

## The soluble antigens of varicella-zoster virus produced in tissue culture

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### INTRODUCTION

Until recently the use of serological tests on patients' sera in suspected cases of chickenpox or zoster infection has been limited because of the difficulty of obtaining suitable antigens. Vesicle fluid from either disease serves as a satisfactory antigen for complement-fixation tests (Brain, 1933; Taylor-Robinson & Downie, 1959), but the difficulty of obtaining adequate supplies for routine work has been a serious handicap. The successful cultivation of varicella virus by Weller & Stoddard (1952) has rendered possible the use of antigens prepared from tissue cultures. Weller (1958) and Weller & Witton (1958) successfully tried this method, but because antigen was apparently present in tissue culture fluids only in very small amounts, concentration of the fluids by ultrafiltration was thought desirable. These authors used bovine amniotic fluid as the tissue culture medium but found that their concentrated antigens were strongly anti-complementary. Indeed, it was necessary to heat their antigens at 60° C. for 30 min. to abolish this effect. Taylor-Robinson & Downie (1959) found that occasional infected tissue culture fluids could be used unconcentrated in complement-fixation tests. Their tissue culture fluids contained horse serum and because of its anti-complementary activity concentration was not a practical measure.

Further work in this laboratory has shown that bovine amniotic fluid is the more suitable medium for the preparation of zoster and chickenpox antigens from tissue cultures. A simple method of concentrating the antigen in such fluids yields preparations which are satisfactory for routine complement-fixation tests. Our concentrated preparations have not proved anti-complementary and, as heating has been unnecessary, they have retained full serological reactivity. In this paper the method of preparation and the results of tests with concentrated antigens are described.

### MATERIALS AND METHODS

#### *Tissue cultures*

These were human amnion cultures prepared by the technique of Zitzer, Fogh & Dunnebacke (1955) and grown in 4 or 6 oz. 'medical flat' bottles.

#### *Media*

Growth medium for amnion cells was 10% horse serum, 10% Hartley's tryptic digest broth and 80% Hanks' balanced salt solution with 100 units/ml. penicillin and nystatin and 100 µg./ml. streptomycin.

The same medium, without nystatin, was used as maintenance medium for the first 3–7 days after the cells had been infected with virus.

Various media were tested for antigen production, including bovine amniotic fluid in different concentrations (Enders, 1953), Parker's 199 (Glaxo Ltd.), Eagle's medium without serum (as described by Paul, 1959) and lactalbumen hydrolysate (L.A.H.). The bovine amniotic fluid (B.A.F.) was not filtered but was tested for sterility, and penicillin and streptomycin were added to give final concentrations of 100 units/ml. and phenol red to give a final concentration of 0.002%. It was used as fresh as possible but was still found to be effective as a medium for antigen production after 9 months' storage at +4°C.

### *Viruses*

Strains from both chickenpox and herpes zoster were used. They were originally isolated from vesicle fluids from patients and were passed in human amnion tissue cultures using infected culture cells as inoculum (Weller, 1953). The passage number is recorded for each strain.

### *Methods of preparing antigens from tissue culture fluids*

Incubation throughout was at 36°–37° C. unless otherwise stated. Only bottles showing confluent sheets of amnion cells were used, and these were infected after 7–21 days of growth. Virus, in the form of infected cells from previous cultures, was added to give at least 500 foci of infection in a 6 oz. bottle. The bottles were incubated until approximately 25% of the cell sheet showed cytopathic changes. This usually took 4–8 days. Maintenance medium was then removed and discarded and medium for antigen production (20 ml./6 oz. bottle) added, and renewed after incubation periods of 2–9 days. Media harvested from individual cultures were combined and kept at 4° C. to the end of each experiment, which was terminated when all cells showed either cytopathic changes or non-specific degeneration. The duration of the experiments varied with the medium used, the temperature of incubation, and with cell cultures from different amnions. The fluid harvested from these cultures was centrifuged at 3000 r.p.m. for 10 min. to deposit cell debris. The supernatant contained no infective virus. The total volume of each pool of supernatant fluid was measured and the material then dialysed in 'Visking' dialysis tubing for 5 days at 4° C. against 10 vol. of distilled water which was changed daily. A precipitate sometimes appeared in the fluid within the tubing. This was found on test to have no complement-fixing activity and was discarded. Following dialysis the soluble indiffusible material was dried from the frozen state. The dry material was stored at +4° C., and for test was dissolved in veronal-buffered saline, pH 7.2, to  $\frac{1}{50}$ th volume of the original fluid. Any insoluble material was discarded, as it has been found to be serologically inactive. The redissolved material has been stored for periods up to 3 months at –20° C. without loss of activity.

