

7-29-1975

# Effects of Antifibroblast Antiserum on Cells Derived from Fibroblast Outgrowth of Human Prostatic Tissues

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<https://doi.org/10.15760/etd.2377>

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AN ABSTRACT OF THE THESIS OF Eva Shang-Lian King for the  
Master of Science presented 29 July 1975.

Title: Effects of Antifibroblast Antiserum on Cells  
Derived from Fibroblast Outgrowth of Human  
Prostatic Tissues

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

  
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Gordon Kilgour

The purpose of this investigation was to provide pure cultures of normal human prostatic epithelium free of fibroblasts in order to study malignant conversion by chemical carcinogens. Normal epithelial cells were needed because this was the cell type implicated in prostatic malignancies of human subjects. Unfortunately fibroblasts grew faster than epithelial cells so that cultures were always overgrown with connective tissue elements. It was considered important to find a method which would eliminate fibroblasts so that normal epithelial cells could grow out in pure culture.

Fibroblast cultures were obtained from human prostatic tissues fed with Eagle's medium containing 20% fetal bovine serum. To prepare antifibroblast antiserum rabbits were injected intravenously with human prostatic fibroblasts which had been transferred through at least 14 tissue culture passages. Complement fixation tests were used successfully to measure reactions of rabbit antifibroblast antiserum with prostatic fibroblasts. It was shown that in the rabbit antifibroblast antiserum reaction with complement and specific antigen, fibroblasts were lysed while prostatic epithelium was unaffected in mixed cell type cultures obtained from explant outgrowth of prostatic tissues. Control tissue cultures treated with normal rabbit serum remained unaffected when rabbit antifibroblast antiserum diluted 1:2 and complement diluted 1:5 were added to test systems.

Ammonium sulfate precipitation followed by DEAE cellulose column chromatography was used to isolate IgG from rabbit antifibroblast antiserum and normal rabbit serum. Only IgG purified from rabbit antifibroblast antiserum and added in the presence of complement had the ability to destroy fibroblasts and not epithelial cells. IgG obtained from normal rabbit serum had no effect.

When rabbit antifibroblast antiserum absorbed with H. Ep. 2 cells and complement were added to cultures of mixed cell types obtained from cellular outgrowth of human prostatic explants similar results were achieved: cells exhibiting fibroblast-like morphology were destroyed while

epithelial cells persisted and continued to multiply.

Thus fibroblasts were lysed after treatment with rabbit antifibroblast antiserum and complement. Epithelial cells remained and proliferated in pure culture thereby providing a model tissue culture system of normal human prostatic epithelium which was available for group studies designed to measure initial events in malignant conversion processes of prostatic tissue.



EFFECTS OF ANTIFIBROBLAST ANTISERUM ON CELLS  
DERIVED FROM FIBROBLAST OUTGROWTH OF  
HUMAN PROSTATIC TISSUES

by

EVA SHANG-LIAN KING

A thesis submitted in partial fulfillment of the  
requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

Portland State University  
1975

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:


The members of the Committee approve the thesis of  
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## ACKNOWLEDGEMENTS

Give thanks to God for blessing me here. I am grateful to my family for letting me have a chance to study here. I am really thankful for the instruction of my advisor, Dr. Thelma N. Fisher and committee members Drs. Earl Fisher, Jr. and Gordon Kilgour. I greatly appreciate Mrs. Donna Ann Fisher for her instruction in tissue culture maintenance techniques.

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## INTRODUCTION

Fibroblasts derived from human fetal tissue (21, 27, 15), human skin (8, 25), chick embryo (26), and murine embryo (12), etc., have been studied extensively by others in order to determine hexosaminidase activity (10), to assess the effects of pH (8) and amino acid composition (15) on growth, to measure dihydrotestosterone formation (25), to study adhesion properties (16, 17), and to isolate glycoproteins from cell surfaces (26). Fibroblasts derived from numerous human prostatic tissues were tested in our study.

For the preparation of antiserum many investigators immunized rabbits via the intravenous route (18, 24), by intramuscular and intravenous injections (7, 15), by intradermal inoculation (22, 9, 18), by alternating intraperitoneal with subcutaneous type injections (5), or by foot pad inoculations (4, 6). Intravenous procedures via marginal ear veins of rabbits provided a satisfactory method for preparing antifibroblast antiserum used in our research.

Because it was necessary to prepare pure cultures of normal prostatic epithelium free of fibroblasts in order to study malignant conversion by chemical carcinogens an effective method to eliminate fibroblast outgrowth from prostatic explants was required. Recovery of epithelium was essential since this cell type was thought to undergo malignant transformation in human subjects. Fibroblasts multiplied more rapidly than epithelial cells thereby overgrowing epithelial cultures. Our purpose was to destroy



fibroblasts so that normal epithelial cells would proliferate freely in pure culture. Subsequently, a cancer research group hoped to study initial events in the malignant conversion process by treating known normal human prostatic epithelial cells with chemical carcinogens. It was obvious to all investigators involved in this field of research that the first enormous problem was to control overgrowth of epithelium by fibroblasts. To make antifibroblast anti-serum in rabbits via intravenous inoculation and to test the effects of antibody in the presence of complement on fibroblasts derived from human prostatic tissue, was the purpose of this study. We hoped to demonstrate that such a system destroyed cells which resembled fibroblasts, thereby allowing typical epithelial cell types to persist and flourish.



## MATERIALS AND METHODS

### Cell Cultures

Fibroblast cultures were isolated from human prostatic tissues removed surgically from patients at Providence Hospital, Portland, Oregon. H. Ep. 2 cells, human epithelium obtained originally from a patient with carcinoma of the larynx, were carried in continuous culture in our laboratory through 280 transfers. Often mixed cultures of fibroblast and epithelial cell types were obtained from prostatic explant outgrowth. These cultures were subjected to antifibroblast antiserum treatment also.

### Procedures for Isolation, Feeding and Harvesting Cell Cultures and the Media Used

Hanks' Balanced Salt Solution containing antibiotics (penicillin and streptomycin) was used to wash prostatic tissues 3 times. Tissues were cut into small 2 mm<sup>2</sup> pieces when held in the third wash solution. Explants were placed in small plastic petri dishes (60 x 15 mm) and allowed to remain at room temperature for approximately 1 hr., after which time Eagle's medium containing 30% fetal bovine serum (Flow Laboratories) was added. Cultures were held in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37°. Hanks' Balanced Salt Solution and Eagle's medium as in Table I and Table II respectively were prepared as indicated in the formulae.

Fibroblast cultures were fed 3-5 times per week with

TABLE I

FORMULA OF HANKS' BALANCED SALT SOLUTION,  
(10X) STOCK SOLUTION

<u>Materials</u>	<u>Amount</u>
<u>Solution A</u>	
NaCl	80 gms
KCl	4 gms
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2 gms

The above reagents were dissolved in about 300 ml distilled water.

<u>Solution B</u>	
KH <sub>2</sub> PO <sub>4</sub>	0.6 gms
Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	0.6 gms
Glucose	10 gms

These compounds were dissolved in approximately 300 ml distilled water.

<u>Solution C</u>	
CaCl <sub>2</sub>	1.4 gms

Calcium chloride was dissolved in 200 ml distilled water and added to Solutions A and B.

<u>Solution D</u>	
Phenol Red	0.2%, 100 ml

The final volume was brought to 1,000 ml and the solution was sterilized by Sietz filtration. Ten times concentrated Hanks' solution was stored at 4° until dilute Hanks' preparation was required.

TABLE II

CONSTITUENTS OF A MODIFIED EAGLE'S MEDIUM AS  
USED TO CULTIVATE HUMAN PROSTATIC TISSUES

<u>Materials</u>	<u>Amount</u>
Hanks' Balanced Salt Solution	85 ml
Fetal Bovine Serum	
(inactivated at 56° for 30 min)	20 ml or 30 ml
Non Essential Amino Acids (100x, Flow Laboratories)	1 ml
Vitamins (100x, Flow Laboratories)	1 ml
Amino Acids (100x, Flow Laboratories)	1 ml
Penicillin [Stock Solution: 20,000 units per ml (Pfizer Laboratories)]	1 ml
Streptomycin [Stock Solution: 20 mg per ml (Pfizer Laboratories)]	1 ml
5% NaHCO <sub>3</sub>	2 ml
Putrescine (1 mg/ml, Sigma Laboratories)	0.25 ml
Glutamine (100x, Flow Laboratories)	1 ml



Eagle's medium containing 20% fetal bovine serum. Trypsin (0.25%, Difco) was used to subculture fibroblasts. Tissues were cultivated in 30 ml plastic tissue culture flasks or in 200 ml milk dilution bottles.

Human prostatic fibroblasts were subcultured for at least 14 passages before they were used experimentally. Cells (approximately  $2.35 \times 10^6$  per ml) were washed twice with 10 ml each of saline, and harvested in a small amount of saline. These cells were frozen and thawed 3 times then inactivated in a water bath at  $56^\circ$  for 30 min. This crude preparation served as antigen which was used to immunize rabbits.

#### Procedures for Rabbit Inoculation and Serum Harvest

The prepared antigen (0.1 ml per inoculation) was injected intravenously into ear marginal veins of rabbits, 3 times per week for 3 weeks. A booster inoculation was given one week later. After a few days, rabbits were bled from the heart in order to obtain 15 ml blood from each animal. Rabbit blood was refrigerated overnight at  $4^\circ$  in a centrifuge tube. Clots were rimmed carefully 24 hr. later and blood was centrifuged twice in order to obtain large serum yields free of red blood cells. Serum was inactivated at  $56^\circ$  for 30 min.

#### Complement

The complement employed throughout consisted of lyophilized guinea pig serum obtained commercially (Flow



Laboratories). Just before use complement was dissolved, in the cold, in 7 ml diluent of which the latter contained 6% sodium acetate and 2% boric acid.

Complement Fixation Tests Employing Rabbit Antifibroblast Antiserum and Fibroblast Cells

Indicator System. The titration of hemolysin as described by Burrell (1) was achieved by using 0.5 ml hemolysin dilutions ranging from 1:1,000 to 1:16,000 with 0.3 ml of a solution made up of complement diluted 1:30, 0.5 ml of a 2% suspension of sheep erythrocytes and 1.7 ml of 0.01 mg% $MgSO_4$  dissolved in physiological saline (Mag saline).

According to Burrell (1) complement was titrated using the following components: two units of an adequate dilution of hemolysin contained in 0.5 ml, 0.1 to 0.5 ml of a fresh solution made up of complement diluted 1:30, 0.5 ml of a 2% suspension of sheep erythrocytes and Mag saline added to provide a final volume of 2.5 ml per tube were included in the titration.

Test System. It was considered necessary to determine adequate concentrations of antigen and antibody required to observe lysis of fibroblasts. A master titration of fibroblasts was achieved by first preparing 2 doubling dilutions starting with a 1:5 dilution of suspended fibroblasts. Rabbit antifibroblast antiserum, heat inactivated 30 min at 56°, was diluted 1:5 and 1:20. Four rows of 13 x 100 mm test

tubes were arranged so that there were 3 tubes per row. Inocula were pipetted as follows: tube 1 of each row---0.5 ml of 1:5 inoculum, tube 2 of each row---0.5 ml of 1:10 inoculum, tube 3 of each row---0.5 ml of 1:20 inoculum. Rabbit antifibroblast antiserum was pipetted as follows: two-tenths ml of undilute serum was added to each tube in the first row; 0.2 ml of serum diluted 1:5 was added to each tube in the second row; 0.2 ml of serum diluted 1:10 was added to each tube in the third row and 0.2 ml of serum was diluted 1:20 and added to each tube in the fourth row. The following controls were included: rabbit antifibroblast antiserum control (0.2 ml of undiluted serum and 0.5 ml of Mag saline), fibroblast antigen control (0.5 ml of fibroblasts diluted 1:5 and 0.2 ml of Mag saline), and a hemolytic system control (0.7 ml of Mag saline). Preparations were incubated 15 min at room temperature, one ml of complement (2 units per ml) was added to each tube. Preparations were held in the refrigerator for 15-18 hr. Tubes were placed in a 37° water bath for 10 min after which time 0.5 ml of hemolysin (2 units) and 0.5 ml of a 2% suspension of sheep erythrocytes were added to each tube. A sheep erythrocytes control containing 0.5 ml of sheep red blood cells suspended in 2.2 ml of Mag saline was included in each titration. Contents of each tube were mixed well and preparations were incubated at 37° for 30 min.



Procedure for Measuring the Effect of Non-Heat-Inactivated Rabbit Antifibroblast Antiserum on Fibroblasts and H. Ep. 2 Cells

Initially growth fluids of fibroblast and H. Ep. 2 cell cultures were removed and appropriate photographs were made. Following photography cultures were fed with Eagle's medium containing 20% fetal bovine serum. To these preparations 0.1 ml of undiluted complement, and 0.1 ml of undiluted rabbit antifibroblast antiserum were added to fibroblast and H. Ep. 2 cell cultures. Treated cultures were incubated in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37°. The next day cultures were examined for evidence of cell lysis and photographs were made to record the results.

Determination of the Most Effective Concentration of Heat Inactivated Rabbit Antifibroblast Antiserum and Complement Necessary to Lyse Fibroblasts Observed in Mixed Fibroblast and Epithelial Cellular Outgrowth from Human Prostatic Explants

Growth fluids were removed and cultures were photographed initially. Then cultures were fed with Eagle's medium containing 20% fetal bovine serum. Undiluted rabbit antifibroblast antiserum (0.2 ml) was added to cultures displaying mixed cell type (fibroblasts and epithelial cells) outgrowth. Other cultures showing outgrowth of mixed cell types were treated with a 0.2 ml rabbit antifibroblast antiserum diluted 1:2. Test cultures were held in a 95% air-5%

CO<sub>2</sub> water-saturated environment at 37° for 15 min, after which time 1 ml of different dilutions of complement (undiluted, 1:5, 1:10, 1:20, 1:30, 1:45; 1:60, 1:75, 1:150) was added to appropriate cultures. Controls consisted of untreated cultures, cultures treated with 0.2 ml undiluted rabbit antifibroblast antiserum, cultures treated with 1 ml complement diluted 1:20, cultures treated with 0.2 ml undiluted heat inactivated normal rabbit serum and cultures treated with 1 ml of complement diluted 1:5. Control and test systems were incubated in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37° overnight then examined and photographed the following day.

