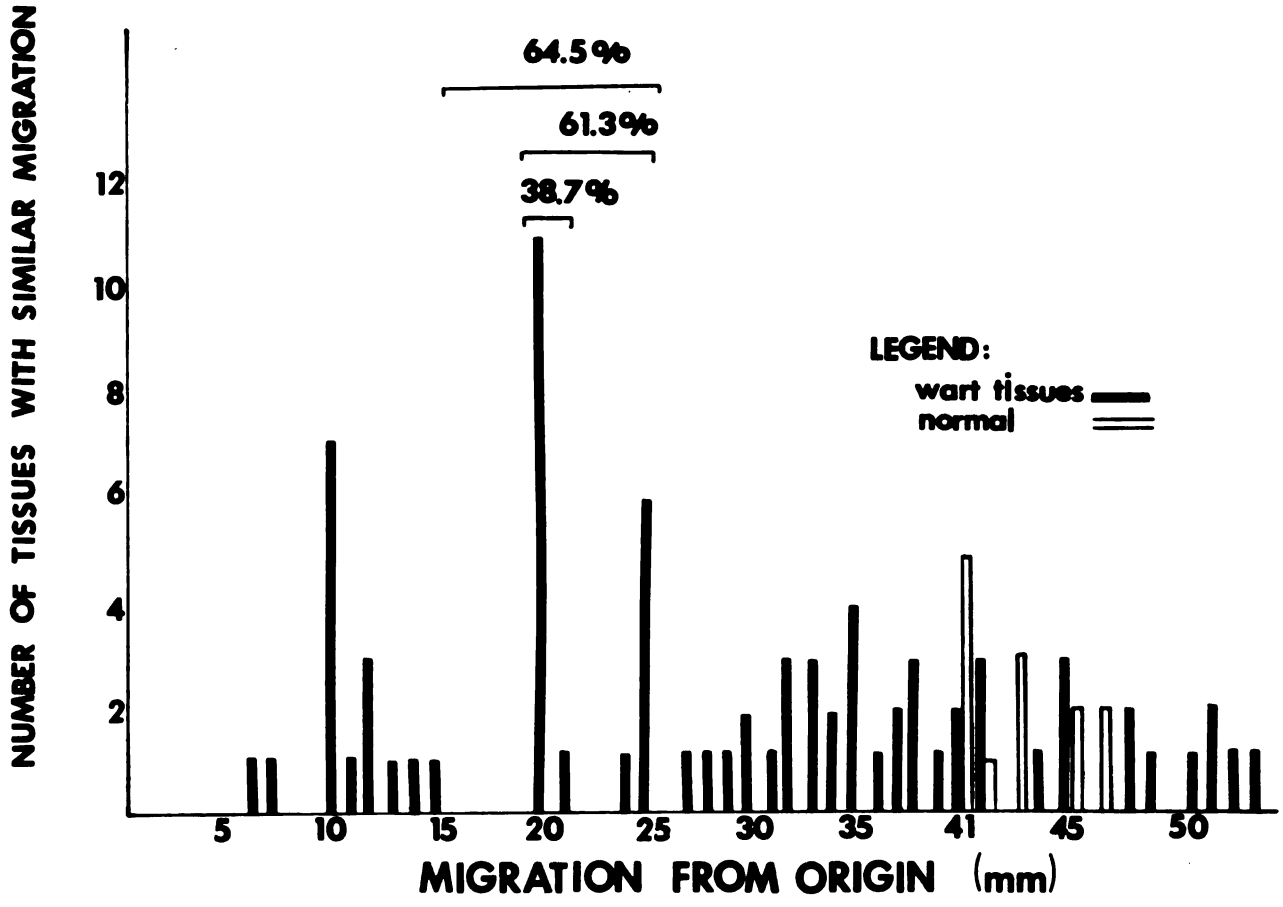


Fig. 2. Migration distribution of proteins from wart and normal skin tissues.

Table 4. Source of normal skin tissues.

Number of specimens	Origin of specimen
13	abdomen
4	breast
2	leg
1	scrotum
4	unknown

Table 5. Protein migration patterns from 24 normal skin tissues examined electrophoretically.

Sample No.	No. of protein zones*	Protein zone location (mm from origin)
1	1	47
2	1	43
3	1	47
4	1	41
5	1	43
6	1	45
7	1	40
8	1	43
9	1	40
10	1	40
11	1	40
12	1	40
13	1	45

*11 of the 24 samples exhibited no protein zones.

analyzed, 13 or 54.1% yielded only one protein zone. Forty-five point nine percent exhibited no protein zones. The migration distances of the protein zones derived from skin tissues are tabulated in Table 5. Fig. 1a illustrates the type of electrophoretic pattern produced from skin fragments.

Fig. 2 compares the migration distribution of protein zones obtained from normal skin tissues and wart tissues. It will be noted that no zones migrating less than 40 mm were detected for any of the 24 normal skin fragments tested. However, a number of proteins with less mobility were noted from the wart tissues.

While the electrophoretic patterns of wart and normal skin tissues were markedly different, the possibility that callus skin might represent a more appropriate control was considered. Therefore, twelve callus tissue samples were electrophoretically analyzed. Table 6 tabulates the origin of callus specimens. Table 7 illustrates that 10 of 12 callus tissues tested (83.3%) yielded more than one protein zone. One half of the samples examined exhibited at least 3 zones. While no protein zones were detected in 2 of the 12 tissues (16.7%), as many as 6 zones were demonstrated from a single callus sample. Table 8 lists the protein

Table 6. Source of callus skin tissues.

Number of specimens	Derivation of specimen
6	metatarsal area
4	toe
2	undesignated area on foot

Table 7. Electrophoretic analysis of 12 callus tissues.

No. of samples	No. of zones produced per sample	% of total tissues studied
2	0	16.7
2	1 only	16.7
10	1 or more	83.3
8	2 or more	66.7
6	3 or more	50.0
4	4 or more	33.3
1	6	3.3

Table 8. Protein migration patterns from 12 callus skin tissues examined electrophoretically.

Sample No.	No. of protein zones*	Protein zone location (mm from origin)
1	3	25-42-47
2	4	12-28-45-50
3	2	26-42
4	1	35
5	3	24-41-47
6	4	22-36-39-44
7	2	22-42
8	4	29-40-44-48
9	6	10-17-23-35-39-44
10	1	45

*2 of the samples did not exhibit protein zones.

zone migration distances obtained from callus tissue electrophoretic patterns. An overall inspection of the data presented illustrates the wide range of migration distances encountered (from 10 to 50 mm).

Fig. 3 (an overlay) gives a comparison of the protein zone migration distances with the number of subjects whose callus tissues yielded similar protein zones. By superimposing this overlay over Fig. 2, a comparison of the migration distribution of proteins from callus samples with wart and normal skin tissues can be made. By such a comparison, it is obvious that callus skin had characteristics in common with both wart and normal skin. While normal skin samples failed to yield any proteins which migrated less than 40 mm, callus specimens demonstrated numerous slow and rapid migrating protein zones. However, the very slow migrating proteins common to wart tissue were not noted with regularity in callus samples. In addition, the 20 mm protein zone observed in 38.7% of wart samples was not observed in either callus or normal skin tissues.

Steps to identify the 20 mm protein zone were undertaken. Wart virus particles in 4 wart tissue pools (2.89 g each) were concentrated in the ultracentrifuge. Each pool consisted of more than 40 individual wart tissues. Three

pools were made up of tissues stored in 50% buffered glycerol at 4°C while the remaining pool consisted of wart tissues stored at -20°C without glycerol.

