# STUDIES ON THE BIOLOGICAL PROPERTIES OF TWO PLAQUE VARIANTS ISOLATED FROM SE POLYOMA VIRUS

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Received for publication March 8, 1960

RECENT communications report growth of SE polyoma virus in tissue cultures of various animal cells, its haemagglutinating property, and the induction of tumours in mice and hamsters (Stewart, Eddy, Gochenour, Borgese and Grubbs, 1957 ; Eddy, Rowe, Hartley, Stewart and Huebner, 1958*a* ; Eddy, Stewart and Berkeley, 1958*b*). The ability of SE polyoma virus to form plaques on mouse embryo cell cultures has also been recently reported (Eddy *et al.*, 1958*a* ; Dulbecco and Freeman, 1959 ; Winocour and Sachs, 1959). The present paper deals with the isolation of 2 kinds of plaques of SE polyoma virus characterized by differences in plaque size. In addition to this difference, the 2 strains derived from these 2 kinds of plaques have been found to differ widely in their haemagglutinating ability, cytopathogenic effect on mouse embryo cells and in their capacity to induce tumours in Swiss mice and hamsters.

## MATERIALS AND METHODS

*Virus.*—SE polyoma virus sent by Drs. S. E. Stewart and B. E. Eddy to Dr. L. Sachs and given by him to us was employed throughout this study. Virus suspensions prepared from this material were kept at 4° while stock virus was kept frozen at −20° or in dried form in the refrigerator.

*Cell cultures.*—Primary cultures of mouse embryo cells were used for growing the virus. Decapitated Swiss mouse embryos from the 16–18th days of gestation were trypsinized in 0·12 per cent trypsin (Difco) solution in phosphate buffered saline (PBS). The trypsinization was carried out in flasks equipped with a magnetic stirrer. The trypsinized cells were collected and washed once and then diluted in lactalbumin hydrolysate medium containing 4 per cent calf serum at pH 7·4. A volume of 1 ml. containing $1 \times 10^6$ cells was used for tube cultures, 5 ml. for 5 cm. diameter plates and 12 ml. for flat bottles. The cultures were ready for use after incubation for 3–4 days, when a complete sheet of cells was formed. Before inoculation of the cultures with virus the medium was changed to Eagle's medium (Eagle, 1955) containing 5 per cent inactivated horse serum at pH 7·4–7·6. A 2 ml. volume of this medium was put in each tube and 10 ml. in each bottle ; no medium changes were made during a period of 2–3 weeks observation.

*Animal tests.*—One-day-old Swiss non-inbred mice and 3–6-day-old Syrian hamsters (*Cricetus auratus*) were used for virus inoculations, and the animals were inoculated subcutaneously with 0·2 ml. of virus suspension and kept up to 6 months under observation for development of tumours, deaths, and antibody production.

Antisera against the virus were obtained by bleeding the animals at various times following inoculation of live virus suspensions. Rabbit antisera were prepared in animals weighing 2 kg. by either 1st injection of 5 ml. virus suspension given intraperitoneally and 4 subsequent injections of 0·5 ml., 2 ml. and 2·5 ml. given intravenously with intervals of 10 days between each injection, and blood drawn one month later, or 20 ml. of virus suspension given intraperitoneally and blood drawn one month later.

It was found that sera received by using the second method contained live virus and therefore could not be used for neutralization tests.

*Virus assay.*—In order to determine virus titres, 3–4 tubes per log dilution were inoculated and used for end-point calculations according to the method of Reed and Muench (1938).

The tubes were observed for the cytopathogenic effect (CPE) of the virus on the cells on the 9th–10th day after inoculation and final readings were made on the 14th–16th day ; strong and slight CPE could be observed and were designated accordingly. These differences in the intensity of the CPE were found to be an important characteristic of the virus strains and will be dealt with later.