*Vesicle fluid antigens*

Vesicle fluids simply diluted in saline can be used as complement-fixing antigens, but in most of the experiments described below the soluble complement-fixing antigen in vesicle fluid was separated from the virus as follows: fluids were collected into Hanks' solution or into phosphate buffered saline (P.B.S.) (Dulbecco & Vogt, 1954). The volume of vesicle fluid was estimated and the whole then diluted with P.B.S. to fill a centrifuge tube (approximately 12.5 ml.) and spun at 12,500 r.p.m. (14,000 g) for 30 min., in a Spinco Model L centrifuge, rotor no. 40. The virus-free supernatant from this treatment was used as complement-fixing antigen and if required could be stored at  $-20^{\circ}$  C. without loss of potency. Two vesicle fluids were used throughout this work as control antigens in all tests of tissue culture material. One fluid (0.5 ml.) was from a severe case of chickenpox in an adult male and the other (1.0 ml.) from a case of zoster in an elderly female.

*Sera*

One or other of two convalescent zoster sera, shown by test to have a high titre of complement-fixing antibody with chickenpox and zoster vesicle fluids, were used in all tests of tissue culture preparations.

*Complement-fixation tests*

The procedure used for carrying out the complement-fixation tests was as described by Taylor-Robinson & Downie (1959).

## RESULTS

*Strain of virus and number of tissue culture passages  
in relation to antigen production*

Three strains of virus from chickenpox cases (Ha, T and M) and one from zoster (Ho) were used. One of the chickenpox strains (T) was tested for antigen production after 34, 41, 49 and 50 passages in tissue culture. This strain did not at this stage readily produce focal lesions with rounding up and necrosis of cells. It tended to produce instead multinucleated cells and syncytial changes with only slow necrosis. The antigen concentration in fluids derived from these tissue cultures was lower than that obtained from cultures of the other three strains. The other strains produced focal lesions with rounding up and necrosis of cells and none had been passed more than 23 times. Strains M and Ho which had been passed only a few times in tissue culture consistently gave good antigens in suitable tissue culture media.

*Frequency of harvesting culture fluid*

Two strains, Ha in 21st passage and T in 49th passage were cultured on the same batch of amnion cells. After replacement of maintenance medium with 50% B.A.F. in Hanks' solution the medium was changed every 2 days in one set of cultures (118A and 119A) and after 6 or 7 days in the other (118B and 119B). The collected fluids were dialysed and dried in the usual way before being

reconstituted for test. The results of complement-fixation tests on these preparations are shown in Table 1. In this and later tables the dilution of the dried antigen is shown in terms of the concentration of pooled tissue culture fluid from which they were prepared. The results indicate that the concentration of antigen was greater in fluids collected weekly. While the total amount of antigen obtained in fluids collected every 2 days was perhaps greater (see Table 2) the larger volume of fluid to be handled made the slightly greater total yield hardly worth while. In subsequent experiments fluids for antigen preparation were only changed weekly, unless it seemed unlikely that the cells would survive that long in the medium used.

Table 1. *Effect of harvesting tissue culture fluids at intervals of 2 days (A) or 6-7 days (B)*

Culture fluid	Fluid conc.	Dilutions of convalescent serum				
		1/8	1/16	1/32	1/64	1/128
Ha 118A	× 25	++++	++++	++++	++++	+++
	× 12.5	++++	++++	++++	+++	+
	× 6.25	++++	++	-	-	.
Ha 118B	× 25	++++	++++	++++	++++	+++
	× 12.5	++++	++++	++++	++++	+++
	× 6.25	++++	++++	+++	+	-
	× 3.125	++++	±	-	-	.
T 119A	× 25	++	±	-	-	-
	× 12.5	-	-	-	.	.
	× 6.25	-	-	-	.	.
T 119B	× 25	++++	+++	++	±	-
	× 12.5	++++	+++	+	-	-
	× 6.25	±	-	-	-	.