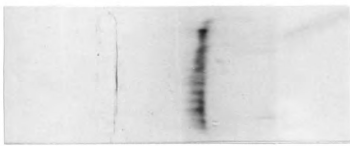
Following electrophoresis, the concentrate from tissues stored in glycerol at 4°C demonstrated an intensely stained protein zone 20 mm from the origin Fig. 2a. An identical electrophoretic pattern was produced by the concentrate of wart tissues stored at -20°C (Fig. 4b).

Electrophoresis was conducted on samples of a wart tissue pool at various stages in the concentration process. An electrophoretic analysis of the original wart pulp failed to demonstrate any protein zones. In contrast, a preparation obtained after the first ultracentrifugation cycle showed a faint protein zone, 20 mm from the origin. After a second ultracentrifugation cycle, the resuspended pellet produced an intensely stained 20 mm protein zone.

A portion of an ultracentrifuged concentrate was tested for purity in an analytical centrifuge. A single boundary was observed, which moved at a uniform rate and indicated the presence of a single component.

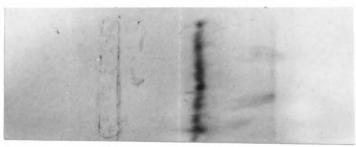
In preliminary attempts to characterize the 20 mm component, ultra-violet absorbance measurements were made on ultracentrifuged preparations. Fig. 5 illustrates the ultra-violet absorbance curves obtained from analysis of

a.



Origin 20 mm

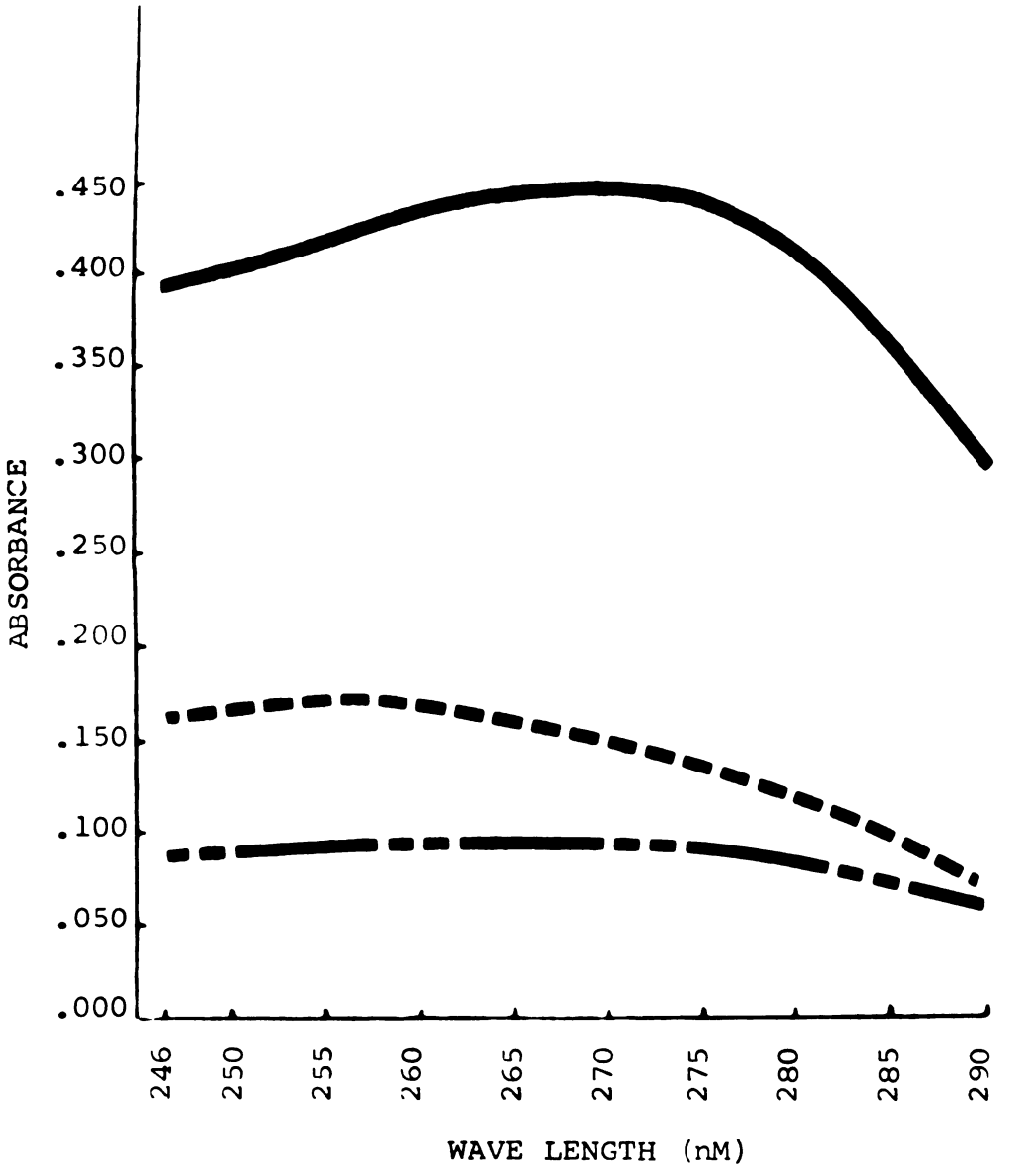
b.



Origin 20 mm

Fig. 4. a. Electrophoretogram of concentrate from glycerin-stored wart tissues.

b. Electrophoretogram of concentrate from -20°C stored wart tissues.



Key:

1:20 dilution of 2 cycle ultracentrifugate
1:100 dilution of 2 cycle ultracentrifugate
1:100 dilution of 4 cycle ultracentrifugate

Fig. 5. Ultra-violet absorbance measurements: 1:20 and 1:100 dilutions of a 2 cycle concentrate; 1:100 dilution of a 4 cycle ultracentrifuged preparation.

2 and 4 cycle ultracentrifuged preparations. The 1:20 dilution of the crude 2 cycle concentrate produced a curve with a 270 nM peak indicating the presence of protein. There was no peak obtained at 260 nM which would indicate the presence of nucleoprotein. Attempts to detect nucleoprotein in this preparation were hampered by the overall protein concentration. From the curve obtained upon further dilution (1:100) of this preparation, it could be concluded that insufficient nucleoprotein was present for adequate UV absorbance. However, it can be observed from Fig. 5 that UV absorbance measurements made on a purified 4 cycle preparation (1:100 dilution) suggested the presence of nucleoprotein by production of a peak in the vicinity of 260 nM.

The presence of nucleoprotein in wart tissue concentrates was confirmed by special staining procedures. Fig. 6 shows the electrophoretic pattern produced by an ultracentrifuged preparation. The 20 mm zone is very distinct. When the zone was stained with a specific nucleoprotein stain, Pyronine Y, a positive reaction was obtained. After applying an Oil Red O-Fat Red 7B staining mixture to a 20 mm zone, the protein zone remained unstained indicating a lack of lipoprotein material (Fig. 7).

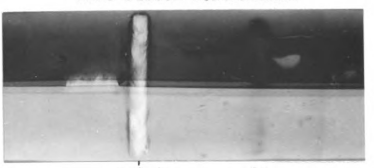
SERUM CONTROL

Origin

WART TISSUE CONCENTRATE

a.

b.