#### Purification of IgG Obtained from Normal Rabbit Serum and Rabbit Antifibroblast Antiserum

Twenty-two ml aliquots of normal rabbit serum and rabbit antifibroblast antiserum were processed in order to obtain IgG fractions from each serum sample. The procedure followed for purification of IgG was as follows:

##### Ammonium Sulfate Precipitation (2)

1. Saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (11 ml) was adjusted to approximately pH 7.8 by the addition of 2 N NaOH. This adjustment was made just prior to precipitation of gamma globulin in order to prevent ammonia release.

2. With constant stirring the 11 ml of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution, pH 7.8, was added slowly, in dropwise



fashion to 22 ml of serum. This procedure salted out those components which were insoluble in a 33.3%  $(\text{NH}_4)_2\text{SO}_4$  solution.

3. Upon completion of  $(\text{NH}_4)_2\text{SO}_4$  addition, suspensions were stirred for 2 to 3 hr.

4. Subsequently, suspensions were centrifuged at room temperature for 30 min at 1,465 x g.

5. Precipitates were dissolved in enough saline to restore volumes of solutions to those of original serum samples.

6. Gamma globulin fractions were purified by a second and a third precipitation. For the second precipitation steps 1 through 5 were repeated. For the third precipitation, steps 1 through 4 were followed.

7. Precipitates from third precipitations were dissolved in borate-buffered saline to a final volume half that, or less than that of original serum samples.

8. Ammonium sulfate was removed from precipitates by dialyzing against borate-buffered saline for several days at 4°. Dialyzates were changed mornings and evenings, and at these intervals a few drops of 5%  $\text{BaCl}_2$  and 3.7%  $\text{HCl}$  were used to check for sulfate ions.

9. After dialysis was completed, solutions were removed from dialysis tubing then centrifuged 35 min 9,750 x g, at 4°.

10. The Lowry (Folin-Ciocalteu) method for protein

estimation was followed by adding standard solutions, saline, sample, reagent A, and reagent B in amounts and sequences as shown in Table III.

Absorbance of reaction mixtures were observed at 500 nm as measured against a distilled water blank. A standard curve was prepared and protein concentrations of samples were read directly from the calibration curve.

#### DEAE Cellulose Column Chromatography (2)

1. A DEAE cellulose column equilibrated in 0.01 M phosphate buffer, pH 7.5 was prepared.
2. Crude gamma globulin fractions were dialyzed against 0.01 M phosphate buffer, pH 7.5, for 24 hr at 4°.
3. Gamma globulin fractions were removed from dialysis bags, centrifuged for 25 min at 1,085 x g, and sediments were discarded.
4. Protein content of supernatant fluids was estimated by the Lowry Method.
5. Nine ml samples of crude gamma globulin were fractionated by DEAE cellulose column chromatography.
6. Samples which displayed the highest protein content were pooled and lyophilized.
7. Dried protein powders were dissolved in adequate amounts of distilled water in order to bring the final protein concentration to 4-5 mg/ml. Protein solutions were dialyzed against 0.01 M phosphate buffer, pH 7.5, for 2 days at 4°.

TABLE III

LOWRY (FOLIN-CIOCALTEAU) METHOD FOR PROTEIN ESTIMATION

	<u>Reagent Blank</u>		<u>Standards</u>						<u>Sample</u>		<u>Reagent Blank</u>
Tube Number:	1	2	3	4	5	6	7	8	9-1*	9-2*	10
mg of protein, standard/tube:		.01	.02	.03	.05	.10	.15	.20			
ml standard (0.5 mg/ml.)	---	.02	.04	.06	.10	.20	.30	.40	---	---	---
ml saline	.40	.38	.36	.34	.30	.20	.10	---	---	---	.40
ml sample	---	---	---	---	---	---	---	---	.40	.40	---
ml Reagent A	2.0 (ALL TUBES)										
	mix, let stand at room temperature for 10 min										
ml Reagent B	.20 (ALL TUBES)										
	mix, let stand at room temperature for 30 min, read at 500 nm										

Reagent blanks, standard dilutions and samples were tested in duplicate.

\* indicated that 0.1 ml sample solution was diluted in 0.9 ml saline (9<sup>-1</sup>) and 0.1 ml 9<sup>-1</sup> sample solution was diluted in 0.9 ml saline (9<sup>-2</sup>).



8. After completing dialysis procedures, solutions were centrifuged 25 min at 1,085 x g. Sedimentation coefficients of protein solutions were determined following analytical centrifugation in a Model E ultracentrifuge.

Complement Fixation Tests Using IgG Obtained from Rabbit Antifibroblast Antiserum and Fibroblast Cells

Indicator System. Methods for titrating hemolysin and complement (1) as described earlier were followed.

Test System. It was necessary to determine appropriate concentrations of reactants for this system. IgG obtained from rabbit antifibroblast antiserum was diluted 1:5 and 1:10 and added to suspensions of human prostatic fibroblasts diluted 1:5, and 1:10 in the presence of complement (2 units per ml), as indicated earlier.

A Comparison of Activities of IgG Obtained from Normal Rabbit Serum and from Rabbit Antifibroblast Antiserum on Human Prostatic Cultures Consisting of Mixed Fibroblast and Epithelial Cell Types

Growth fluids were removed and initial photographs were made of all cell cultures. Cultures were fed with Eagle's medium containing 20% fetal bovine serum to which 0.2 ml of undiluted IgG purified from rabbit antifibroblast antiserum was added. Test systems were held in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37° for 15 min. One ml of



complement diluted 1:5 was added to each culture. A control cell culture received 0.2 ml undiluted IgG obtained from normal rabbit serum and 1 ml of a solution made up of complement diluted 1:5. Preparations were examined and photographed.

An Attempt to Remove Antibodies of Epithelial Cells from Non-Heat-Inactivated Rabbit Antifibroblast Antiserum by Treating Antiserum with H. Ep. 2 Cells

For absorption studies 2-200 ml bottle cultures of H. Ep. 2 cells approximately 5 days old were used for these experiments. Growth fluids were removed and 3.5 ml rabbit antifibroblast antiserum were added to each culture. Cells were scraped from glass by means of rubber spatulas. Preparations were held 30 min in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37°. Cells were centrifuged 15 min at 3,020 x g. Supernatant fluids were retained while sedimented fractions were discarded. Presumably this supernatant fluid contained antifibroblast antiserum from which epithelial components of H. Ep. 2 cells had been removed by the absorption process. To test the effect of absorbed antifibroblast antiserum on cell cultures, growth fluids were removed from cultures of prostatic explants displaying mixed cell type outgrowth. Preparations were photographed then fed with Eagle's medium containing 20% fetal bovine serum and 0.2 ml undiluted but absorbed antifibroblast antiserum. After 15 min incubation at 37° 1 ml of a solution made up of

complement diluted 1:5 was added to preparations. Test systems were cultivated overnight in a 95% air-5% CO<sub>2</sub> water-saturated environment at 37°. The following day cultures were examined and photographed in order to document observed effects.

## RESULTS

### Complement Fixation Tests with Rabbit Antifibroblast Antiserum and Fibroblast Cell Cultures Derived from Human Prostatic Tissues

It was determined by titration that 0.5 ml of a 1:500 dilution of hemolysin contained 2 units of complement. This amount proved to be the highest hemolysin dilution which gave complete sheep red blood cell hemolysis. Complement was titrated also. Two full units of a solution of complement diluted 1:75 and contained in 1 ml was found to be the least amount of complement which provided complete lysis of sheep red blood cells.

The test system was carried out as described in Table IV.

When a 1:5 dilution of suspended fibroblast cells and a 1:10 dilution of rabbit antifibroblast antiserum were included in test systems, the greatest amount of complement fixation was observed. From such observations it was evident that antibody specific for fibroblasts was present in the rabbit antiserum.

### Visual Effects of Non-Heat-Inactivated Rabbit Antifibroblast Antiserum on Fibroblasts and H. Ep. 2 Cells

After treating human prostatic fibroblasts with rabbit antifibroblast antiserum in the presence of complement, growth of fibroblasts was limited and cell lysis occurred so that fibroblasts disappeared from cultures in 24 to 72 hr.



TABLE IV

AN ESTIMATION OF THE AMOUNT OF RABBIT ANTIFIBROBLAST  
ANTISERUM REQUIRED TO FIX COMPLEMENT IN THE  
PRESENCE OF VARYING DILUTIONS OF  
FIBROBLAST CELL SUSPENSIONS

Rabbit Antifibroblast Antiserum Dilutions	Fibroblast Cell Suspension Dilutions		
	1:5	1:10	1:20
Undiluted	+	+	+
1:5	++	+	+
1:10	++++	+++	-
1:20	++	-	-

Symbols:

+ = no hemolysis

- = hemolysis



Rabbit antifibroblast antiserum and complement had no visible effect on H. Ep. 2 cells. The latter were known to be malignant epitheloid cell types. These findings suggested that rabbit antifibroblast antiserum added in the presence of complement brought about a specific reaction with fibroblasts which caused them to lyse; however, such activity was not demonstrable for H. Ep. 2 cells. Photographs displaying these effects were documented and recorded in Figure 1.

Determination of Effective Concentrations of Heat Inactivated Rabbit Antifibroblast Antiserum and Complement as Used to Lyse Fibroblasts Present in Cultures of Mixed Cell Types Derived from Human Prostatic Explants

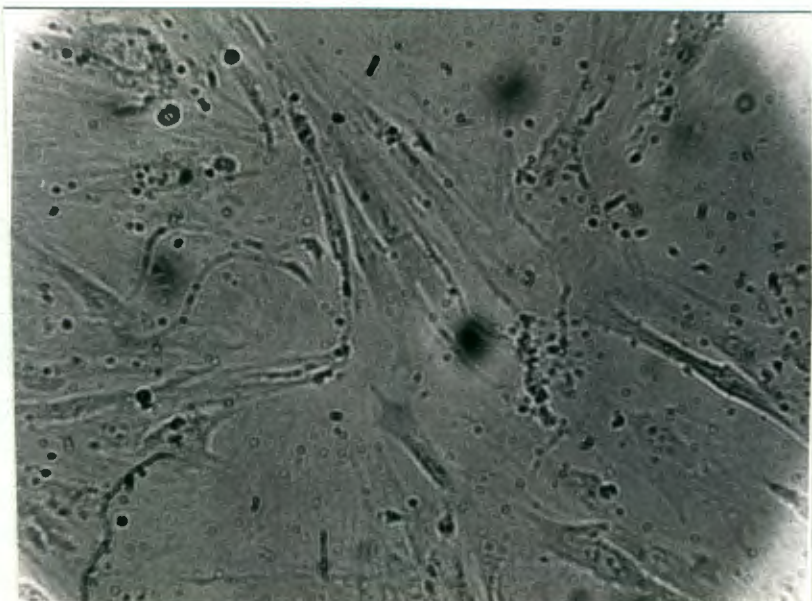
As seen in Table V, 1 ml of complement diluted 1:5 and 0.2 ml of antifibroblast antiserum diluted 1:2 provided the best conditions for fibroblast cell lysis when these reagents were used. Antifibroblast antiserum represented pooled serum collected from 6 immunized rabbits as obtained early during these studies.

Depending on controls, rabbit antifibroblast antiserum alone or complement alone or normal rabbit serum with complement gave negative results throughout. It was apparent that it was necessary to add complement in the reaction mixture in order to achieve lysis of fibroblasts when antigen reacted with antibody. Also, it appeared that rabbit antifibroblast antiserum was specific for cells demonstrating fibroblast like morphology but not for cells resembling epithelium.

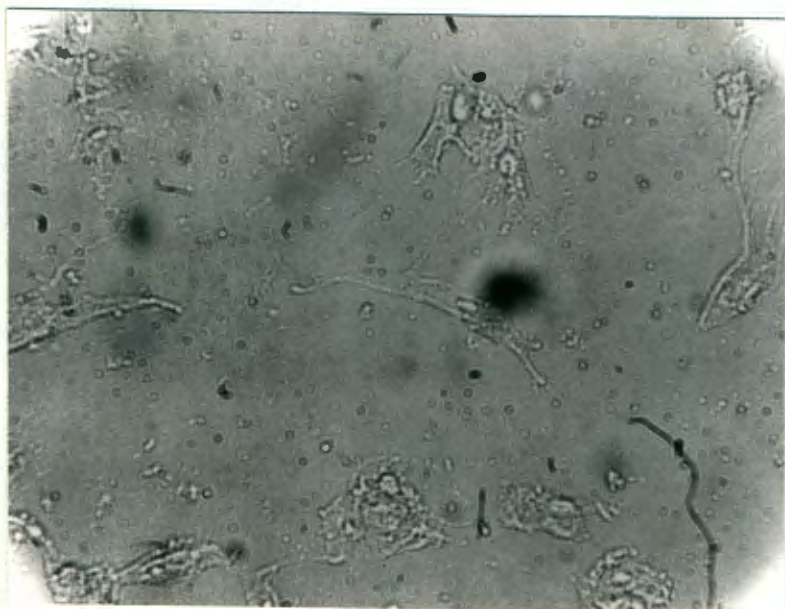
Figure 1. A comparison of fibroblast and H. Ep. 2 cell cultures before and after treatment with antifibroblast antiserum and complement.

Fibroblast cultures (12th passage) were obtained initially from human prostatic tissue while H. Ep. 2 cells (epithelial like) were isolated originally from a patient known to have carcinoma of the larynx. Photographs, taken with a Zeiss Standard W. L. Research Microscope equipped with Polaroid attachments, were made just before treatment of cultures (Figs. 1a and 1c) with antifibroblast antiserum (0.1 ml of undiluted preparation) and complement (0.1 ml of undiluted preparation). Twenty-five hours following treatment with antifibroblast antiserum plus complement, photographs were recorded again (Figs. 1b and 1d). Photographs shown in Figs. 1a and 1b were made of fibroblast cultures before and after antifibroblast treatment respectively while photographs displayed in Figs. 1c and 1d were made of H. Ep. 2 cells before and after antifibroblast treatment respectively. Magnification x205.