#### *Effect of the medium on antigen production*

To test this a number of bottle cultures of amnion cells were grown and infected with the same virus inoculum. After a few days the medium was changed and different media used for antigen production. The most satisfactory medium was 20-50% B.A.F. in Hanks' solution in which uninfected cells would remain healthy for 2-3 weeks. The medium required to be changed only once a week, although in some experiments it was changed more often. Synthetic media such as Parker's 199 or Eagle's medium without serum did not support growth of cells so well, so that the fluid had to be changed every 2 or 3 days to maintain cells in a viable state. Little or no antigen was produced unless B.A.F. in a concentration of 10% or greater was added to these media. Data on various antigens prepared in tissue cultures of the four strains of virus are summarized in Table 2. It should be noted that the bulk of the dried materials prepared from culture fluids was probably derived from the constituents of the medium; material prepared from fluids from uninfected cultures contained approximately the same amount of indiffusible matter as that from virus-infected cultures, and little more than that obtained from the B.A.F. which supplied the bulk of the indiffusible material in the medium.

Moreover, the dry weight of material obtained from each ml. of culture fluid was not related to serological activity. All these preparations after reconstitution were tested in dilutions against dilutions of convalescent zoster serum. In Table 2, however, the titres obtained with 1/8 dilution of serum serve to show their relative reactivities.

Table 2. *Antigens prepared from tissue culture fluids*

Virus strain	No. of passages	Medium	Batch No.	Days* of culture	Volume of culture fluid	Mg./ml.†	Titre‡
T	34	B.A.F. undiluted	110	15	151	0.43	× 8
T	41	Eagle's medium	111	3	60	0.24	Neg. × 30
T	41	B.A.F. undiluted	112	9	71	0.38	× 10
T	45	0.1% L.A.H. 5% Hartley's broth in Hanks' solution	115	4	76	0.41	Neg. × 30
T	49	50% B.A.F. in Hanks' solution	119A	20	865	0.24	× 25
T	49	50% B.A.F. in Hanks' solution	119B	22	208	0.28	× 12.5
T	50	50% B.A.F. in Hanks' solution	120A	11	117	0.22	Neg. × 25
T	50	Parker's 199	120B	13	154	0.07	Neg. × 25
Ha	13	Eagle's medium	113	3	131	0.24	× 20
Ha	21	50% B.A.F. in Hanks' solution	118A	17	442	0.28	× 6
Ha	21	50% B.A.F. in Hanks' solution	118B	14	193	0.26	× 3
Ha	23	50% B.A.F. in Hanks' solution	125A	8	75	0.40	× 6
Ha	23	Parker's 199	125B	8	111	0.10	× 12
M	5	50% B.A.F. in Hanks' solution	142	15	138	0.18	× 1.5
M	4	50% B.A.F. in Hanks' solution	165/27°	17	120	0.95	× 12
M	4	50% B.A.F. in Hanks' solution	165/31°	17	120	0.77	× 6
M	4	50% B.A.F. in Hanks' solution	165/36°	9	80	0.74	× 3
M	4	50% B.A.F. in Hanks' solution	165/38°	6	60	0.19	× 3
M	4	50% B.A.F. in Hanks' solution	165/39.5°	6	60	0.48	× 12
M	2	50% B.A.F. in Hanks' solution	143A	8	73	0.12	× 25
M	2	Parker's 199	143B	6	56	0.08	Neg. × 25
Ho	2	50% B.A.F. in Hanks' solution	160A	8	80	0.12	× 3
Ho	2	50% B.A.F. in Hanks' solution	160B	2	19	0.20	× 3
Ho	2	10% B.A.F. in Hanks' solution	160D	6	59	0.07	× 3
Ho	2	10% B.A.F. in Parker's 199	160F	4	39	0.10	× 3
Ho	2	10% B.A.F. in Eagle's medium	160H	8	77	0.09	× 12
Ho	2	Eagle's medium	160J	8	73	0.06	Neg. × 25
Ho	3	20% B.A.F. § in Hanks' solution	169	11	257	0.83	× 1.5
Control material from uninfected tissue cultures		50% B.A.F. in Hanks' solution	119C	20	35	0.16	Neg. × 25
		50% B.A.F. in Hanks' solution	119D	15	20	0.14	Neg. × 25
		50% B.A.F. in Hanks' solution	125C	8	58	0.12	Neg. × 25
		B.A.F. undiluted	.	.	10	0.32	Neg. × 25

\* Duration of culture from the time when medium for antigen production was added.

† Dry weight of indiffusible material from each ml. of tissue culture fluid.

‡ Serological reactivity of material in terms of lowest concentration of culture fluid reacting with standard immune serum diluted 1/8.