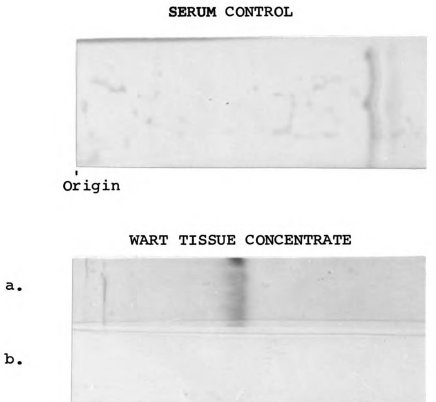


Origin 20 mm

a. Reaction with counterstain.

b. Reaction without counterstain.

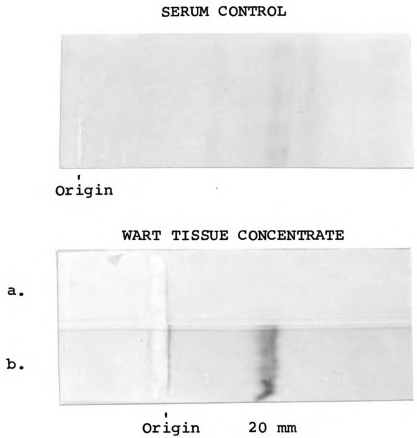
Fig. 6. Stain for nucleoprotein applied to 20 mm protein zone.



a. Reaction with counterstain.

b. Reaction without counterstain.

Fig. 7. Stain for lipoprotein applied to 20 mm protein zone.



a. Reaction without counterstain.

b. Reaction with counterstain.

Fig. 8. Stain for glycoprotein applied to 20 mm protein zone.

Application of the Periodic Acid Schiff's staining procedure failed to detect glycoprotein in a 20 mm protein zone (Fig. 8). As will be observed in Figs. 6, 7, and 8, appropriate control strips were included.

For further identification of the 20 mm protein zone, another 4 cycle concentrate was prepared from a wart tissue pool. A portion of the pellet was viewed in the electron microscope. Fig. 9 demonstrates the many viral particles observed, with no debris. Morphologically the virus particles appear identical with the wart virus (44).

Fig. 1c illustrates the intensely stained protein zone, 20 mm from the origin, which was produced by an aliquot of the wart virus preparation. When another similarly stained protein zone was eluted and this material examined under the electron microscope, virus particles were observed. This is shown in Fig. 10. Although this zone was produced following electrophoresis of a less purified concentrate, many virus particles were found.

Similar eluates, obtained from areas preceding and following the protein-containing fraction, were examined by electron microscopy. The eluate that contained the origin of application exhibited a reduced number of viral particles. An eluate preparation which preceded the 20 mm protein zone but did not contain the origin of application

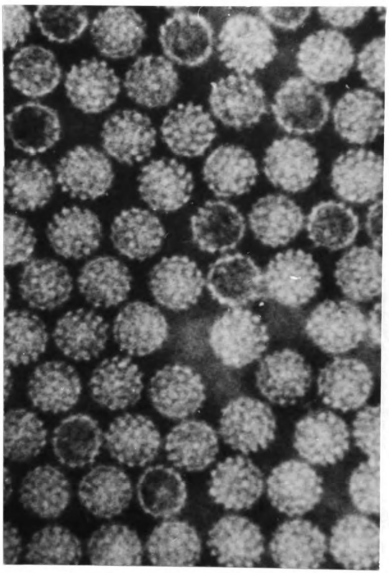


Fig. 9. Wart tissue pools following ultracentrifugation yielded numerous viral particles as demonstrated by electron micrographs.

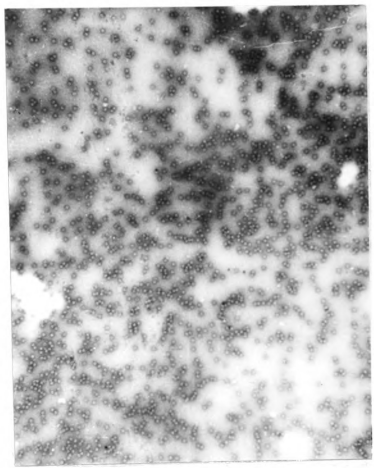


Fig. 10. Electron microscopy of eluted 20 mm protein zone.

showed only an occasional viral particle. Only rarely were viral particles observed from the eluate of the area that followed the 20 mm protein zone.

To demonstrate that the 20 mm zone was due to the virus and not to some other protein, normal skin tissue samples were ground as before but in a suspension of concentrated wart virus particles instead of 0.7 M urea borate buffer. Thus, a virus suspension was added to normal tissue. Following electrophoretic tests on these samples, the 20 mm protein zone was again detected. These tests prove that the 20 mm zone was produced by large amounts of viral protein.

To prove that the protein zone was due to virus and not to viral associated globulins, a virus of the same diameter but not related to the wart virus was tested electrophoretically. An *E. coli* T_3 bacteriophage suspension was concentrated in the ultracentrifuge and the sediment resuspended in distilled water. Fig. 11 shows an electron micrograph of the concentrated bacteriophage preparation.

The electrophoretogram of this virus preparation is illustrated in Fig. 12a. Although a single protein zone occurred, 25 mm from the origin, a number of channels existed horizontally throughout the zone. It was noted that when the 3 x 25 mm filter strip was used for application of this virus prior to electrophoresis, a certain amount of

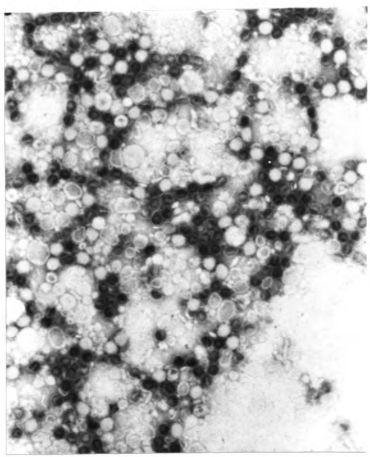
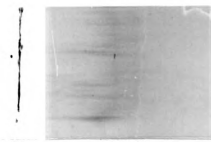


Fig. 11. Electron microscopy of *E. coli* T_3 bacteriophage concentrated preparation.

a.



b.

Fig. 12. Electrophoretograms of *E. coli* T_3 bacteriophage.

a. Channelling effect produced by application of 20 microliter sample on 25 x 3 mm filter strip.

b. Five microliter portion of sample applied to narrow 25 mm slit cut in agar in place of filter strip.

virus was hindered from migration by the paper fibres, and channelling of the sample resulted.

To avoid the channelling effect, it was necessary to apply the sample to a narrow 25 mm slit cut in the agar in place of the filter strip. The protein zone on this pattern appears faint since only 5 microliters of sample could be applied to the agar slit. Fig. 12b demonstrates the 25 mm zone without channelling.

The results obtained from electrophoresis of individual wart tissues indicated that the wart virus was frequently being encountered. The possibility that homologous sera might contain antibodies to these tissue viral particles was investigated. As a control for such a study, it was necessary to prove that antibodies to normal skin components were not also present in the sera of such subjects. Immuno-electrophoretic procedures were conducted. Eleven normal skin homogenates were subjected to electrophoresis. Following migration, the separated components were allowed to diffuse against homologous serum samples. In all eleven instances in which such tests were undertaken, no antibodies to normal skin protein could be demonstrated.

Immuno-electrophoretic procedures were then done to determine if antibodies to the wart virus in tissue could be detected in the serum of subjects from whom warts had

been obtained. Six wart tissue homogenates were submitted to electrophoresis. Following migration, separated fractions were diffused against homologous serum samples. Antibodies to tissue wart viral particles were not demonstrated in serum samples from these subjects.