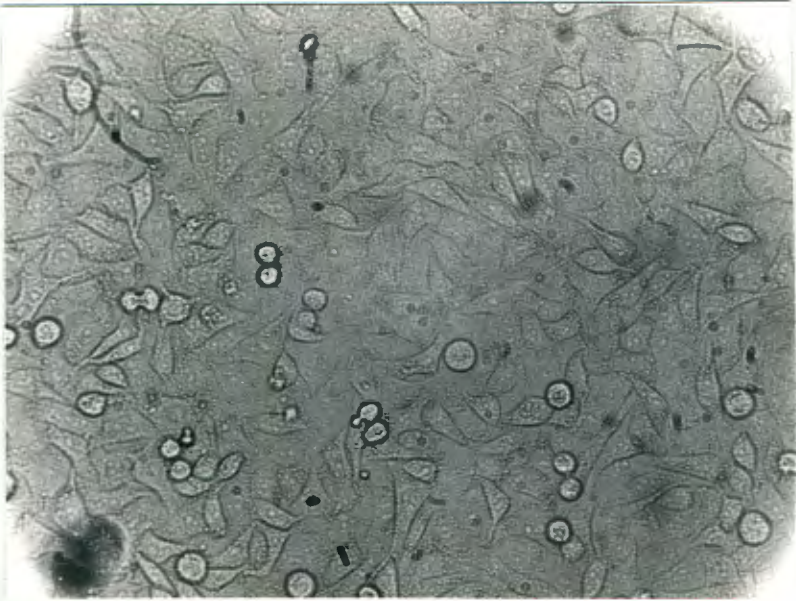




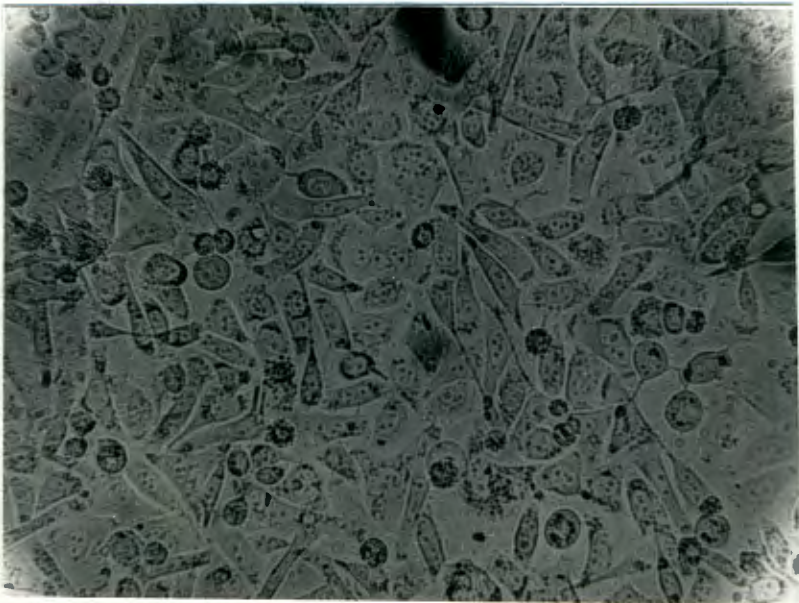
(1a)



(1b)



(1c)



(1d)



TABLE V

RESULTS OF CONTROL SYSTEMS AND DIFFERENT DILUTIONS OF RABBIT ANTIFIBROBLAST ANTISERUM WITH COMPLEMENT ON CULTURES COMPOSED OF MIXED FIBROBLASTS AND EPITHELIAL CELLS DERIVED FROM HUMAN PROSTATIC TISSUES

TEST SYSTEMS					CONTROLS SYSTEMS			
Rabbit Antifibroblast Antiserum Dilutions	Complement Dilutions				Cells Alone	Rabbit Antifibroblast Antiserum Alone	Complement Alone	Normal Rabbit Serum + Complement
	Undiluted	1:5	1:10	1:20				
Undiluted	+	+	++	+++	-	-	-	-
Diluted 1:2	+++	++++	++	++	-	-	-	-

+ to ++++ indicated degree of cell lysis when 0.2 ml of undiluted or rabbit antifibroblast antiserum diluted 1:2 and 1 ml complement concentrations ranging from undiluted to 1:20 were added to the mixed cultures of fibroblasts and epithelium obtained from human prostatic tissues. Higher dilutions of reactants were not made because antiserum and cell cultures were limiting.

Many epithelial cells remained in cultures following treatment of mixed cell type outgrowth with antifibroblast antiserum and complement. These results were observed and recorded in photographs shown in Figure 2.

After many experimental assays using different dilutions of rabbit antifibroblast antiserum and complement, it was concluded that undiluted rabbit antifibroblast antiserum plus complement diluted 1:150 or rabbit antifibroblast antiserum diluted 1:5 plus complement diluted 1:75 provided very little evidence of fibroblast cell lysis. Because quantities of mixed cell cultures consisting of fibroblasts and epithelial cells and the supply of rabbit antifibroblast antiserum were limiting other dilutions of reactants could not be carried out in these studies. Of the various dilutions of rabbit antifibroblast antiserum and complement, rabbit antifibroblast antiserum diluted 1:2 supplemented with complement diluted 1:5 provided the best and most consistent effects in terms of promoting lysis of fibroblasts.

#### Purification of IgG Recovered from Rabbit Antifibroblast Antiserum and Normal Rabbit Serum

By reference to the standard curve for protein estimation, prepared according to the Lowry method and shown in Figure 3, the protein content of the reconstituted fraction of antifibroblast antiserum after  $(\text{NH}_4)_2\text{SO}_4$  precipitation was calculated to be 0.071 mg protein per 0.4 ml reaction mixture or 0.18 mg per ml. The protein concentration of



Figure 2. Photographic demonstration of selective effects of antifibroblast antiserum and complement on cultures of mixed cell types arising from explants of human prostatic tissues.

In these studies various concentrations of antifibroblast antiserum and complement were employed in order to find the most effective reagent proportions necessary to achieve fibroblast destruction. Procedures for culture treatment, including control systems, were recorded in the Materials and Methods section. All cells were grown in Eagle's medium containing 20% fetal bovine serum. Mixed cell type cultures were composed of cells exhibiting epithelial and fibroblast-like morphologies. All antifibroblast antiserum was heat inactivated at 56° for 30 min. Magnification x160.

Identification of photographs: All photographs designated with the letter "a" as from (1a) through (14a) were those cultures composed of mixed cell types which were photographed at zero time before treatment. Photographs designated with the letter "b" were made of preparations treated in the various ways listed below.

(1b) Untreated control culture of mixed cell types photographed 23 hr after zero time.

(2b) Mixed cell type culture photographed 23 hr after treatment with undiluted antifibroblast antiserum only:



an antiserum control.

(3b) Mixed cell type culture photographed 23 hr after treatment with complement only, diluted 1:20: a complement control.

(4b) Mixed cell type culture photographed 23 hr after treatment with undiluted normal rabbit serum and complement diluted 1:5: a normal rabbit serum control.

(5b) Mixed cell type culture photographed 23 hr after treatment with undiluted antifibroblast antiserum and undiluted complement.

(6b) Mixed cell type culture photographed 28 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:5.

(7b) Mixed cell type culture photographed 28 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:10.

(8b) Mixed cell type culture photographed 28 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:20.

(9b) Mixed cell type culture photographed 28 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:30.

(10b) Mixed cell type culture photographed 23 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:45.

(11b) Mixed cell type culture photographed 23 hr after

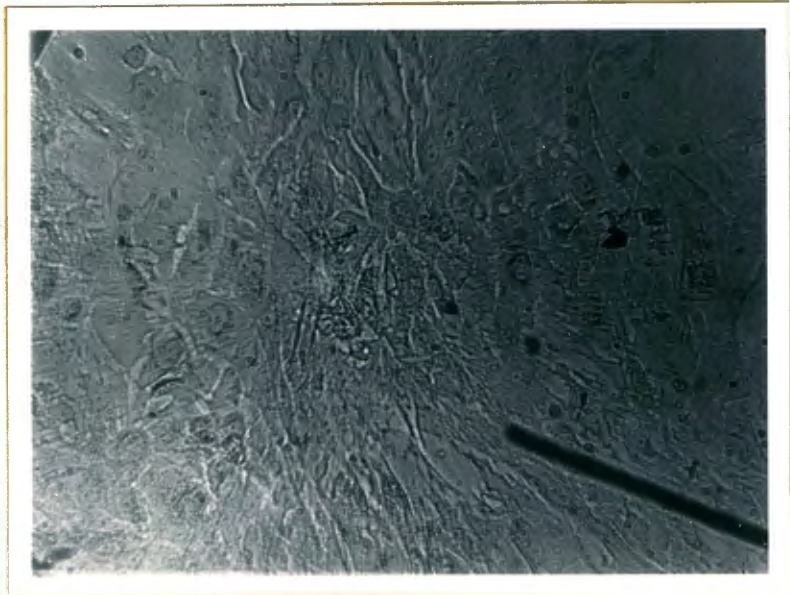
treatment with undiluted antifibroblast antiserum and complement diluted 1:60.

(12b) Mixed cell type culture photographed 23 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:75.

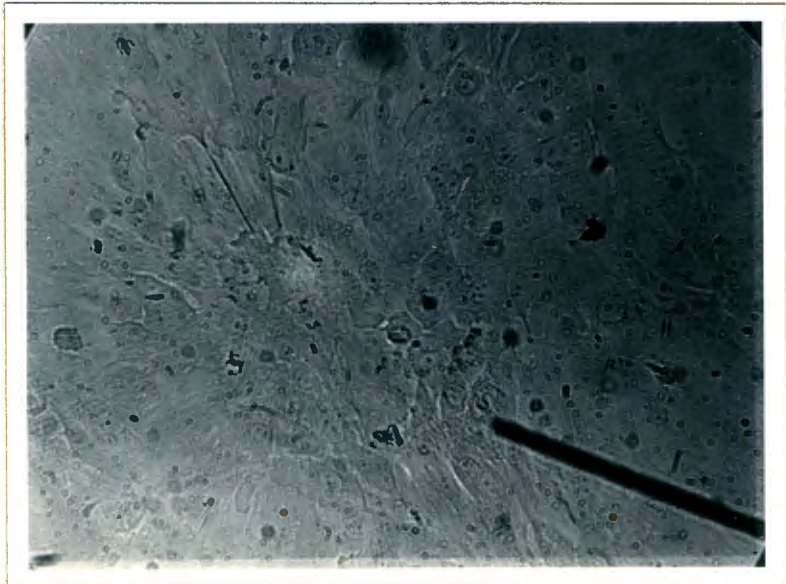
(13b) Mixed cell type culture photographed 23 hr after treatment with undiluted antifibroblast antiserum and complement diluted 1:150.

(14b) Mixed cell type culture photographed 23 hr after treatment with antifibroblast antiserum diluted 1:2 and complement diluted 1:5.

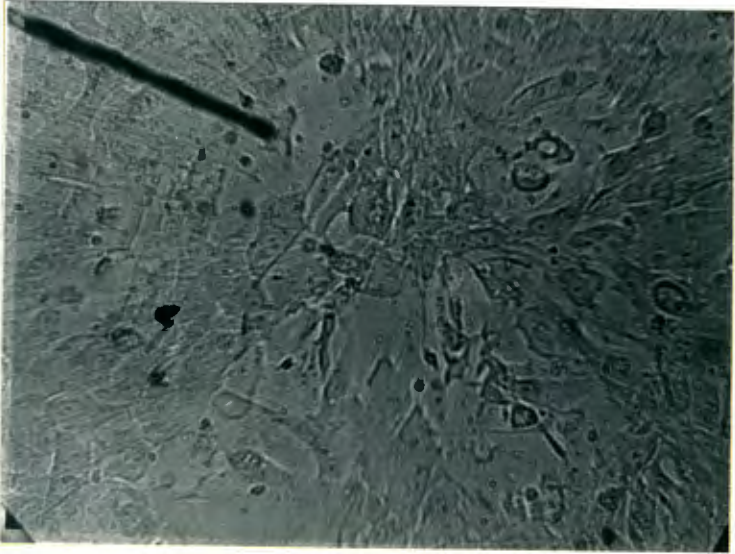
(1a)



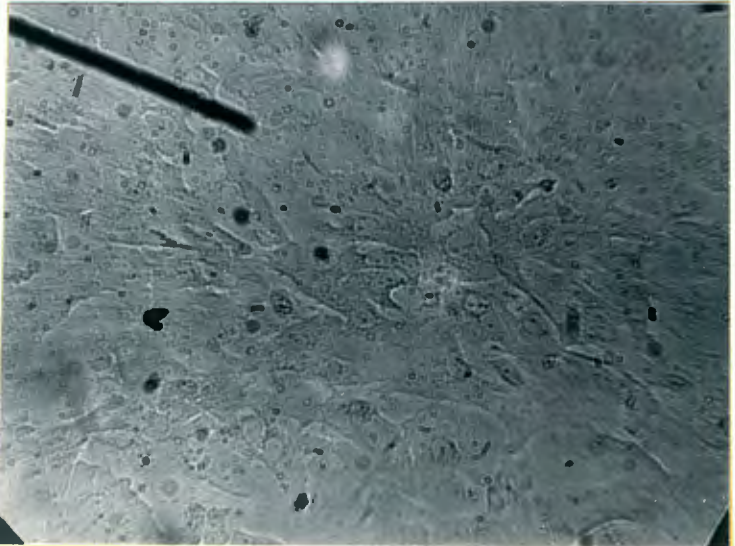
(1b)



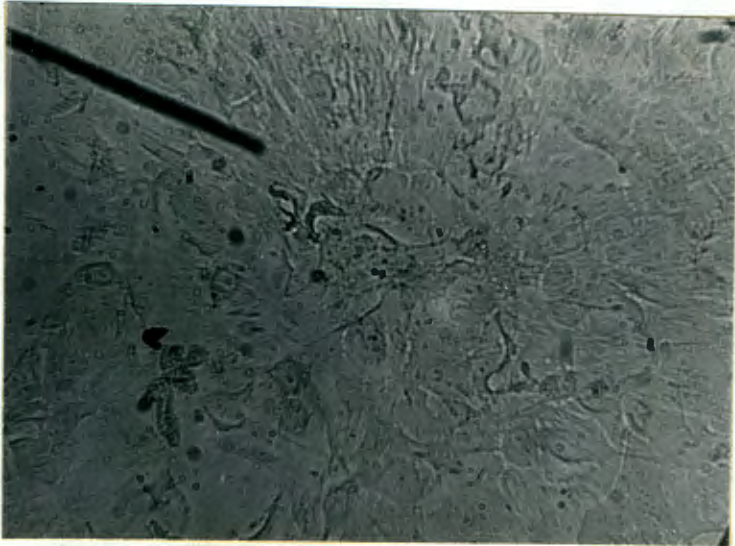




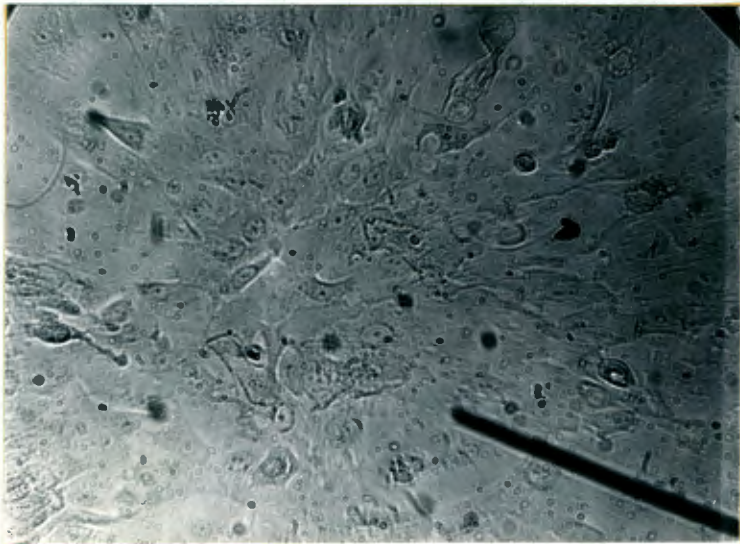
(2a)