§ The B.A.F. used in this experiment contained 1.7 mg./ml. indiffusible material as compared with the B.A.F. used in all other experiments which contained 0.32 mg./ml. indiffusible material.

### *Temperature of incubation*

One experiment was made to find out if greater antigen production might occur at temperatures other than 36° C. All the bottle cultures of amnion cells were infected in the usual way with the same dose of virus, and incubated at 36° C. for a further 4 days. The medium was then changed to 50% B.A.F. in Hanks' solution and the cultures then incubated at 27°, 31°, 36°, 38° or 39.5° C. The infection progressed more rapidly at temperatures above 36° C. and these bottles

reconstituted for test. The results of complement-fixation tests on these preparations are shown in Table 1. In this and later tables the dilution of the dried antigen is shown in terms of the concentration of pooled tissue culture fluid from which they were prepared. The results indicate that the concentration of antigen was greater in fluids collected weekly. While the total amount of antigen obtained in fluids collected every 2 days was perhaps greater (see Table 2) the larger volume of fluid to be handled made the slightly greater total yield hardly worth while. In subsequent experiments fluids for antigen preparation were only changed weekly, unless it seemed unlikely that the cells would survive that long in the medium used.

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	× 6.25	++++	++	-	-	.
Ha 118B	× 25	++++	++++	++++	++++	+++
	× 12.5	++++	++++	++++	++++	+++
	× 6.25	++++	++++	+++	+	-
	× 3.125	++++	±	-	-	.
T 119A	× 25	++	±	-	-	-
	× 12.5	-	-	-	.	.
	× 6.25	-	-	-	.	.
T 119B	× 25	++++	+++	++	±	-
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		50% B.A.F. in Hanks' solution	125C	8	58	0.12	Neg. × 25
		B.A.F. undiluted	.	.	10	0.32	Neg. × 25

\* Duration of culture from the time when medium for antigen production was added.

† Dry weight of indiffusible material from each ml. of tissue culture fluid.

‡ Serological reactivity of material in terms of lowest concentration of culture fluid reacting with standard immune serum diluted 1/8.

§ The B.A.F. used in this experiment contained 1.7 mg./ml. indiffusible material as compared with the B.A.F. used in all other experiments which contained 0.32 mg./ml. indiffusible material.

### *Temperature of incubation*

One experiment was made to find out if greater antigen production might occur at temperatures other than 36° C. All the bottle cultures of amnion cells were infected in the usual way with the same dose of virus, and incubated at 36° C. for a further 4 days. The medium was then changed to 50% B.A.F. in Hanks' solution and the cultures then incubated at 27°, 31°, 36°, 38° or 39.5° C. The infection progressed more rapidly at temperatures above 36° C. and these bottles

were discarded after 6 days (three medium changes) but the bottles incubated at 27° and 31° C. did not show infection of all cells even after 15 days (six medium changes) when the experiment was discontinued. Tests on the antigens prepared from the culture fluids are shown in Table 3, and other data are to be found in Table 2. The results suggest that antigen production is rather better at 38° C. than at 36° C., but that outside this range antigen production is unsatisfactory.

As a result of these experiments the method finally adopted for the production of complement-fixing antigen in tissue cultures was to use as inoculum a strain of virus which had been passed up to 8 or 10 times in tissue culture, to use a medium of 50% B.A.F. in Hanks' solution, to incubate cultures at 36–37° C., and to harvest fluid every 7 days.

Table 3. *Effect of temperature of incubation on antigen production in tissue culture*

Antigen	Temp. of incubation (° C.)	Fluid conc.	Dilutions of zoster convalescent serum				
			1/8	1/16	1/32	1/64	1/128
M 165A	27	× 25	++	++	+	—	—
		× 12·5	++	—	—	—	—
		× 6·25	+	—	—	—	—
		× 3·125	—	—	—	—	—
M 165B	31	× 25	++++	++++	++	—	—
		× 12·5	++++	++++	++	±	—
		× 6·25	++++	++	±	—	—
		× 3·125	+	—	—	—	—
M 165C	36	× 25	++++	++++	+++	++	—
		× 12·5	++++	++++	++++	++	—
		× 6·25	++++	++++	+++	—	—
		× 3·125	+++	±	—	—	.
M 165D	38	× 25	++++	++++	++++	++++	+++
		× 12·5	++++	++++	++++	+++	+
		× 6·25	++++	++++	+++	—	—
		× 3·125	+++	++	—	—	.
M 165E	39·5	× 25	++++	±	—	—	—
		× 12·5	+++	—	—	—	—
		× 6·25	—	—	—	—	—
		× 3·125	—	—	—	—	—