It occurred to us that there might be plasma proteins attached to the virus in warts which accounted for the migration of viral particles to the 20 mm distance. Prior to testing wart tissues for plasma protein, callus skin samples were studied as a control.

Fig. 13 illustrates a typical electrophoretogram and immuno-electrophoretic pattern produced by callus tissue. A human serum sample was included in the immuno-electrophoretic procedure to aid in the identification of any plasma proteins detected. The immuno-precipitate detected in callus tissue occupied a position which corresponded to albumin in the serum control. Table 9 summarizes the findings obtained when 12 callus tissues were subjected to electrophoresis and examined for plasma protein. It will be noted that 7 of the 12 callus tissues (58.8%) demonstrated one immuno-precipitate when diffused against anti-human serum. It will also be observed that no immuno-precipitate occurred within a distance 41 mm from the origin.

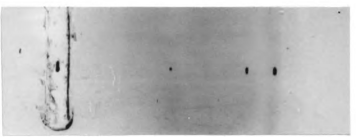


Fig. 13. Demonstration of plasma protein in callus tissue.

a. Electrophoretogram of callus tissue sample showing three protein zones, 24, 41, and 47 mm from origin (0).

b. Immuno-electrophoretogram demonstration antigen-antibody reaction by callus tissue protein and anti-human serum (1). Human serum control reactions are shown (2).

Table 9. Immuno-electrophoretic analysis of 12* callus tissues for plasma protein.

Sample No.	No. of protein zones produced	Location of protein zones (mm from origin)	No. of immuno-arcs produced	Location of immuno-arcs (mm from origin)
1	3	25-42-47	1	45
2	0		1	46
3	3	24-41-47	1	45
4	4	22-36-39-44	1	41
5	2	22-42	1	44
6	4	29-40-44-48	1	42
7	1	45	1	44

*Plasma proteins were not detected in 5 tissues.

Table 10. Immuno-electrophoretic analysis of 6 wart tissues for plasma protein.

Dimension of wart tissue used (mm)	Presence of plasma protein
1 x 1 x 1	-[1]
1 x 1 x 1	-
1 x 1 x 1	-
4 x 4 x 2	+[2]
4 x 4 x 2	+
4 x 4 x 2	+

[1]No plasma proteins detected.

[2]Plasma proteins present.

Immuno-electrophoretic analysis of 6 wart tissues were then conducted to determine if plasma proteins were present. The results of these tests are shown in Table 10. In the procedure, electrophoretically separated anti-human serum proteins were allowed to diffuse toward channels containing homogenates of individual warts. Following incubation, plasma proteins were detected in 50% of the wart tissues tested. It will be noted that positive reactions were obtained only from the tissues of larger dimensions. Fig. 14 demonstrates the type of immuno-precipitate produced when anti-human serum was reacted with wart tissue homogenates in this manner. When this reverse type of immuno electrophoretic procedure (separated antiserum components diffused against antigenic material) was used, it was impossible to determine the migration distance of plasma protein represented in wart tissue.

However, it was more important to determine if plasma proteins were represented in the 20 mm zone. Therefore, tests to detect plasma protein were conducted on wart virus concentrates which yielded only the 20 mm protein zone. The cellulose acetate double diffusion technic was selected for this investigation because the supporting membrane allows free diffusion of large molecules, such as serum proteins, without absorption (25). As can be seen in Fig. 15a, no

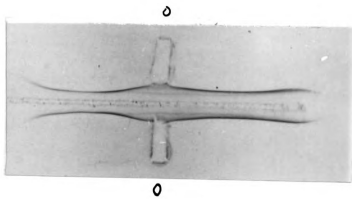
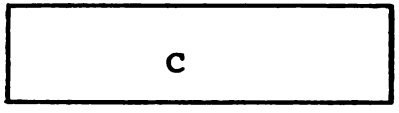
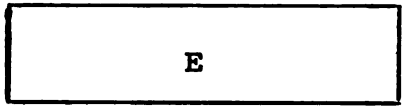


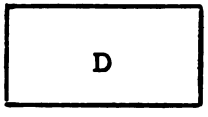
Fig. 14. Demonstration of plasma protein in wart tissue sample. Anti-human serum applied at site (O) was electrophoresed and a wart tissue homogenate placed in the central trough.





a.

b.



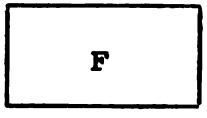


Fig. 15. Cellulose acetate double diffusion procedures employing
a. Anti-human serum (C) and wart virus concentrate (D)
b. Rabbit-produced wart virus antisera (E) and normal human serum (F)

immuno-precipitates were produced following diffusion of anti-human serum (C) against the wart virus concentrate (D). The results indicated an absence of plasma protein in the concentrated preparation. In addition, no immuno-precipitates were produced following diffusion of the rabbit-produced wart virus antiserum (E) against normal human serum (designated F in Fig. 15b), substantiating the lack of plasma protein in the immunizing wart virus preparation.

Fig. 16 shows the results obtained when cellulose acetate diffusion technics were employed to study the reactivity of the wart virus concentrate (A) with its rabbit-produced homologous antiserum (B). It will be noted that 2 distinct immuno-precipitates were produced (c and d). In addition, one questionable precipitate (illustrated by a dotted line, e) was found. Impurity of the concentrate is not implied as multiple antigenic components of several viral agents have been reported (7, 27, 28).

Fig. 17 illustrates the cross reactivity observed when rabbit-produced wart virus antiserum (A) was simultaneously diffused against a callus extract (B) and the concentrated wart virus preparation (C) in a cellulose acetate diffusion procedure. The immuno-precipitate shared by the wart virus preparation and the callus skin tissue indicates that a tissue protein was included in the viral suspension used

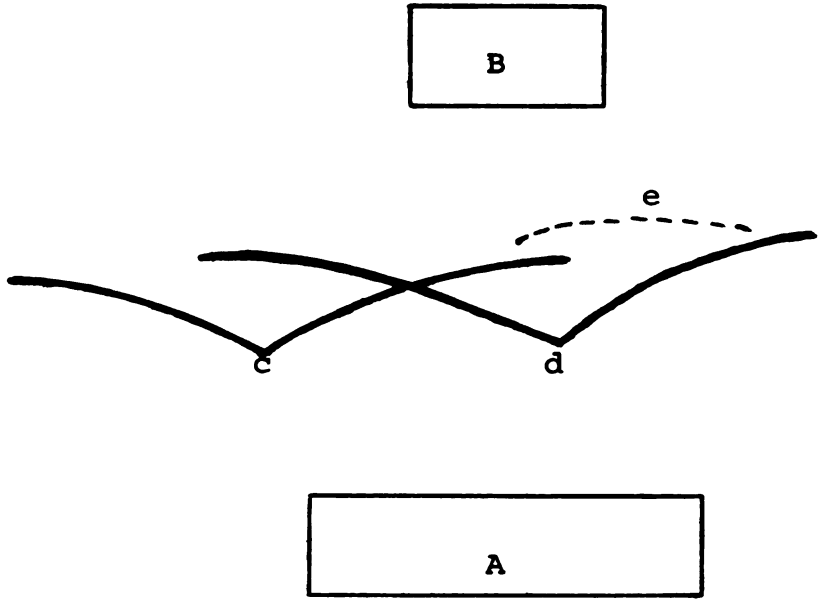


Fig. 16. Cellulose acetate double diffusion reaction of rabbit-produced wart virus antisera (A) and concentrated wart virus preparation (B). Distinct immuno-precipitates are represented by solid lines (c and d) while the questionable immuno-precipitate is indicated by dotted lines (e).