(2b)

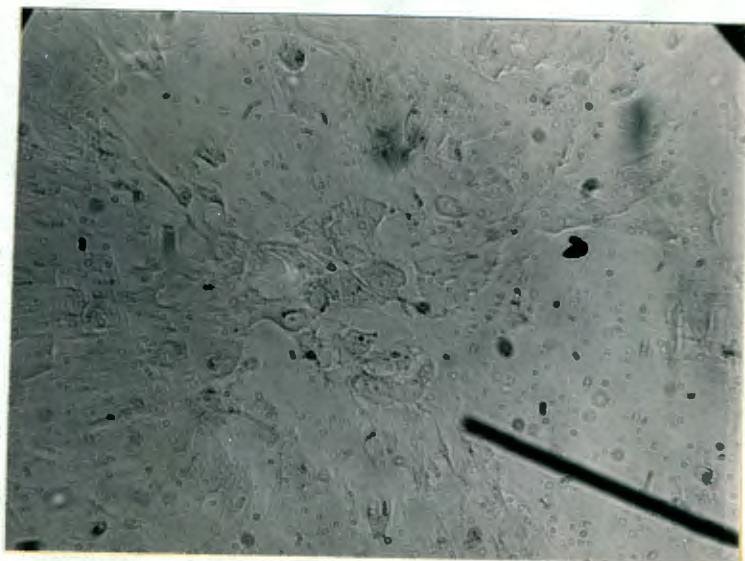


(3a)

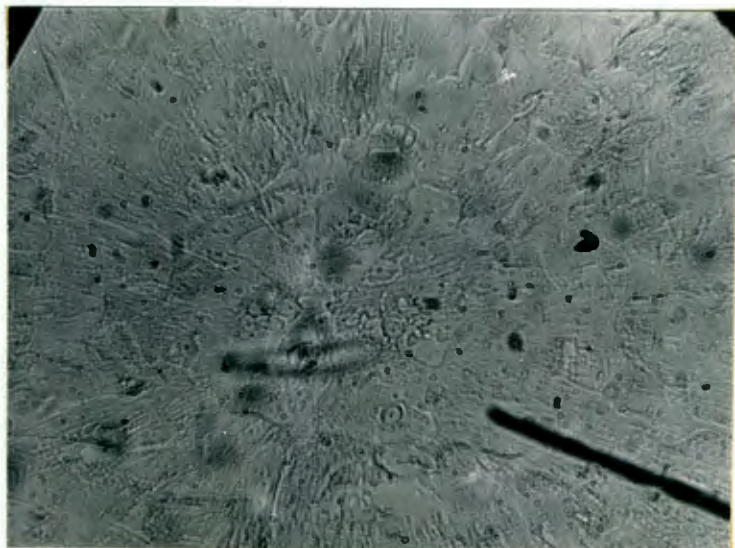


(3b)



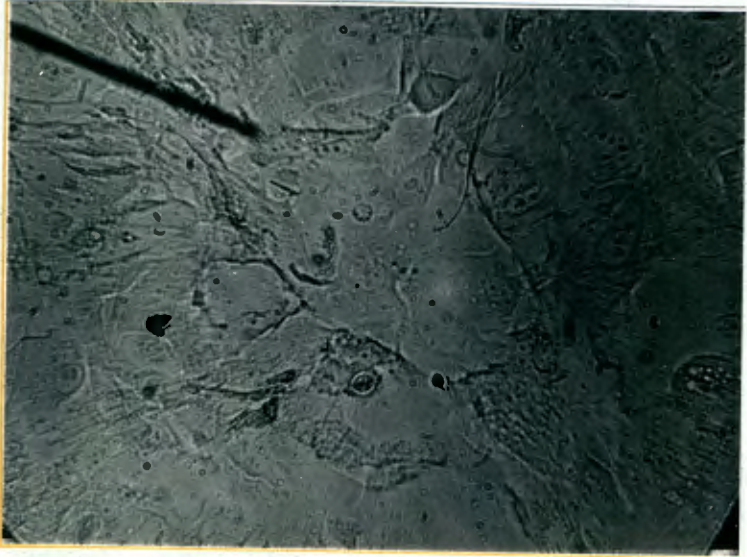


(4a)

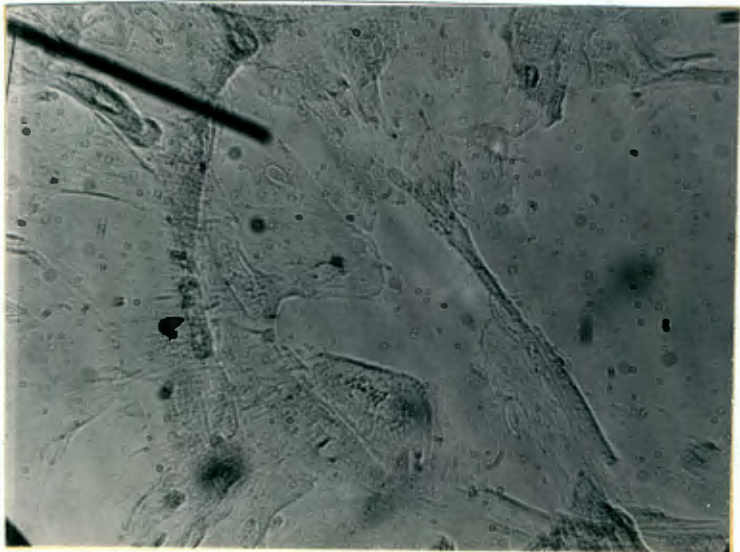


(4b)



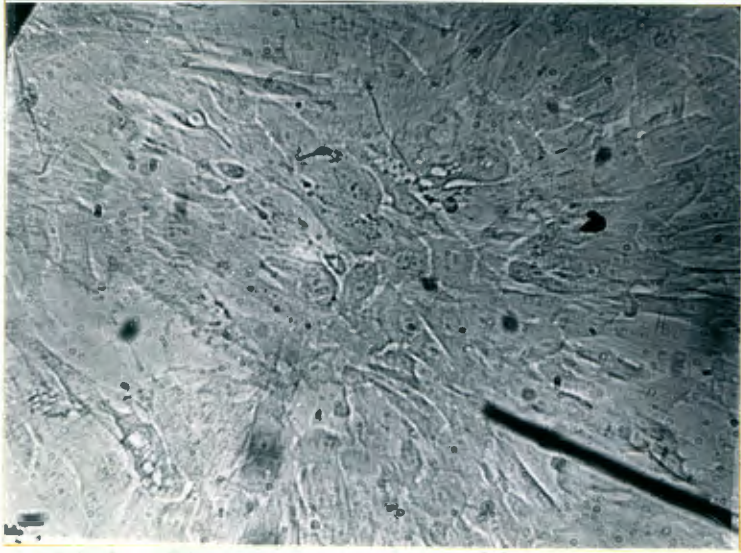


(5a)

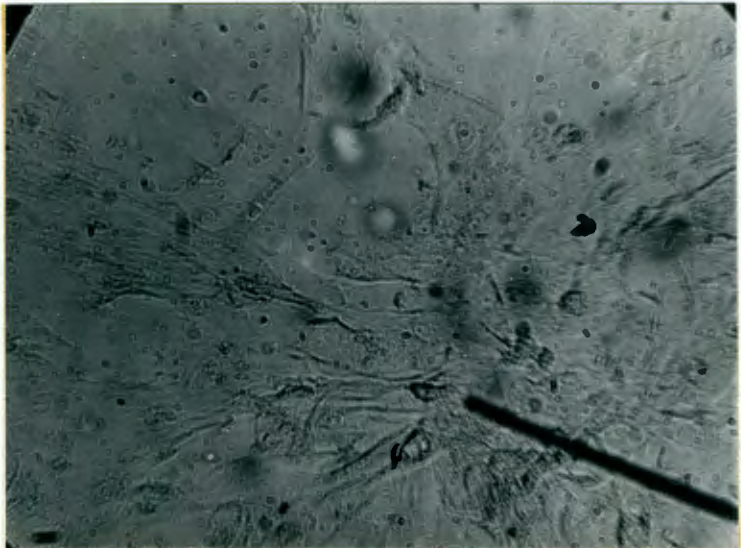


(5b)

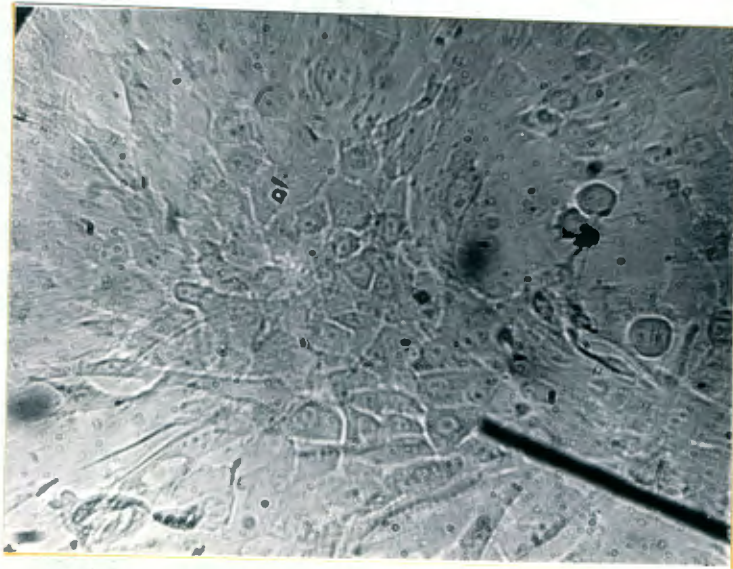
(6a)



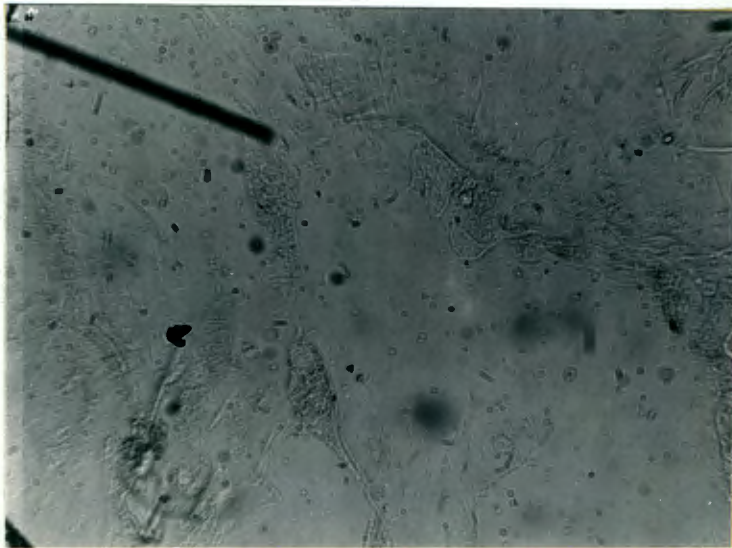
(6b)







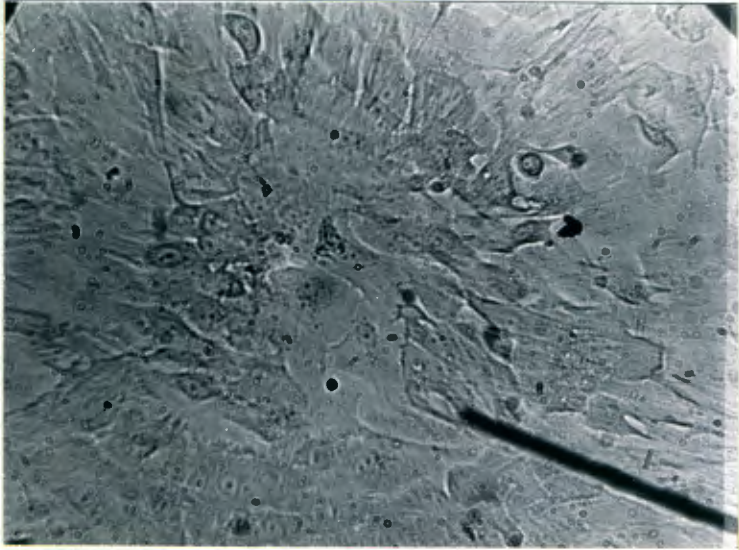
(7a)



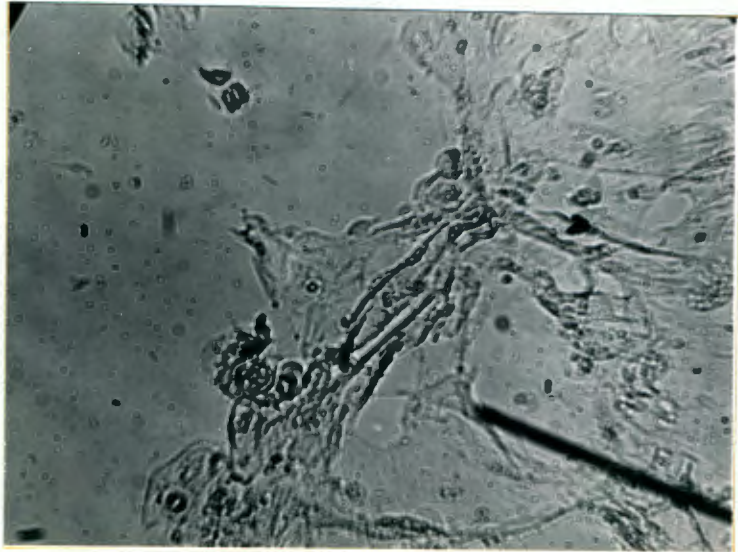
(7b)