*Effect of heat at 60° C. on serological reactivity of antigens*

Weller and his colleagues found their concentrated tissue culture antigen so anticomplementary that heating at 60° C. was necessary to abolish this effect. In our experience, while occasional preparations have been anticomplementary, all those prepared by the method given above have been satisfactory. Heating at 60° C. seemed likely to lessen the specific activity of the virus antigen and indeed such an effect was readily demonstrable. The complement-fixing antigen in vesicle fluid is equally affected by heating at 60° C. for 30 min. (Table 4).

*Serological reactivity of cell extracts*

In tissue cultures little free virus can be found in the fluid freed from cells so that cells or cell fragments have to be used as inoculum for successive cultures (Weller, 1953). As much of the virus appears to remain associated with the cells it seemed that antigenic material might be obtained in high concentration by extraction of infected cells. Weller & Witton (1958) have found that antigen could be obtained in this way. We have attempted the extraction of antigen from infected cells using various methods of disruption. These included freezing and thawing, treatment with distilled water, trypsin, and sonic vibration. However, our attempts did not yield antigen in amount which justified further efforts in this direction.

Table 4. *Effect of heating at 60° C. for 30 min. on the reactivity of tissue culture and vesicle fluid antigens*

Antigen	Conc. of culture fluid	Dilutions of convalescent serum				
		1/8	1/16	1/32	1/64	1/128
Tissue culture antigen, No. 142 unheated	× 25	++++	++++	++++	++++	++++
	× 12.5	++++	++++	++++	++++	++++
	× 6.25	++++	++++	++++	++++	++++
	× 3.125	++++	+++	++	±	-
	× 1.5	+++	±	-	-	.
Tissue culture antigen, No. 142 heated	× 25	++++	+++	++	+	-
	× 12.5	+++	+	-	-	-
	× 6.25	++	-	-	-	-
	× 3.125	-	-	-	-	.
Vesicle fluid unheated	Dilution of fluid					
	1/25	++++	++++	++++	++++	++++
	1/50	++++	++++	++++	++++	+++
	1/100	++++	++++	+++	++	-
Vesicle fluid heated	1/200	+	±	-	-	-
	1/25	++++	+++	-	-	-
	1/50	++++	-	-	-	-
	1/100	±	-	-	-	-
	1/200	-	-	-	-	-

*Use of tissue culture antigen in diagnostic tests*

In order to assess the value of the tissue culture antigen for diagnostic tests a number of sera were examined in complement-fixation tests using a tissue culture antigen and a zoster vesicle fluid antigen in parallel. The two antigens were first titrated against dilutions of our standard antiserum; dilutions of each of comparable serological reactivity were then chosen for test against chickenpox and zoster sera. Fifty sera from acute and convalescent chickenpox and zoster patients were tested in serial twofold dilutions against a constant dilution of each antigen. The results were very similar with both antigens in that serum titres were in all cases practically identical. The results on sera from three cases of atypical zoster are shown in

Table 5. Patient A was a male, aged 33 years, who had developed an eruption on the left side of his hard palate, upper lip and left side of his nose. Patient B was a female, aged 14 years, diagnosed as suffering from geniculate zoster who later developed left facial paralysis. Patient C, a female aged 57 years, also had a clinical diagnosis of geniculate zoster followed by left-sided deafness and facial paralysis. Varicella-zoster virus was grown in tissue culture from the lesions in case A, but virus isolation was not successful in case B and suitable material for such examination was not submitted to this laboratory from case C. The results of complement-fixation tests on sera from these three patients are shown in Table 5.