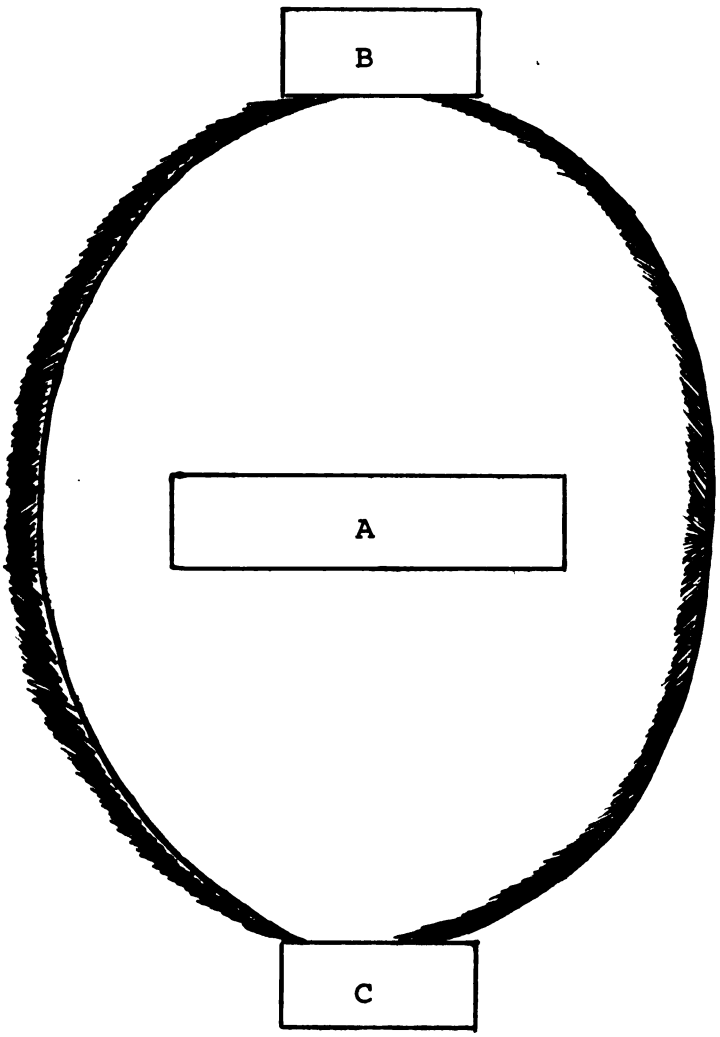


Fig. 17. Cellulose acetate double diffusion reaction produced by wart virus antisera (rabbit) (A) reacted simultaneously with callus extract (B) and a concentrated wart virus preparation (C).

for immunization.

The performance of cellulose acetate diffusion procedures is not without difficulty. The proper degree of moisture on the sample applicator strips as well as on the cellulose acetate strip is difficult to maintain. Since these technical problems are not encountered with the micro-modified Ouchterlony technic, this procedure was used for remaining serologic studies.

First, the serologic relationship of a concentrated wart virus preparation and its homologous antiserum was evaluated. By observation of Figs. 18, 19, 20 and 21, it is apparent that with this technic only a single, intense immuno-precipitate was produced when concentrated wart viral particles were diffused against homologous antiserum (rabbit). Fig. 18 also demonstrates that in contrast with cellulose acetate diffusion results, no cross reactivity occurred between the rabbit-produced wart virus antiserum (center well) and the callus tissue extract (B). Contrary to cellulose acetate diffusion studies, these results indicate that no tissue proteins were present in the wart virus preparation used for immunization.

The micro-modified Ouchterlony procedure was also used in an attempt to detect wart viral particles in two individual wart tissues. Fig. 19 shows that no immuno-precipitates were

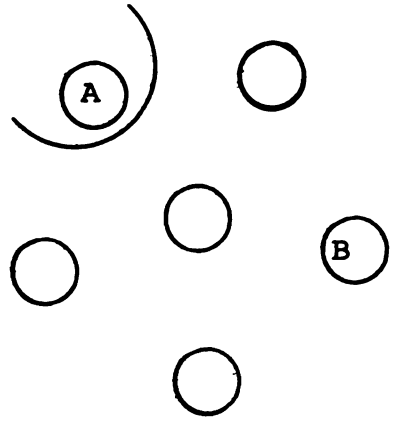


Fig. 18. Micro-modified Ouchterlony double diffusion of concentrated wart virus preparation (A), and callus extract (B). Wart virus antiserum (rabbit) was placed in the center well.

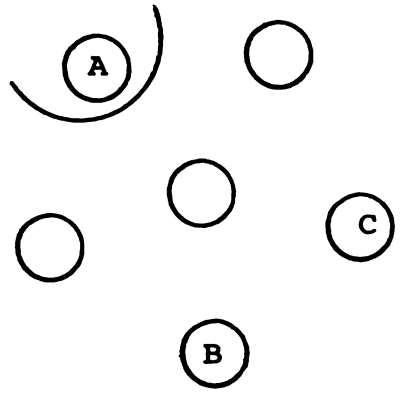


Fig. 19. Micro-modified Ouchterlony double diffusion of concentrated wart virus preparation (A), and tissue homogenates of warts (B) and (C) from which positive tissue culture results were obtained. Wart virus antiserum (rabbit) was placed in the center well.

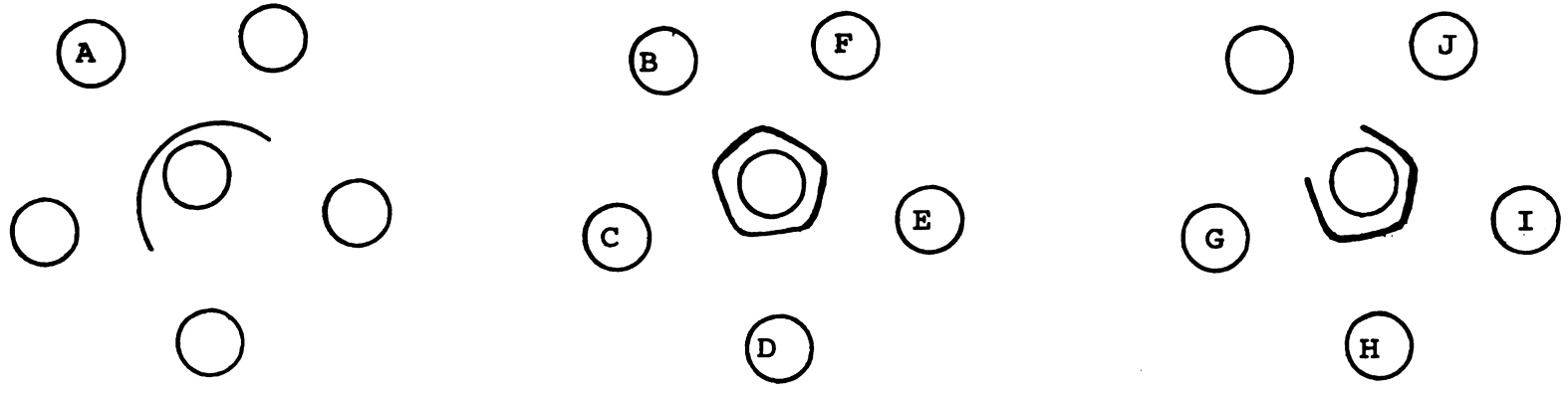


Fig. 20. Micro-modified Ouchterlony double diffusion of wart virus antisera (rabbit) (A), and sera from individuals with a history of warts (B), (C), (D), (E), (F), (G), (H), (I), (J). A concentrated wart virus preparation was placed in the center wells.