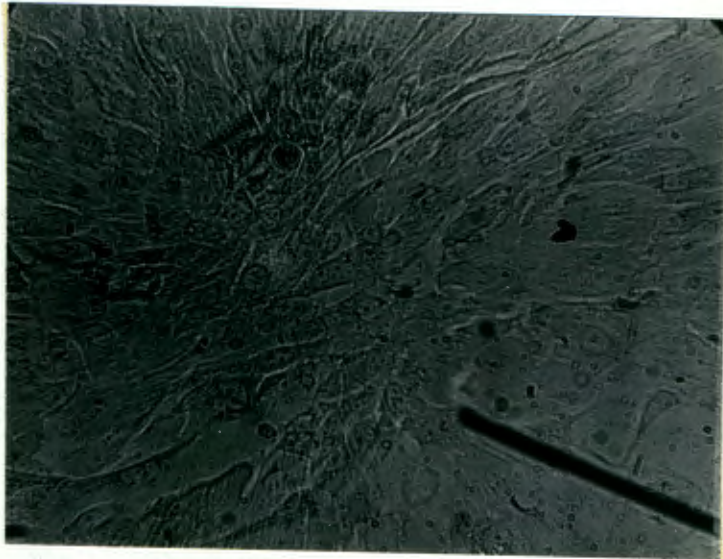
(8a)



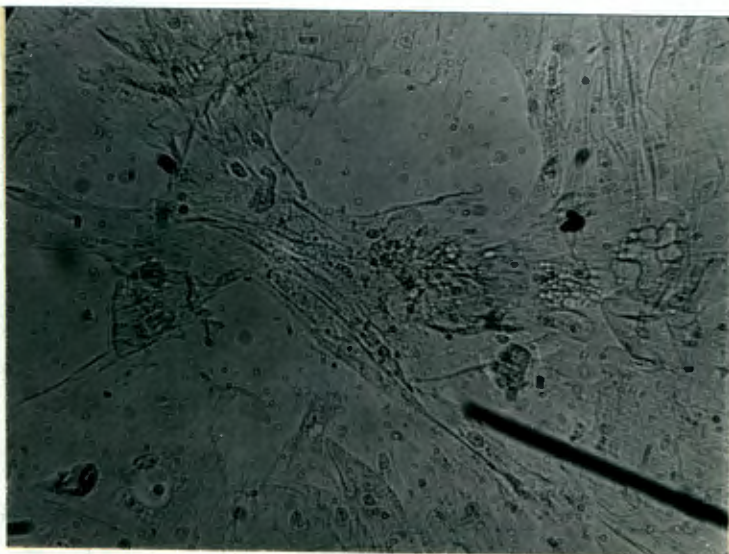
(8b)



(9a)

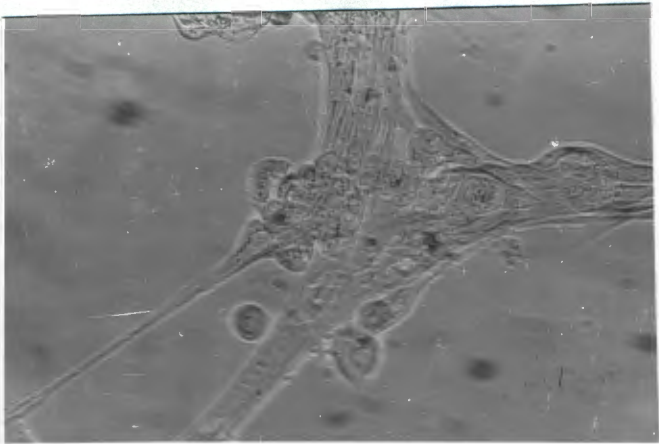


(9b)



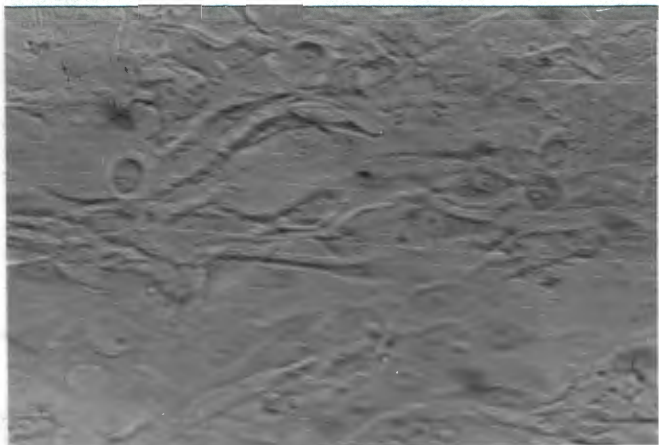


(10a)



(10b)

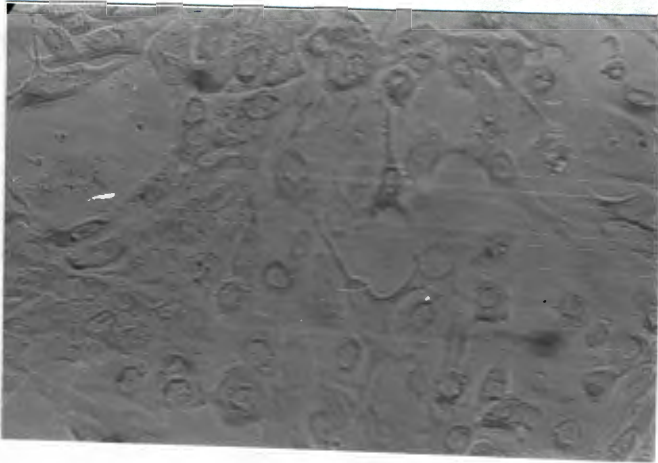




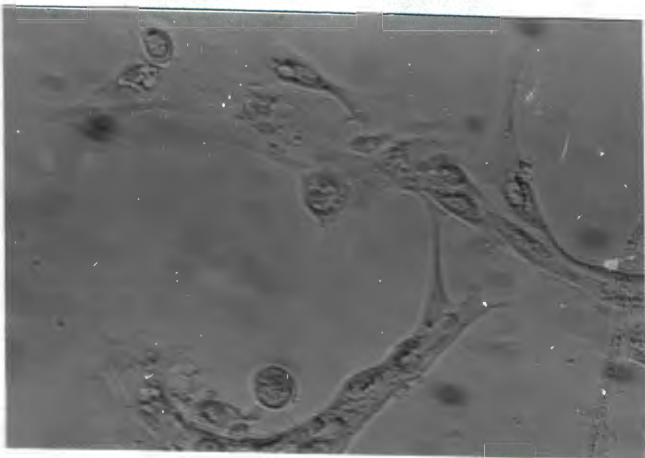
(11a)



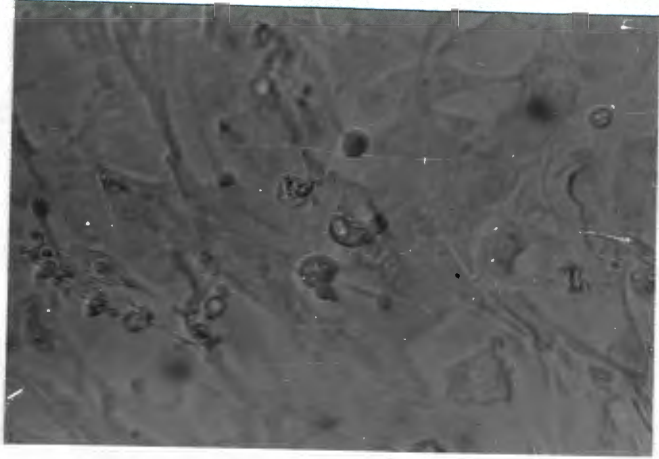
(11b)



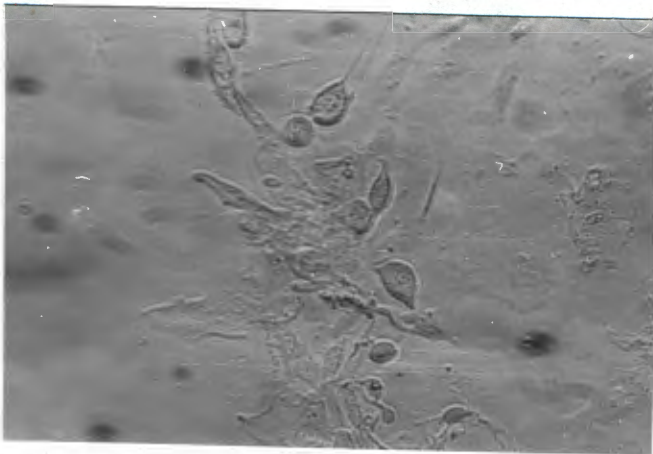
(12a)



(12b)



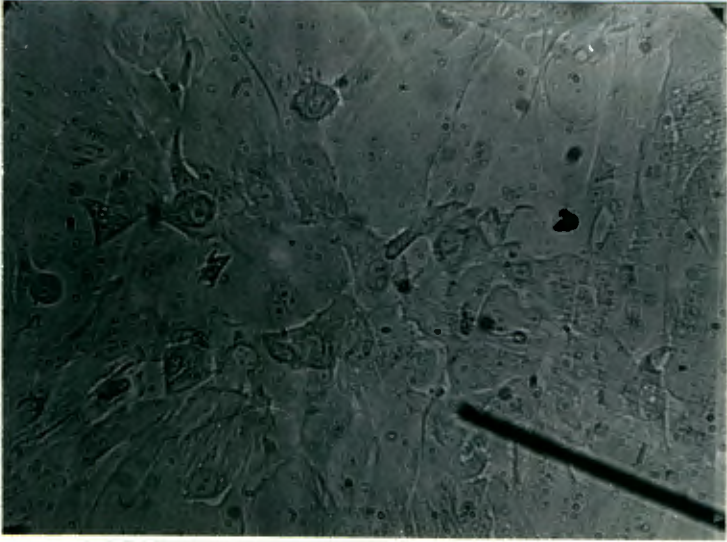
(13a)



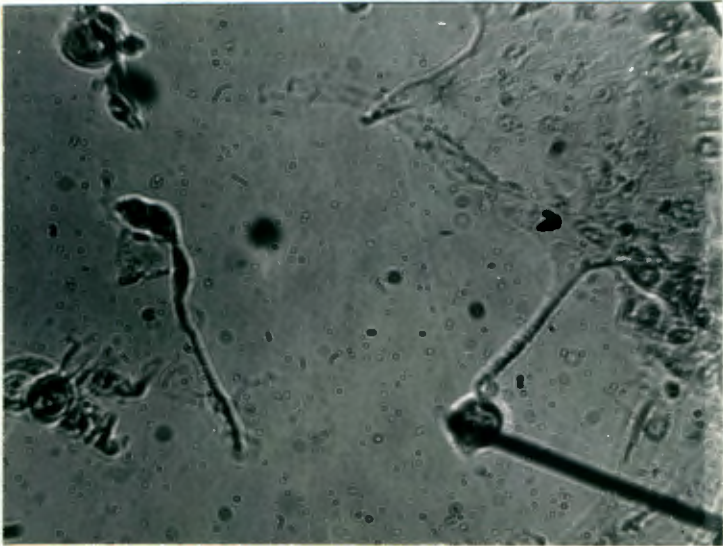
(13b)



(14a)



(14b)



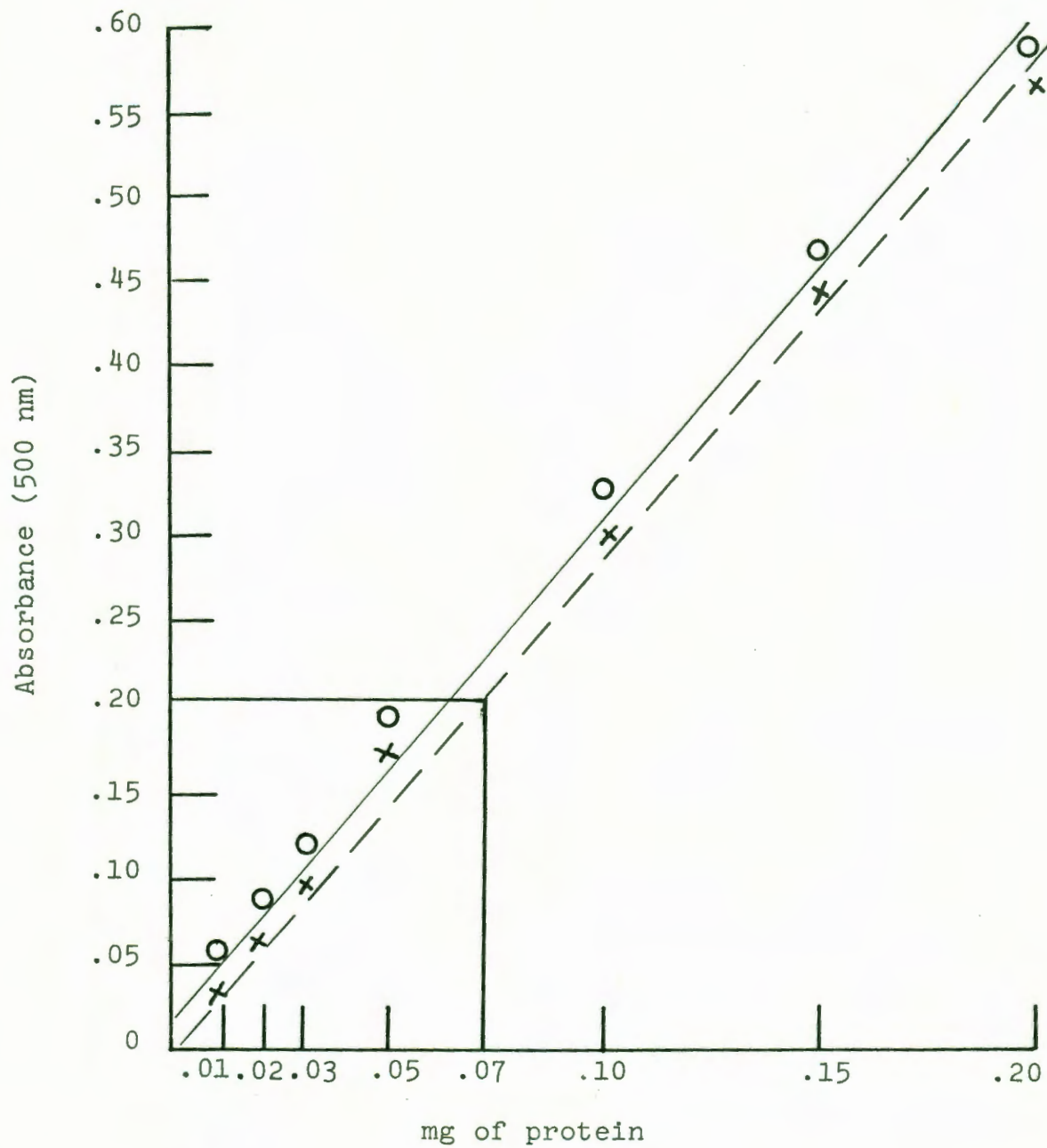


Figure 3. Standard curve as prepared by the Lowry method for protein estimation and used to calculate protein content of rabbit antifibroblast antiserum and that of fractions derived from it.

purified IgG recovered from antifibroblast antiserum was found to be 18 mg per ml.