Infectivity titres were also determined by measuring the haemagglutinins (HA) in each inoculated tube on the 21st day after inoculation when the tests were completed. After preliminary studies (Gotlieb and Leventon, unpublished) the following method for detection of HA was used : 0·1 ml. of supernatant was removed and treated with 0·15 ml. of 0·01 N $NaIO_4$ at 37° or 44° for 30 min. and 0·15 ml. of 40 per cent glucose solution added to neutralize the periodate. Serial 2-fold dilutions were made in phosphate saline at pH 7·2–7·4 ; 0·5 ml. of 0·4 per cent guinea-pig red blood cells were added to 0·5 ml. of virus dilution and the test read by the pattern method after overnight settling of the cells at 4°.

For the haemagglutination-inhibition (HI) test, sera were inactivated at 56° for 30 min. and 2-fold serial dilutions were made in phosphate buffer saline. To 0·25 ml. of appropriate serum dilution, 0·25 ml. of 4–8 HA units of virus were added and incubated together for 1 hr. at 37°. To this 0·5 ml. of red blood cells were added and the mixture kept at 4° and read as indicated above. In order to remove non-specific HA and serum inhibitors, sera for the HI test were pre-treated with 1 : 10 RDE solution (N.V. Philips Roxane, " Duphar " Amsterdam, Holland) at a 1 in 10 serum dilution. Five parts of RDE were incubated with 1 part of serum overnight at 37° and then heated to 56° to inactivate the RDE.

Neutralization tests in tube cultures were carried out by inoculating 4 tubes with 0·2 ml. of a mixture of a standard amount of virus and appropriate serum dilution. Before inoculation, the mixtures were incubated for 1 hr. at 37°. Serum titres were based on 50 per cent tissue culture end-point calculations.

Virus assays on plates were carried out as follows : the tissue culture plates were washed once with Hanks' solution and inoculated with 0·3 ml. of virus suspension and incubated at 37° for 1 hr. for adsorption. Then the cultures were washed $\times 3$ to remove non-adsorbed virus and overlayed with a mixture composed of 4 ml. of 2 per cent agar and 4 ml. of Youngner's medium (1956) modified to contain 10 per cent horse serum and 0·05 per cent lactalbumin. On the 12–14th day of incubation 0·5 ml. of 0·05 per cent neutral red solution was added and the number of plaques read after 6 hr. at 37°. All plates were incubated in a 5 per cent $CO_2$ incubator.

Since pH, and the bicarbonate concentration of the medium had a marked effect on the CPE of the virus and on the production of HA, media with 2 different bicarbonate concentrations were used as well as with different pHs. The details will be given later.

## RESULTS

### *Isolation and differentiation of the two strains*

The SE polyoma virus was carried in mouse embryo cell culture tubes for 10 passages. Tests for HA, CPE and results of animal inoculations were found to be in accord with those described for SE polyoma virus (Stewart *et al.*, 1957 ; Eddy *et al.*, 1958*a* ; Eddy *et al.*, 1958*b* ; Eddy *et al.*, 1958*c* ; Stewart, *et al.*, 1958). At the 10th passage, plating of the material was carried out. Plaques 1–4 mm. diameter appeared on the 11th–14th day after inoculation. Two kinds of plaques differing in size were observed and isolated. On subsequent plating out, pure populations of large and small plaques could be obtained. The small plaques were of an average size of 1–1·5 mm. in diameter while the large plaques were 3–4 mm. in diameter. Fig. 6 shows the appearance of large and small plaques at various virus dilutions. In the small plaques a necrotic centre was more obvious when observed under the microscope while the large plaques were more diffuse and not all the cells at the site of the plaque were destroyed. From these 2 kinds of plaques, 2 lines of virus were carried and further studied for their various properties *in vitro* and *in vivo*. In Table I, the 2 strains are compared as to their plaque sizes, intensity of CPE, HA titres and their infectivity as measured