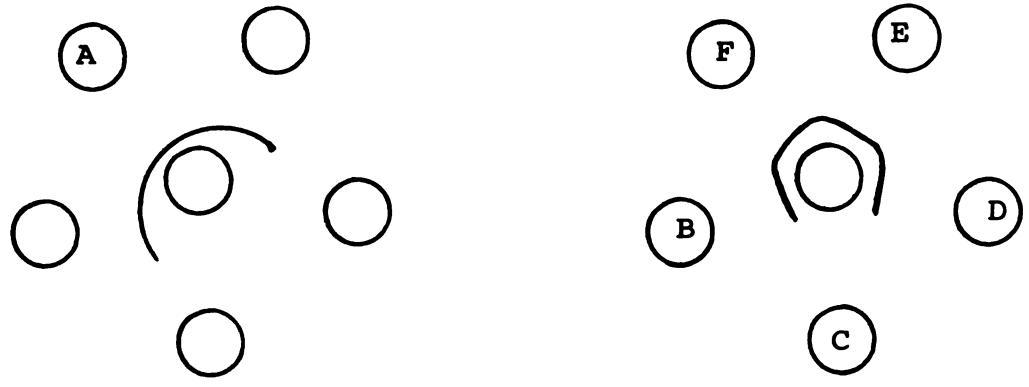


Fig. 21. Micro-modified Ouchterlony double diffusion of wart virus antisera (rabbit) (A), and random sera from individuals not known to have warts (B), (C), (D), (E), and (F). A concentrated wart virus preparation was placed in center wells.

produced upon diffusion of the rabbit-produced wart virus antiserum (center well) against wart tissues designated B and C. A positive control was included by placing the wart virus concentrate (A) in a peripheral well.

Fig. 20 characterizes the results obtained following the diffusion of 9 sera from individuals with histories of warts (peripheral wells) against concentrated wart virus (center well). Immuno-precipitates were produced which were identical with that produced by the wart virus and its homologous anti-serum. Wart virus antiserum (rabbit) designated (A) was included for a positive control. As part of a negative control procedure 5 sera, from individuals not known to have warts, were collected. These sera were diffused against the concentrated viral suspension. Fig. 21 illustrates the results obtained from this diffusion procedure. Unexpectedly, sera (designated B, D, E and F) produced identical immuno-precipitates. One serum (designated C), from a 3 year-old child, failed to produce an immuno-precipitate. The serum samples yielding positive reactions were all from adult individuals. An additional control procedure (illustrated by Fig. 22) was also performed in which the serum samples from subjects with warts (A, B, C, D, E, F, G, H, I) were diffused against a pool of normal

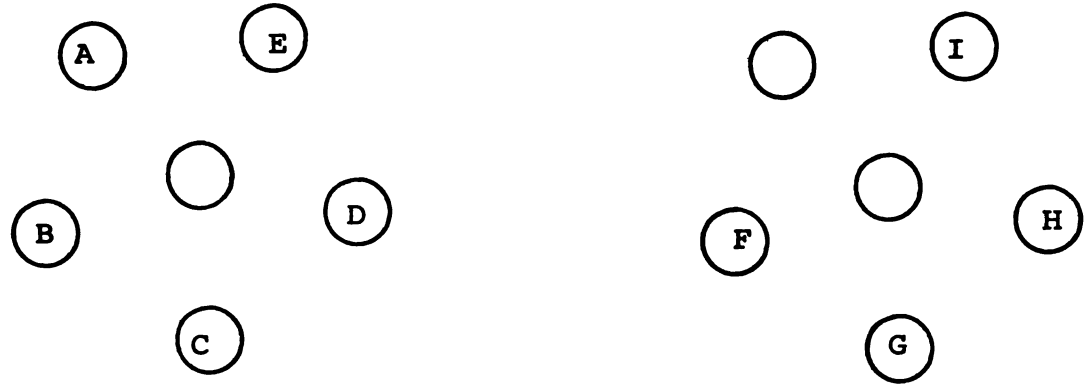


Fig. 22. Micro-modified Ouchterlony double diffusion of sera from individuals with histories of warts, (A), (B), (C), (D), (E), (F), (G), (H) and (I). A pool of normal skin homogenates was placed in the center well.

skin homogenates (in center wells). No immuno-precipitates were produced which proved that previously detected antibodies were not produced by a normal skin component.

The E. coli T_3 bacteriophage was electrophoretically compared to the wart virus because the two agents are the same size. Since simple diffusion reactions are also influenced by molecular size (25), micro-modified Ouchterlony procedures were used to further compare the reactions produced by the two viruses in the presence of their rabbit-produced antisera. Fig. 23 illustrates the reaction obtained when the bacteriophage preparation (center well) was diffused against homologous antisera (A). It will be noted that 3 distinct immuno-precipitates were produced. By comparison, the wart virus and its antisera (Fig. 18) yielded only one distinct precipitate when studied by this technic. Several possibilities exist for the multiple immuno-precipitates produced by the bacteriophage-antisera preparations. For example, a number of virus have been found to consist of multiple antigenic components (7, 27, 28). Also virus-immunizing preparations may contain antigenic materials (unrelated to the virus) which are carried over from the growth medium. It was not possible in this study to determine which of the 3 immuno-precipitates represented the infectious viral component reacted with its specific antibody.

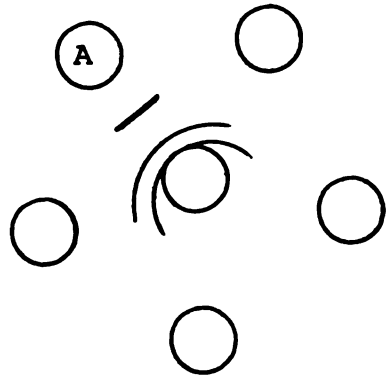


Fig. 23. Micro-modified Ouchterlony double diffusion of E. coli T_3 bacteriophage antisera (rabbit) (A), and a concentrated bacteriophage preparation placed in the center well.

DISCUSSION

In this investigation, different tissues such as normal skin, callus and warts were examined electrophoretically. The type of pattern obtained varied with the tissue studied, i.e., patterns from different tissues were not comparable with regard to number and relative mobility of zones detected. Normal skin patterns uniformly exhibited a single, rapidly migrating (greater than 40 mm) protein zone, while callus tissue patterns demonstrated approximately equal numbers of fast and slow migrating zones. Wart tissue showed a predominance of zones which moved less than 40 mm from the origin.

Our observation that distinct electrophoretic patterns were produced by different tissues substantiates the results of a number of previous investigators. Both Demling in 1952 (9) and Kessel in 1959 (17), as well as Scheiffarth and co-workers in 1961 (31), showed that the same type of pattern was produced when similar tissues of a given species were tested. Their work illustrated that the number and quantity of proteins detected, as well as the migration pattern, varied with the tissue under investigation.

The aim of the current study was to detect a virus in tissue, therefore human wart was chosen for analysis. Since

warts occur in epidermal tissue, normal skin samples were electrophoretically analyzed for control. Wart and callus skin are composed of keratin, so these tissues were also compared.

The results of these comparisons illustrated that the wart and callus electrophoretic patterns differed markedly from those of normal skin. Since Alfonzo in 1963 (2) found that pathological changes as well as tissue origin were observed in patterns from malignant soft tissue, it was predictable that epidermal changes in tissue proteins would be observed in the migration patterns of wart and callus tissue.