As calculated from an appropriate Lowry standard curve for protein estimation shown in Figure 4 the protein concentration of the reconstituted fraction of normal rabbit serum after  $(\text{NH}_4)_2\text{SO}_4$  precipitation used in these experiments was found to be 0.066 mg per 0.4 ml reaction mixture or 0.17 mg per ml. Purified IgG isolated from normal rabbit serum contained 17 mg protein per ml.

Purification of Gamma Globulin Isolated from Rabbit Antifibroblast Antiserum by DEAE Cellulose Column Chromatography

As seen in Figure 5, a single peak representing partially purified gamma globulin was separated from rabbit antifibroblast antiserum and removed from the antiserum by DEAE cellulose column chromatography. High ultraviolet (UV) absorbing (280 nm) spectrophotometric readings suggested that significant amounts of protein were present in samples recovered from the peak area displayed in Figure 5. The average protein concentration of eluted solutions showing UV absorption at 280 nm was calculated to be 0.29 mg per ml.



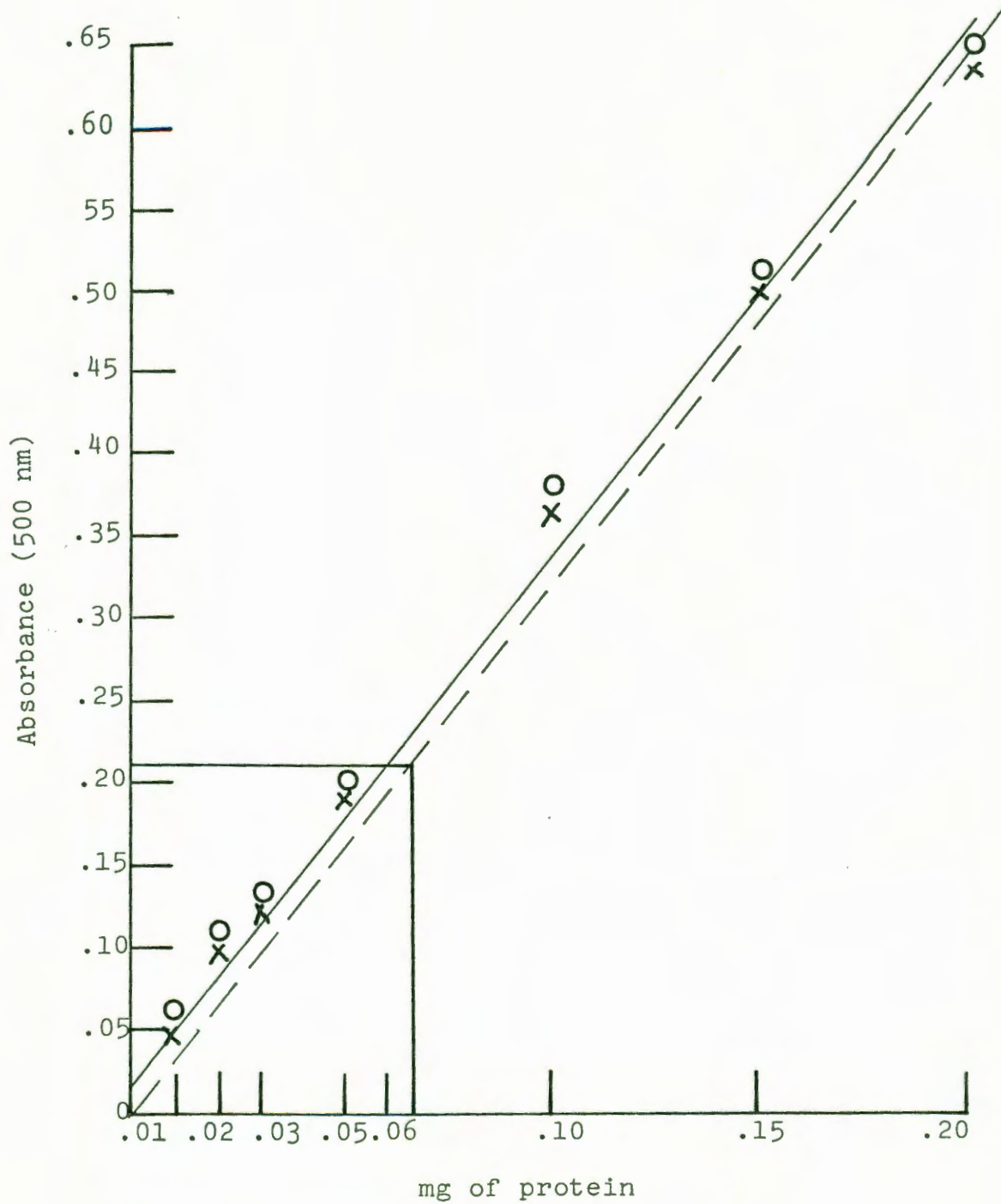


Figure 4. Standard curve as prepared by the Lowry method for protein estimation and used to calculate protein content of normal rabbit serum and that of fractions derived from it.

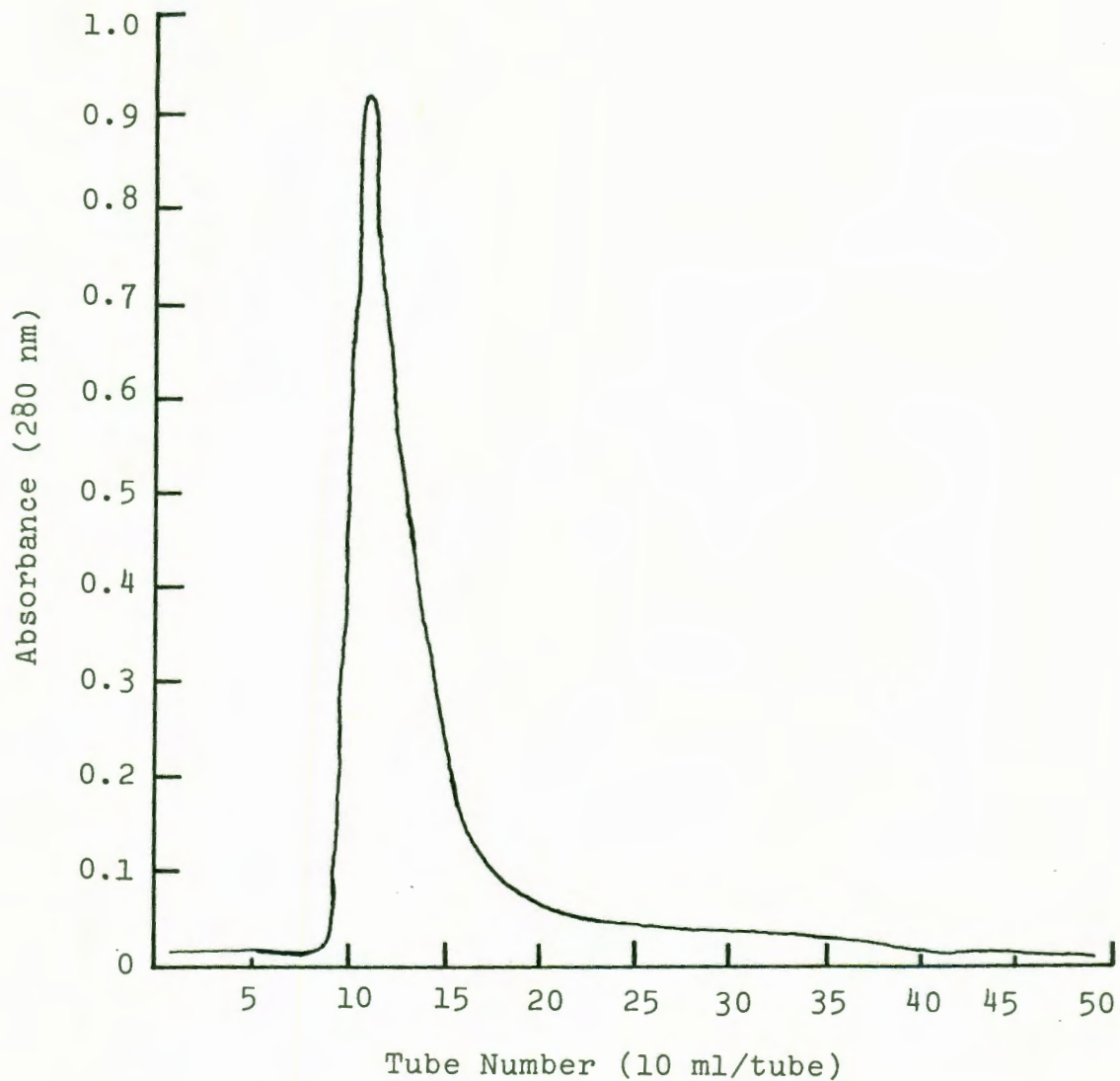


Figure 5. Tracing of a partially purified peak of gamma globulin isolated from rabbit antifibroblast antiserum by DEAE cellulose column chromatography.

Data recorded in Figure 5 represented UV absorption values (read at 280 nm) vs volume. DEAE cellulose column was equilibrated in 0.01 M phosphate buffer, pH 7.5.

Analytical Ultracentrifugation Information Used to Characterize Gamma Globulin Isolated from Rabbit Antifibroblast Antiserum by DEAE Cellulose Column Chromatography

Partially purified gamma globulin recovered from anti-fibroblast antiserum was centrifuged in the Model E Spinco Analytical Ultracentrifuge as stated in the Materials and Methods section in order to obtain sufficient information for sedimentation coefficient calculations. Photographs were made at 8 min intervals and scale readings were measured in cm as recorded in Table VI.  $\log_{10}x$  values were determined by using appropriate  $\log_{10}$  tables.

Using  $\log_{10}$  values vs time in min, as recorded in Table VI it was possible to determine the slope of the line. A plot of the slope was recorded in Figure 6.

$$\text{Equation: } s = \frac{1}{w^2 r} \times \frac{dr}{dt} = \frac{2.303}{60 w^2} \left( \frac{d \log x}{dt'} \right) \quad (3)$$

$$\frac{d \log x}{dt'} = \frac{0.8338 - 0.7850}{80} = 6.1 \times 10^{-4}$$

$$\text{at } 56,000 \text{ rpm, } \frac{2.303}{60 w^2} = 1.116 \times 10^{-9} \quad (3)$$

The sedimentation coefficient of IgG obtained from normal rabbit serum was reported by others to be approximately 6.6. However such measurements were made under totally standard conditions. Observations for the rabbit antifibroblast antiserum IgG fraction were not made under



TABLE VI

DATA REQUIRED FOR THE CALCULATION OF THE SEDIMENTATION  
COEFFICIENT OF IgG ISOLATED FROM RABBIT  
ANTIFIBROBLAST ANTISERUM

Frame Number	Time (interval in min)	Scale Reading (cm)	Log <sub>10</sub> x
1-1	8	6.095	0.7850
1-2	8	6.17	0.7903
1-3	-	-----	-----
1-4	8	6.32	0.8007
1-5	8	6.42	0.8075
2-1	8	6.57	0.8176
2-2	8	6.67	0.8241
2-3	-	-----	-----
2.4	8	6.72	0.8274
2-5	8	6.82	0.8338

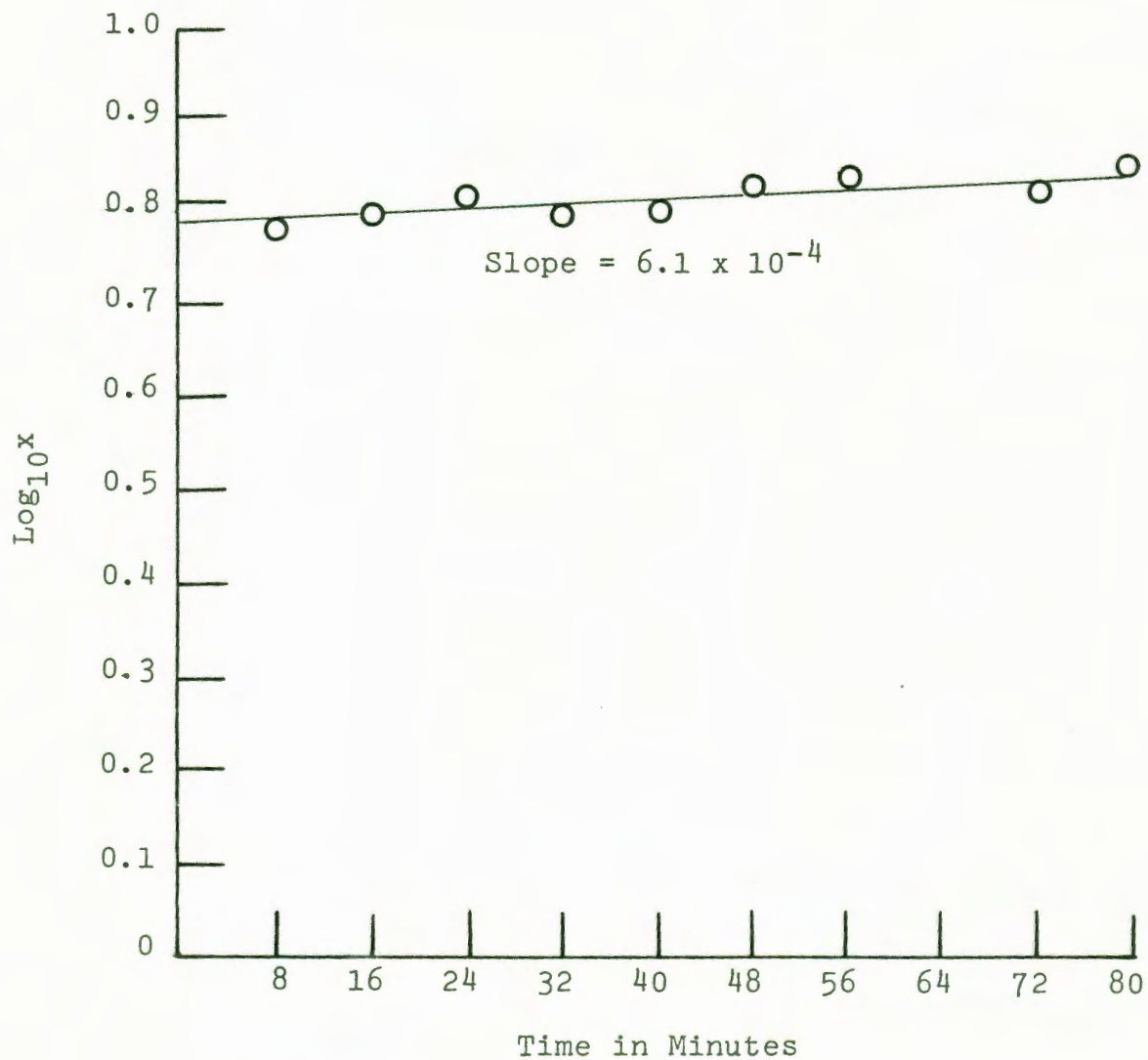


Figure 6. Plot of  $\log_{10}x$  versus  $t$  as used for the calculation of a sedimentation coefficient for gamma globulin (IgG) isolated from antifibroblast antiserum.

rigidly controlled conditions which probably accounted for the small difference in S values. From these data it was evident that the gamma globulin fraction obtained from rabbit antifibroblast antiserum was IgG.