It will be observed that the serological tests supplied confirmatory evidence for the clinical diagnosis in these cases. The tissue culture antigens and vesicle fluid

Table 5. *Sera from three patients tested against vesicle fluid and tissue culture antigens*

Serum	Days from onset of illness	Serum dilutions					
		1/4	1/8	1/16	1/32	1/64	1/128
Vesicle fluid antigen diluted 1/100							
A1	3	-	-	-	-	.	.
A2	17	++++	++++	++++	++++	++++	++
B1	3	-	-	-	-	.	.
B2	20	++++	++++	++++	++++	++++	+++
C1	15	++++	++++	++++	++++	+++	-
C2	30	++++	++++	++	±	-	-
C3	49	+++	+++	+	-	-	-
Tissue culture antigen Z169 concentrated tenfold							
A1	3	++	-	-	-	.	.
A2	17	++++	++++	++++	++++	++++	+++
B1	3	-	-	-	-	.	.
B2	20	++++	++++	++++	++++	++++	+++
C1	15	++++	++++	++++	++++	++	-
C2	30	++++	++++	+++	±	-	-
C3	49	++++	+++	++	-	-	-

antigen gave comparable results in the concentrations used. In case C the falling titre from the fifteenth to the thirtieth and forty-ninth day after onset of illness is consistent with previous observations (Taylor-Robinson & Downie, 1959).

*Precipitating antigen in tissue culture fluids*

It has previously been found in this laboratory that there was a lack of parallelism between the complement-fixing titre of convalescent sera and the ability of these sera to produce precipitation lines on Ouchterlony plates with vesicle fluid antigens. Moreover, there was a suggestion that vesicle fluids might differ in antigen content when measured by the two techniques (Taylor-Robinson & Rondle, 1959). Further evidence that serological reactivity demonstrable by the two techniques did not depend on identical antigen antibody-systems was obtained in the course of the present work.

For satisfactory demonstration of precipitation lines by the slide technique of Nizamuddin & Dumbell (1959) it was necessary to use the dried material from infected tissue culture in solutions which represented 100-fold concentration of the original fluids. The reactivity of such solutions in precipitation tests did not always correspond to activity as measured by the complement-fixation technique. For example, preparations M165A–M165E (Table 3) were tested by precipitation in solutions which represented 125-fold concentration of the original culture fluid. M165A and M165B which had a low titre by complement fixation gave a more distinct precipitation than M165C and M165D which gave higher titres by complement fixation. These observations confirm the previous results in indicating that the antigens demonstrable by the two techniques are not identical. This problem is still under investigation.

#### DISCUSSION

In the laboratory diagnosis of atypical cases of zoster or chickenpox, vesicle fluid may not be available for the isolation of the virus in tissue culture. In such cases clinical diagnosis may be confirmed by the examination of paired sera for antibodies. This examination is most readily effected by the complement-fixation test, using vesicle fluid from known cases of chickenpox or zoster as antigen. The Ouchterlony precipitation technique may also be used, although this is less sensitive and convalescent chickenpox sera may fail to give a positive result.

Suitable vesicle fluids for serological tests may not always be readily available. However, a constant supply of usable antigen may be prepared from tissue cultures of varicella-zoster virus by the method outlined above. In the tissue culture fluids from which the antigens were prepared no infective virus was demonstrable. These tissue culture antigens when tested with chickenpox and zoster convalescent sera give results comparable with those obtained with a good preparation of vesicle fluid. The complement-fixing activity in either vesicle fluid or tissue culture antigen is markedly reduced by heating at 60° C. for 30 min.

The antigen-antibody systems concerned in precipitation tests and complement-fixation do not appear to be identical. In tissue cultures of human amnion infected with varicella virus and incubated at different temperatures it was found that the greatest concentration of complement-fixing antigen was present in cultures incubated at 36°–38° C., while precipitating antigen was more readily demonstrated in cultures incubated at 27° C. or 31° C. These observations confirm the earlier results obtained with convalescent sera which failed to show comparable titres when tested by the two serological techniques (Taylor-Robinson & Rondle, 1959).

#### SUMMARY

It has been found that antigens suitable for routine tests for complement-fixing or precipitating antibodies in the sera of suspected cases of chickenpox or zoster can be readily prepared from tissue cultures of human amnion infected with zoster-varicella virus.

Useful antigens were obtained when infected cells were incubated at 36°–38° C. in bovine amniotic fluid diluted with an equal volume of Hanks' solution.

Virus strains gave a good yield of antigen after two or more passages in tissue culture but one strain in its fiftieth passage did not.

Harvested culture fluids require 5- to 20-fold concentration for complement-fixation tests and 100- to 200-fold for precipitation tests; concentration of culture fluids was readily effected by drying from the frozen state after removal of salts by dialysis. Tissue culture antigens gave results by complement-fixation tests which were comparable to those given by a good vesicle fluid.

Some evidence was obtained that the antigens responsible for precipitation were not identical with those fixing complement with convalescent sera.

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