As the wart tissues were tested, it became clear that a zone 20 mm from the origin was detected which was not found in callus or normal skin samples. The 20 mm protein zone was present after concentration of wart tissue while other zones were not. Electron microscopic examination of the concentrates, as well as eluted 20 mm protein zones, demonstrated particles identical with those described as the wart virus by Williams et al in 1961 (44).

The purity of the concentrate was evident since large numbers of viral particles were observed with no debris, and these particles produced only one protein zone upon

electrophoresis. Single component composition was also substantiated by analytical ultracentrifugation data. Immunologically, only one immuno-precipitate was produced when the concentrated preparation and wart virus antisera were subjected to micro-modified Ouchterlony diffusion technics.

There were two exceptions. Two distinct immuno-precipitates were produced when the concentrated virus preparation and homologous antisera were diffused by cellulose acetate technics. The presence of more than one precipitate produced by virus and antisera does not prove heterogeneity. Several investigators have shown that some viruses consist of multiple antigenic components. In 1958, Brown and Crick (7) demonstrated that two antigens were associated with the virus of foot and mouth disease. In the same year, Polson et al (28) showed that type 1 poliomyelitis virus contained three antigenic components. Pereira in 1960 (27) illustrated that with certain adenovirus, four antigens are related.

While no cross reactivity of wart virus antisera and callus extracts was observed with Ouchterlony technics, an identical immuno-precipitate was produced when cellulose acetate procedures were performed. The possibility that a

tissue protein existed in close proximity to the virus particles used for immunization and the comparative sensitivity of the two diffusion technics remains to be clarified.

The presence of nucleoprotein in the concentrated wart virus preparation, suggested by ultra-violet absorption studies, was confirmed by application of a specific stain to the 20 mm protein zone. Application of other specific stains failed to demonstrate lipoprotein or glycoprotein in the zone.

Earlier investigators demonstrated the presence of plasma protein in tissue. In 1961, Scheiffarth et al (31) illustrated that plasma proteins as well as tissue proteins occurred in a wide variety of tissues. Albumin, gamma globulins and other differentiated plasma proteins were detected in normal skin tissue by Fisher in 1965 (12). Therefore, it was necessary to show that a plasma protein carrier was not responsible for the migration of the wart virus. By immunodiffusion technics used in this study, the 20 mm protein zone was shown to be devoid of plasma protein thereby refuting this possibility. In addition, the electrophoresis of a similar-sized virus (E. coli T_3 bacteriophage) which had never been associated with plasma

proteins demonstrated that a carrier was not required for viral movement in an electrical field.

This investigation demonstrated that virus can be studied by agar-gel electrophoresis. Two viruses, the wart virus and T_3 bacteriophage, were shown to migrate distances of 20 and 25 mm respectively. However, the ability of viruses to migrate in an electrical field has been shown by other investigators. Lauffer and Ross studied the alfalfa mosaic virus electrophoretically as early as 1940 (19). Other plant viruses, such as southern bean mosaic virus (23), tobacco ringspot virus (10), and the watermelon virus (41) have also been studied by this procedure. Within the last ten years, electrophoretic analysis of animal viruses have included the human enteroviruses conducted by Polson and Deeks (29). The polyoma virus of mice, another oncogenic virus similar to the wart virus, was electrophoretically characterized by Thorne et al in 1965 (38). Although these investigators used electrophoresis to study certain viruses, they did not employ agar-gel substrates for migration, as was done in the present study. Earlier research involved either free or density-gradient electrophoresis procedures. In addition, concentrated viruses from tissue cultures were

used for testing, while this study showed that viral protein in tissue could also be detected by electrophoresis.

In the present study, the 20 mm protein zone was detected in 38.7% of the wart tissues. There may be several reasons why a higher rate of detection was not obtained. First, the number of viral particles in wart tissue has been related to the age of the tissue mass, with the greatest numbers occurring in those of 6 to 12 months duration (5). In tissues used here, the length of time required for wart development was not known. Second, as with electron microscopy, a minimum number of virus must be present for electrophoretic detection to be possible. Barrera-Oro et al have shown that in order to observe viral particles by the electron microscope, counts of 1 x 10^7/mg are necessary (5). The number of particles required to produce a protein zone may be greater than that required for visualization. In this investigation, the number of viral particles released from an individual wart tissue were diluted in buffer solution. No concentration of these particles was done prior to electrophoresis.

By electron microscopy, concentrated preparations from wart tissue pools were shown to consist of particles morphologically identical to the wart virus. Results

obtained by the double diffusion of the viral concentrate and 9 sera from individuals with histories of warts substantiated that the agent was the wart virus. One immunoprecipitate was produced by each of the 9 sera tested. This precipitate was identical to that produced by the virus and homologous antisera from immunized rabbits. The 9 human sera did not cross-react with normal skin extracts showing that the antigen-antibody reaction was specific. When 5 sera from individuals not known to have warts were tested with the wart virus, 4 of 5 yielded a precipitate identical to that produced by the wart virus and the other 9 sera. Positive reactors were adults while the non-reactor was a 3 year old child. It would be of value to use this antigen to study the incidence of wart virus antibodies in the general population.

Electrophoresis was found a much faster and simpler procedure than the time-consuming, complex and expensive procedures of electron microscopy and ultracentrifugation. However, incorporation of all three technics would be more conclusive.

In this study, a protein zone derived from wart tissue and migrating 20 mm was shown to be composed of the wart virus. Further application of electrophoresis to the study of other oncogenic viruses was indicated.

SUMMARY

1. Normal skin, callus and wart tissue were examined by an agar-gel electrophoresis technic. While only 54.1% of the normal skin samples yielded a protein zone, 83.3% of the callus tissues exhibited protein zones. Protein zones were demonstrated in 87.1% of the wart tissues tested.

2. No zones migrating less than 40 mm were detected for any of the normal skin fragments tested. By comparison, a number of proteins with less mobility were noted from wart tissues. Callus skin tissues exhibited proteins common to both normal skin and wart tissues.

3. In wart tissue, one protein zone was noted to occur with greater frequency (38.7%) than any other. This protein zone migrated 20 mm from the origin. The 20 mm zone was not observed in electrophoretic patterns from either normal skin or callus tissue.

4. Concentration of wart tissue by ultracentrifugation resulted in the disappearance of all but the 20 mm zone. Analysis of the concentrate showed a single moving boundry.

5. Electron microscopy of the concentrate and eluted 20 mm protein zones revealed numerous wart viral particles.

6. Special staining technics as well as ultra-violet absorption studies indicated the presence of nucleoprotein in the 20 mm zone.

7. Immunodiffusion technics demonstrated that no plasma protein was associated with the concentrated wart virus. The virus was shown to be capable of migration in an electrical field without a protein carrier. Electrophoresis of *E. coli* T_3 bacteriophage, a similar-sized virus, demonstrated a single protein zone 25 mm from the origin, illustrating that plasma protein was not a prerequisite for migration.

8. One precipitate was produced by the diffusion of concentrated wart virus and homologous antiserum in Ouchterlony procedures. When this antigen and antiserum were diffused on a cellulose acetate medium, two distinct precipitates and one questionable one were produced.

9. A precipitate was produced by the diffusion of a callus extract against the wart virus antiserum in a cellulose acetate procedure. This precipitate was identical with that produced by the diffusion of wart virus and homologous antiserum. No reaction was noted upon the diffusion of callus material and the wart virus antiserum when tested by Ouchterlony technics.

10. Antibodies to the wart virus were detected in the sera of 9 subjects who had histories of wart infection and 4 subjects who were not known to have warts.