#### Purification of Gamma Globulin Isolated from Normal Rabbit Serum by DEAE Cellulose Column Chromatography

Again a single peak of partially purified gamma globulin, as shown in Figure 7, was obtained from normal rabbit serum. High UV absorbing values when measured spectrophotometrically at 280 nm were obtained. The average concentration of UV absorbing substance in peak solutions was calculated to be 0.27 mg per ml.

#### Analytical Ultracentrifugation Data Used to Characterize Gamma Globulin Isolated from Normal Rabbit Serum by DEAE Cellulose Column Chromatography

As reported for similiar studies with IgG obtained from rabbit antifibroblast antiserum partially purified gamma globulin recovered from normal rabbit serum was centrifuged in the Model E Spinco Analytical Ultracentrifuge. Photographs were made at 8 min intervals and scale readings were measured in cm as recorded in Table VII.  $\log_{10}x$  values were determined by using appropriate  $\log_{10}$  tables.

Using  $\log_{10}$  values vs time in min, as recorded in Table VII the slope of the line was determined. A plot of the slope was recorded in Figure 8 and the S value was



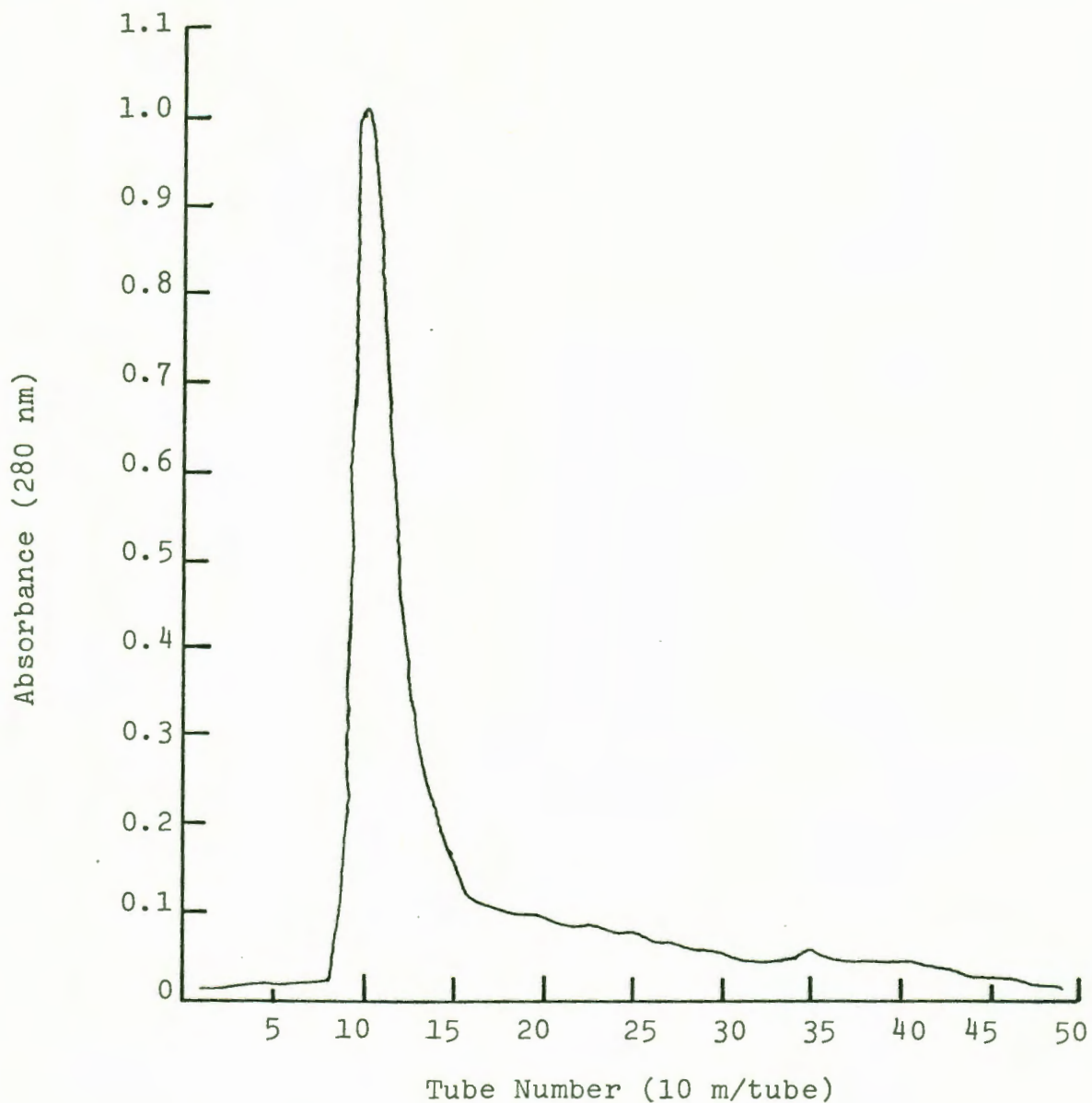


Figure 7. Tracing of a partially purified peak of gamma globulin isolated from normal rabbit serum by DEAE cellulose column chromatography.

Data recorded in Figure 7 represented UV absorption values (read at 280 nm) vs volume. DEAE cellulose column was equilibrated in 0.01 M phosphate buffer, pH 7.5.

TABLE VII

DATA REQUIRED FOR THE CALCULATION OF THE SEDIMENTATION  
COEFFICIENT OF IgG ISOLATED FROM NORMAL  
RABBIT SERUM

Frame Number	Time (interval in min)	Scale Reading (cm)	Log <sub>10</sub> x
1-1	8	5.995	0.7778
1-2	8	6.08	0.7839
1-3	8	6.15	0.7889
1-4	8	6.22	0.7938
1-5	8	6.295	0.7990
2-1	8	6.38	0.8048
2-2	8	6.47	0.8109
2-3	8	6.52	0.8142
2-4	8	6.62	0.8209
2-5	8	6.695	0.8257

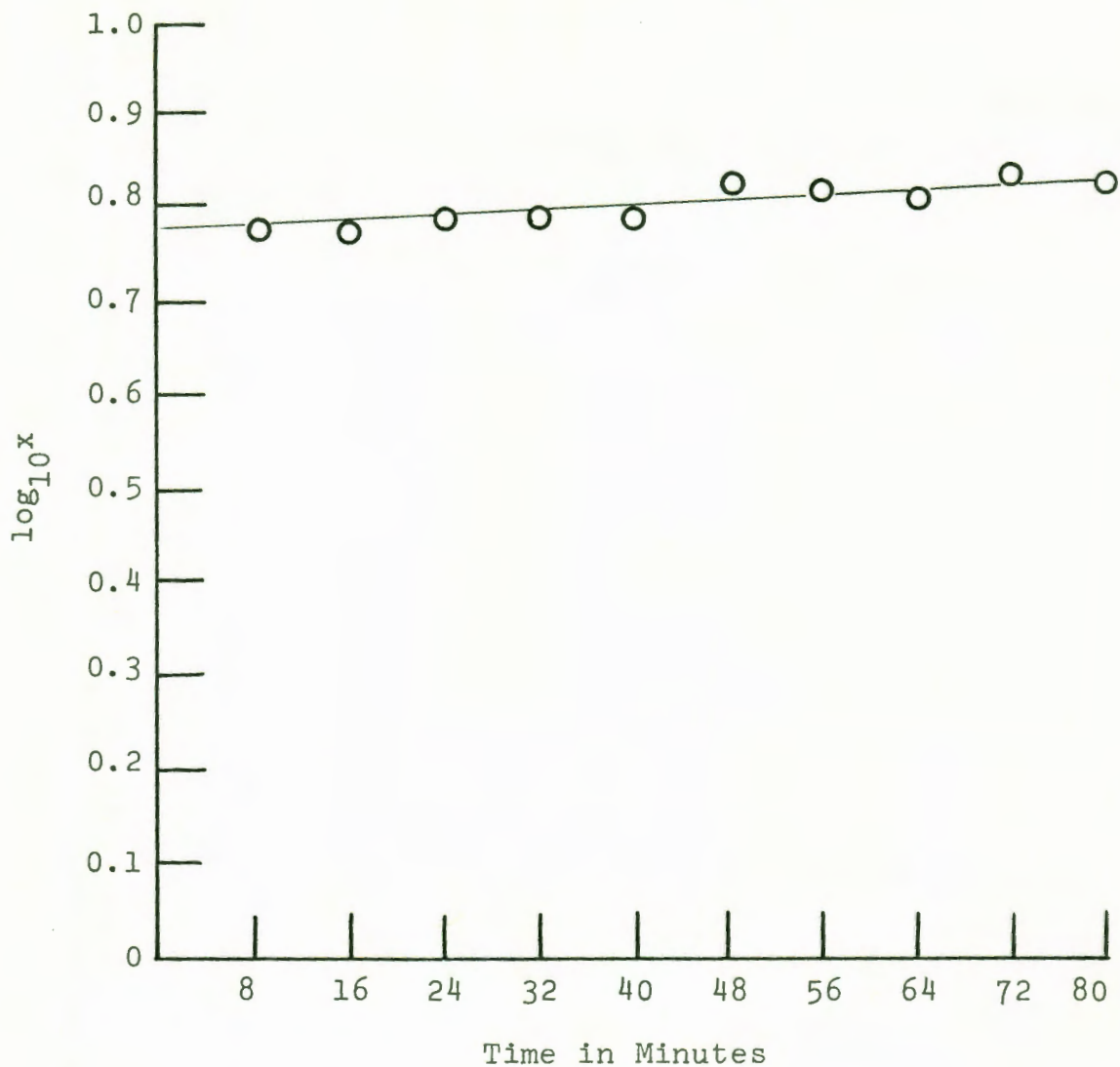


Figure 8. Plot of  $\log_{10}x$  versus  $t$  as used for the calculation of a sedimentation coefficient for gamma globulin (IgG) isolated from normal rabbit serum.



determined as follows:

$$\begin{aligned} \frac{d \log x}{dt'} &= 5.99 \times 10^{-4} \\ s &= \frac{2.303}{60 \text{ } w^2} \left( \frac{d \log x}{dt'} \right) \\ &= (1.116 \times 10^{-9}) (5.99 \times 10^{-4}) \\ &= 6.68 \times 10^{-13} \\ &= 6.68 \text{ s} \end{aligned}$$

The data indicated that the purified gamma globulin fraction obtained from normal rabbit serum was IgG, as was the fraction characterized from rabbit antifibroblast antiserum.

Complement Fixation Tests Using IgG Isolated from Rabbit Antifibroblast Antiserum as Antibody and Human Prostatic Fibroblasts as Antigen

Indicator System. Two units of hemolysin contained in 0.5 ml of a 1:500 dilution gave complete hemolysis of sheep red blood cells as used in these assays. Two full units of complement diluted 1:75 and contained in a 1 ml volume was the least amount of complement which could be used in the reaction mixture which gave complete lysis of sheep erythrocytes.

Test System. The complement fixation test with IgG and fibroblasts was carried out by the same procedure as that described for antifibroblast antiserum and fibroblasts using concentrations of constituents of the indicator system as detailed above. Results were recorded in Table VIII.

In the experiment reported in Table VIII, undiluted fibroblast antigen suspension and undiluted IgG purified from rabbit antifibroblast antiserum provided the greatest amount of complement fixation. It was evident that IgG possessed the function of antibody which could react with fibroblast cells to promote cell lysis.

Effects of IgG Obtained from Rabbit Antifibroblast Antiserum and Normal Rabbit Serum on Mixed Human Prostatic Cultures Composed of Fibroblasts and Epithelial Cells

As shown in Figure 9 only IgG isolated from rabbit antifibroblast antiserum had the ability to destroy fibroblasts, while epithelial cells persisted. IgG from normal rabbit serum like untreated normal rabbit serum did not have any effect on fibroblasts or epithelium. Thus formation of IgG in antifibroblast antiserum must have occurred in specific response to the fibroblast antigen.

Effects of Non-Heat Inactivated Rabbit Antifibroblast Antiserum Absorbed with H. Ep. 2 Cells on Human Prostatic Cultures Composed of Fibroblasts and Epithelial Cells

Photographs taken before and after treatment as shown

TABLE VIII

A COMPLEMENT FIXATION REACTION WITH IgG ISOLATED FROM RABBIT  
ANTIFIBROBLAST ANTISERUM AND SUSPENSIONS OF HUMAN PROSTATIC  
FIBROBLASTS SERVING AS TEST SYSTEM REACTANTS

IgG Dilutions	Fibroblast Antigen Dilutions		
	Undiluted	1:5	1:10
Undiluted	++++	+++	++
1:5	----	neg.	neg.
1:10	----	neg.	neg.

## Symbols:

+ to ++++: Degree of complement fixed in the test system  
neg.: Negative or no complement fixed in test system  
----: Not done at these dilutions because of insufficient amount of fibroblast antigen preparation.

Undiluted fibroblast antigen contained  $2.35 \times 10^6$  cells per ml. Undiluted IgG antibody contained 0.29 mg protein per ml.

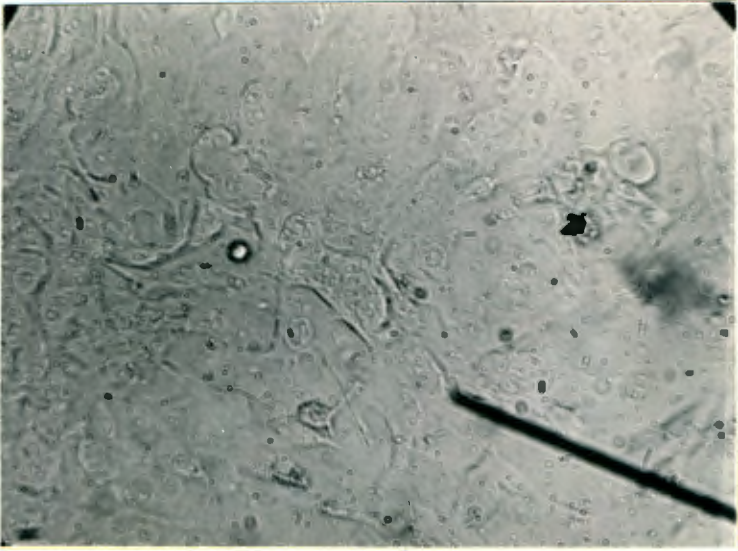


Figure 9. Observation of lytic effects by purified IgG isolated from rabbit antifibroblast antiserum on fibroblasts derived from human prostatic explant outgrowth composed of fibroblasts and epithelial cells.

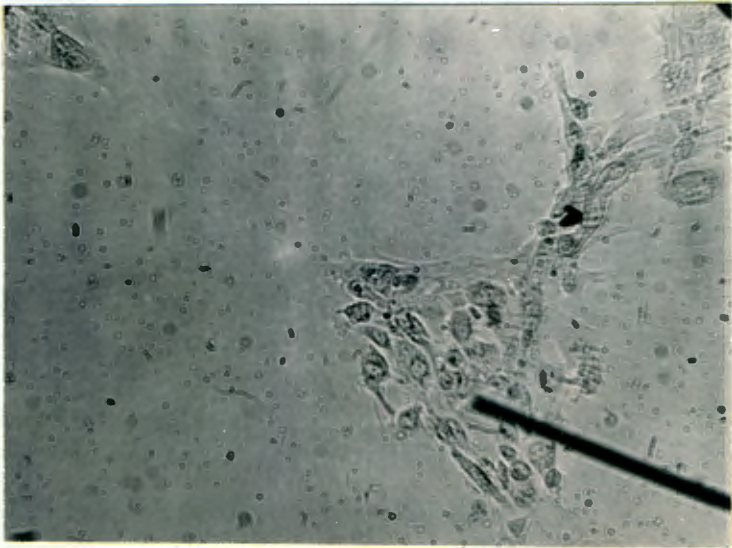
Mixed cultures of fibroblasts and epithelial cells derived from explant outgrowth of human prostatic tissues were photographed (Magnification xl60) at zero time before treatment and 23 hr following treatment. In the interim preparations were cultivated in Eagle's medium containing 20% fetal bovine serum at 37° in a 95% air-5% CO<sub>2</sub> water-saturated environment. Photographs 1a and 2a represent both preparations before treatment.

1b: This culture was allowed to react with complement and IgG isolated from rabbit antifibroblast antiserum according to routine procedures described in Materials and Methods. The preparation was photographed 23 hr after treatment.

2b: The culture was allowed to react with complement and IgG isolated from normal rabbit serum, according to procedures described. The preparation was photographed 23 hr after treatment.

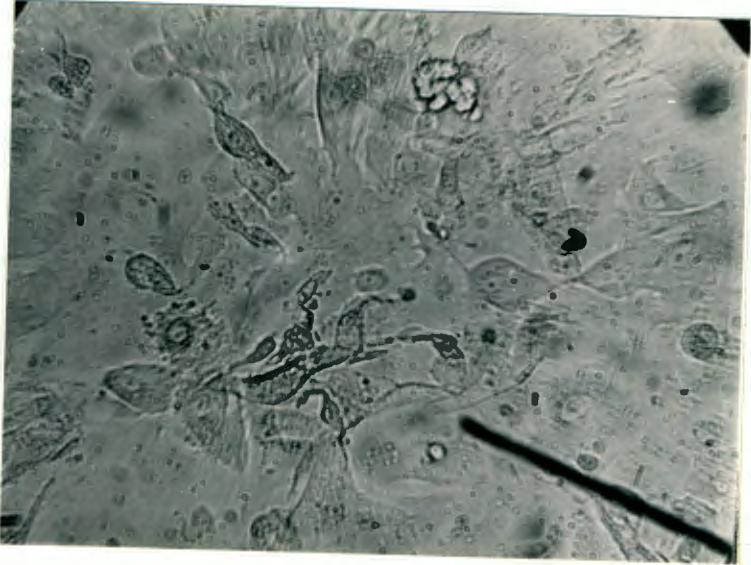


(1a)

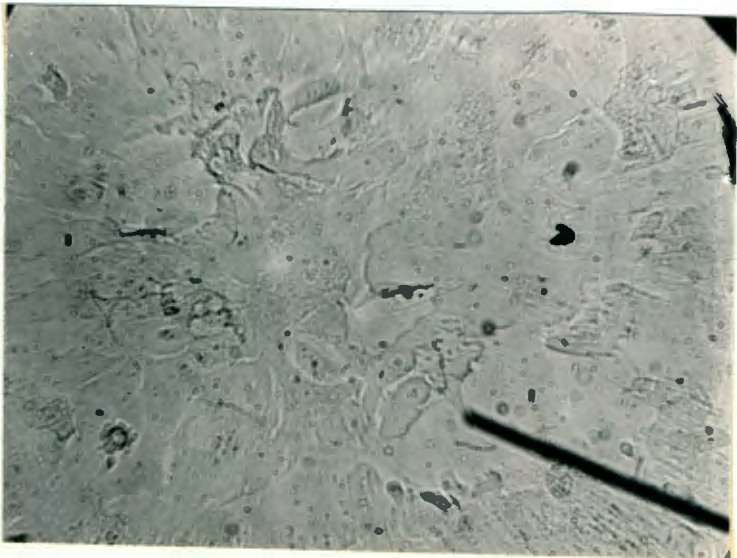


(1b)





(2a)



(2b)

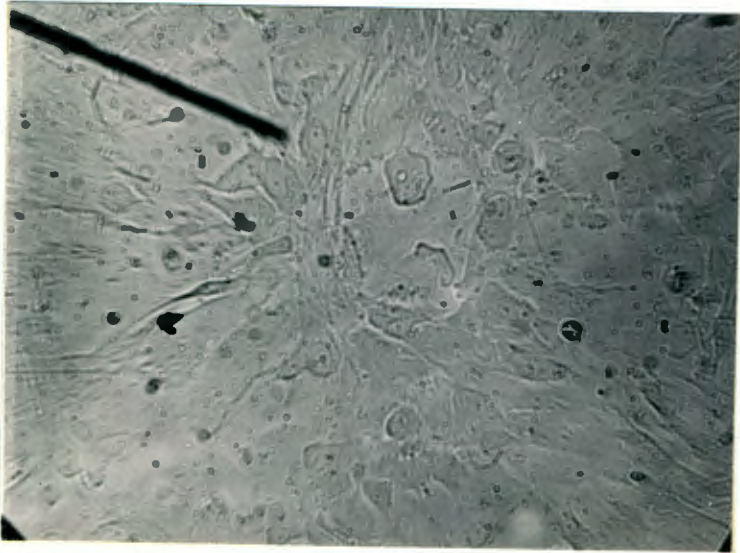


in Figure 10 indicated that rabbit antifibroblast antiserum absorbed earlier with H. Ep. 2 cells (derived from human epidermoid carcinoma of the larynx) destroyed cells which resembled fibroblasts morphologically but had no effect on cells displaying characteristics of epithelium. Antiserum appeared to remain specific for fibroblasts. It was of considerable interest to speculate as to whether or not there were certain different receptor site components on cell surfaces which accounted for the different antigenic responses of fibroblasts and epithelial-like-cells.

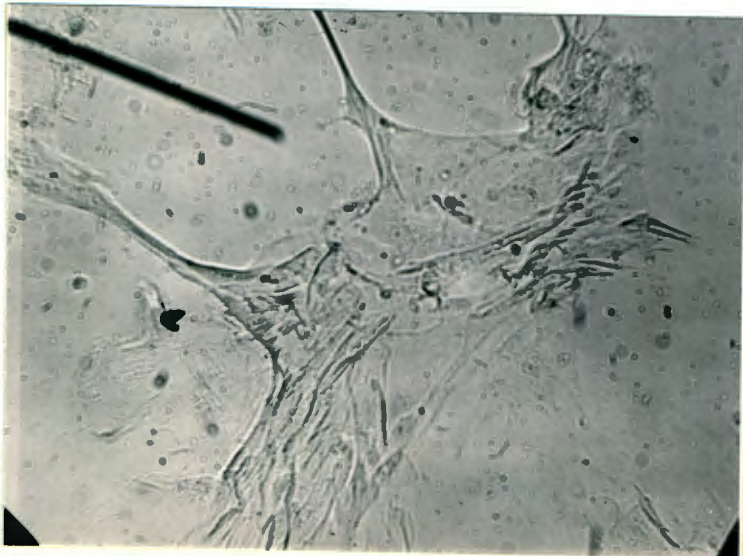
Figure 10. Effect of complement and non-heat-inactivated, H. Ep. 2-cell absorbed, rabbit antifibroblast antiserum on cellular outgrowth of mixed cell types obtained from human prostatic explant tissue.

The preparation was photographed (Magnification x160) before (1a) and 23 hr after (1b) treatment with reactants. Cells were cultivated in Eagle's medium containing 20% fetal bovine serum.

1b: Photograph of culture 23 hr after treatment with complement diluted 1:5 and undiluted-H. Ep. 2-cell-absorbed rabbit antifibroblast antiserum.



(1a)



(1b)



## DISCUSSION

Experimental results presented in this thesis showed that rabbit antifibroblast antiserum in the presence of complement had the capacity to destroy human prostatic cells resembling fibroblasts morphologically, while cells displaying characteristic epithelial-like morphology were unaffected. Control systems containing normal rabbit serum supplemented with complement or rabbit antifibroblast antiserum alone had no effect on fibroblasts and epithelium derived from prostatic tissues. These data suggested that antifibroblast antiserum was specific for the fibroblast antigen. The observation that fibroblasts and epithelial cells might have certain differences in antigenicity was extended further when it was shown that antifibroblast antiserum absorbed in the presence of H. Ep. 2 epithelial-like cells lysed fibroblasts only and not epithelial cells found growing in mixed cell type cultures derived from human prostate tissue. Concentrations of rabbit antifibroblast antiserum diluted 1:2 in the presence of complement diluted 1:5 provided the most effective reaction mixture for observing lysis of fibroblasts. When fibroblasts and epithelial cells found in cultures of prostatic explant outgrowth were treated with antibody plus complement, fibroblasts lysed and disappeared, while epithelium remained intact and multiplied.

In general, ammonium sulfate precipitation followed by DEAE cellulose column chromatography were found to be very

useful methods for isolation of IgG from various serum sources (20, 23); these methods were frequently used in enzyme purification studies also (19, 14). IgG isolated from rabbit antifibroblast antiserum caused fibroblast lysis. Concentration studies were not carried out to determine whether purified IgG or crude antifibroblast antiserum was most effective in promoting fibroblast lysis. It was not known if IgG was partially denatured in the purification process. Such questions require further investigation.

According to data reported in this thesis, quantities of IgG purified from rabbit antifibroblast antiserum and normal rabbit serum were very close in value, while sedimentation coefficients varied slightly. Variability must be due to the fact that standard conditions were not maintained when such determinations were made. In regard to IgG activity, perhaps responses obtained with IgG as compared with crude rabbit antifibroblast antiserum differed as a result of partial inactivation of IgG protein during purification processes of gamma globulin. Our findings demonstrated the usefulness of the complement fixation test in observing lysis of fibroblasts when subjected to antifibroblast antiserum. The test was shown to be rapid and specific. The mechanism of complement fixation has to do with the fact that an antibody-complement complex binds to surface receptors of antigens (11) after which the reaction, such as lysis, occurs. Complement binding sites are located on heavy chains of



antibody molecules found just before and after the s-s regions of antibody. It is known that IgG combines with complement in this way also. Thus fibroblasts are lysed and disappear after treatment of cells with antifibroblast anti-serum and complement while epithelial cells are maintained and continue to proliferate in pure culture. This procedure allows investigators working in the field of cancer carcinogenesis to use such epithelial cultures as model systems in order to study initial events of the malignant conversion process.



## SUMMARY

Antifibroblast antiserum was prepared in rabbits by immunizing 6 animals with human prostatic fibroblasts cultivated and transferred through 14 passages in tissue culture. By routine complement fixation procedures complement was fixed by antifibroblast antiserum complexed with suspensions of fibroblast cells. Likewise, when rabbit antifibroblast antiserum and complement were added to cultures of mixed cell types obtained from cellular outgrowth of prostatic explant tissues, fibroblasts were destroyed but epithelial cells remained intact in culture and continued to multiply. Normal rabbit serum used for controls had no effect on tissue cultures.

Ammonium sulfate precipitation followed by DEAE cellulose column chromatography was adopted to purify IgG from rabbit antifibroblast antiserum and normal rabbit serum. IgG obtained from rabbit antifibroblast antiserum and supplemented with complement eliminated growth of fibroblasts but not epithelium. IgG obtained from normal rabbit serum and tested in the presence of complement had no effect on cultures of prostatic fibroblasts and epithelial cells.

Following rabbit antifibroblast antiserum absorption with H. Ep. 2 cells, absorbed antiserum in the presence of complement retained the capacity to lyse fibroblasts but not epithelial cells.

Thus fibroblasts were destroyed and after treatment with rabbit antifibroblast antiserum and complement,

epithelial cells persisted intact and multiplied in pure culture. As a result of these research efforts a model system was provided so that other investigators could study initial events in the conversion of normal prostatic epithelium to malignant cells.

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