BIBLIOGRAPHY

1. Adams, M. H., 1950. Methods of study of bacterial viruses. Meth. in Med. Res. 2:3-73.

2. Alfonzo, E., 1963. Electrophoretic patterns of tumor tissue proteins. J. Clin. Path., 16:375-376.

3. Almeida, J. D., Howatson, A. F., and Williams, M. G., 1962. Electron microscope study of human warts; Sites of virus production and nature of inclusion bodies. J. Invest. Derm., 38:337-345.

4. Almeida, J. D., Goffe, A. P., 1965. Antibody to wart virus in human sera demonstrated by electron microscopy and precipitin tests. Lancet 2:1205-1207.

5. Barrera-Oro, J. G., Smith, K. O., and Melnick, J. L., 1962. Quantitation of Papova virus particles in human warts. U. S. National Cancer Institute, 29:583-595.

6. Bivins, J. A., 1953. The growth in the developing chicken embryo of a filtrable agent from verruca vulgaris. J. Invest. Derm., 20:471-481.

7. Brown, F. and Crick, J., 1958. Application of agar gel diffusion and immuno-electrophoresis to the study of foot-and-mouth disease; in Abstracts of Communications Delivered at Paper Sessions, 7th int. Congr. Microbiol., p. 152 Almqvist & Wiksel, Uppsala.

8. Ciuffo, G., 1907. Innesto positivo con filtrato di Verruca Volgare. Gior, ital, d. mal. vener., 48:12-17.

9. Demling, L., 1952. Elektrophoretische aufspaltung von organeiweiss. Klinische Wochenschrift, 30:74.

10. Desjardens, P. R., Senseney, C. A., and Hess, G. E., 1953. Further studies on the electron microscopy of purified tobacco ringspot virus. Phytopath. 43:687-690.

11. Felsher, Z., 1947. Virus studies on cutaneous material. Inoculation of the chick chorioallantoic membrane with material from cutaneous material. J. Invest. Derm., 8:123-124.

12. Fisher, J. P., 1965. Soluble substances of human stratum corneum. J. Invest. Derm., 44:43-50.

13. Fisher, J. P., 1966. Soluble substances and antigens of human epidermis, (Analysis of electrophoresis and immunoelectrophoresis). J. Invest. Derm., 47:336-350.

14. Hanks, J. H., and Wallace, R. E., 1949. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exper. Biol. & Med., 71:196-200.

15. Hayashi, Hajime, 1961. Studies on the viral etiology of verruca vulgaris. Ph'd. Thesis, Michigan State University.

16. Jadassohn, J., 1896. Sind die Verrucae vulgares ubertragbar? Verhandle. Dermat. gesellsch., 5:497-501.

17. Kessel, M., 1959. Darstellung und identifizierung electrophoretische trennbarer organproteine auf agar-gel. Clinica. Chimica. Acta., 4:148-153.

18. Kingery, L. B., 1921. The etiology of common warts. - Their production in the second generation. J.A.M.A., 76:440-442.

19. Lauffer, M. A., and Ross, A. F., 1940. Physical properties of alfalfa mosaic virus. J. Am. Chem. Soc., 62:3296-3300.

20. Lillie, R. D., 1965. Histopathologic Technic and Practical Histochemistry 3rd Ed., pg. 270, Blakiston Division, McGraw-Hill Book Co., Inc., New York, N.Y.

21. McManus, J. F. A., and Mowry, R. W., 1960. Staining Methods: Histologic and Histochemical, pg. 126, Paul B. Hooker, Inc., Medical Division of Harper and Brothers, New York, N.Y.

22. Mendelson, C. G., and Kligman, A. M., 1961. Isolation of wart virus in tissue culture. Successful reinoculation into humans. Arch. Derm., 83:559-562.

23. Miller, G. L. and Price, W. C., 1946. Physical and chemical studies on the southern bean mosaic virus. III Electrophoretic and nucleic acid studies. Arch. Biochem. 11:333-337.

24. Noyes, W. F., 1964. Structure of the human wart virus. Virology 23:65-72.

25. Ouchterlony, O., 1968. Handbook of Immunodiffusion and Immuno electrophoresis pg. 3-59, Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan.

26. Pease, D. C., 1964. Histological Technics for Electron Microscopy, 2nd Ed., Academic Press, New York.

27. Pereira, H. G., 1960. Antigenic structure of non-infectious adenovirus materials. Nature (Lond.) 186:571-572.

28. Polson, A., Ehrenberg, A. and Cramer, R., 1958. Specific precipitin reactions of the virus of poliomyelitis in gels. Biochem. biophys. Acta 29:622-629.

29. Polson, A., and Deeks, D., 1962. Zone electrophoresis of Enteroviruses. J. Hyg., 60:217-234.

30. Rudall, K. M., 1962. Advances in Protein Chemistry, Vol. VII pg. 253, Academic Press Inc., New York, N.Y.

31. Scheiffarth, F., Gotz, H., and Schernthaner, G., 1961. Immunoelectrophoretische studien zur frage der identitat menschlicher organ und serumproteine. Clinica. Chimica. Acta., 6:481-492.

32. Serra, A., 1908. Ricerche istologiche e sperimentali sul condiloma acuminato - I papillomi del capo e la verruca volgare. Gior. ital. d. mal. vener., 49:11-16.

33. Siegel, B. V., and Novy, F. G., 1955. Cultivation studies on wart suspensions of verruca vulgaris and condyloma acuminatum. J. Invest. Derm., 25:265-268.

34. Siegel, B. V., 1956. Contaminant filtrable agent derived from a human wart. J. Invest. Derm., 27:379-381.

35. Siegel, B. V., 1960. Electron microscopic studies of the virus of human warts. J. Invest. Derm. 35:91-93.

36. Strauss, M. J., Shaw, E. W., Bunting, H., and Melnick, J. L., 1949. "Crystalline" virus-like particles from skin papillomas characterized by intranuclear inclusion bodies. Proc. Soc. Exp. Biol. & Med., 72:46-50.

37. Strauss, M. J., Bunting, H., and Melnick, J. L., 1950. Virus-like particles and inclusion bodies in skin papillomas. J. Invest. Derm., 15:433-444.

38. Thorne, H. V., House, W., and Kisch, A. L., 1965. Electrophoretic properties and purification of large and small plaque-forming strains of Polyoma virus. Virology, 27:37-43.

39. Ulbrich, A. P., Arends, N. W. and Koprince, D., 1957. Warts: Discussion with special reference to therapy by total enucleation. J. Am. Osteopathic Assoc., 56:345-349.

40. Uriel, J., 1964. The characterization reactions of the protein constituents following electrophoresis or immuno-electrophoresis in agar. In Immuno-Electrophoretic Analysis-Applications to Human Biological Fluids pg. 30-57. Edited by P. Graber and P. Burtin, Elsevier Publishing Co., New York, N.Y.

41. van Regenmortal, M. H. V., 1960. Zone electrophoresis and electron microscopy of a watermelon mosaic virus from South Africa. Virology 12:127-129.

42. Virus Subcommittee of the International Nomenclature Committee, 1963. Recommendations on virus nomenclature. Virology, 21:516-517.

43. Wile, U. J., and Kingery, L. B., 1919. The etiology of common warts. Preliminary report of an experimental study. J.A.M.A., 73:970-973.

44. Williams, M. G., Howatson, A. F., and Almeida, J. D., 1961. Morphological characterization of the virus of the human common wart (Verruca vulgaris). Nature 189:895-897.

45. Wilner, B. I., 1965. A Classification of the Major Groups of Human and Other Animal Viruses 3rd Ed., pg. 32, Burgess Publishing Co., Minneapolis, Minn.