TABLE I.—*Characteristics of Two Strains of SE Polyoma Virus*

| Strain No. | Characteristics of strains: Plaque size (mm.) | CPE* | HA titre/0·1 ml.† | Infectivity titre ($ID_{50}$/0·1 ml.) as measured by: CPE | HA | Strain designation |
|---|---|---|---|---|---|---|
| 5552 | 1·0–1·5 | +++ | 0 | 6·5 | 3·0 | C⁺ H⁻ |
| 4671 | 1·0–1·5 | +++ | 80 | 6·0 | 4·0 | |
| 7753 | 1·0–1·5 | +++ | 240 | 6·0 | 4·5 | |
| 5551 | 5–4 | + | 960 | 3·5 | 5·7 | C⁻ H⁺ |
| 6781 | 5–4 | + | 3840 | 4·5 | 6·2 | |
| 8192 | 5–4 | + | 1920 | 5·5 | 6·0 | |

* + = slight.
+++ = strong.
† HA titres are expressed as the reciprocal of the highest dilution showing partial haemagglutination.

by CPE or HA. It will be seen from Table I that various fluids derived from the 2 kinds of plaques differed in their CPE in tubes and HA titres. The 3 fluids obtained from small plaques were highly cytopathogenic and had low HA titres, ranging from 0–240. On the other hand, the 3 fluids derived from large plaques were only slightly cytopathogenic and exhibited very high HA titres in the range of 960–3840. Fig. 7 shows the differences in the intensity of the CPE between the 2 strains. Accordingly the two strains were designated $C^+ H^-$ and $C^- H^+$. The infectivity titres of each of the 2 strains was measured according to its CPE and HA characteristics; the highest titre obtained, either by CPE or HA was subsequently used for the 50 per cent end point-calculations.

In order to determine whether the strains vary in their antigenicity, cross HI and neutralization tests were carried out using mouse and rabbit immune sera. The results of the cross HI tests are shown in Table II and those of the cross-neutralization in Table III. In comparison, results obtained with the parent SE polyoma strain are included. Only slight antigenic differences were found in the cross HI titres between the 2 virus strains as well as with the parent SE polyoma strain with mouse immune sera. This was also true for the cross neutralization tests. Essentially similar results were obtained in the cross HI tests using rabbit antisera; thus, it appears that the 2 strains derived from the parent SE polyoma virus did not change in their antigenic characters.

*Growth curves*

Growth curves of the 3 strains were carried out in bottles; a group of 14–16 bottles with mouse embryo cells were used after a complete sheet of cells was formed. Before seeding with virus, the sheet was washed once with Hanks'

TABLE II.—*Cross HI Titres of the Two Strains of Virus with Mouse Immune Sera*

| Type of strain | HA units used | HI titre of pre-immunization serum | HI titre of antiserum against: $C^+ H^-$ | $C^- H^+$ |
|---|---|---|---|---|
| $C^+ H^-$ | 4 | 4,000 | 16,000 | 8,000 |
| $C^- H^+$ | 4 | 4,000 | 24,000 | 32,000 |
| Parent SE polyoma | 4 | 2,000 | 16,000 | 16,000 |

TABLE III.—*Cross Neutralization of the Two Strains Against Mouse Antisera*

| Type of strain | Number of $ID_{50}$ used | Serum dilution end-point of: Pre-immunization serum | Antiserum against: $C^+ H^-$ | Antiserum against: $C^- H^+$ | Antiserum against: Parent SE polyoma |
|---|---|---|---|---|---|
| $C^+ H^-$ | 100 | 100 | 3,200 | 1,600 | 1,600 |
| | 10,000 | 100 | 400 | 200 | 100 |
| $C^- H^+$ | 100 | 100 | 3,200 | 3,200 | 3,200 |
| | 10,000 | 100 | 200 | 200 | 300 |
| Parent SE polyoma | 100 | 100 | 3,200 | 1,600 | 1,600 |
| | 10,000 | 100 | N.D. | 100 | 100 |

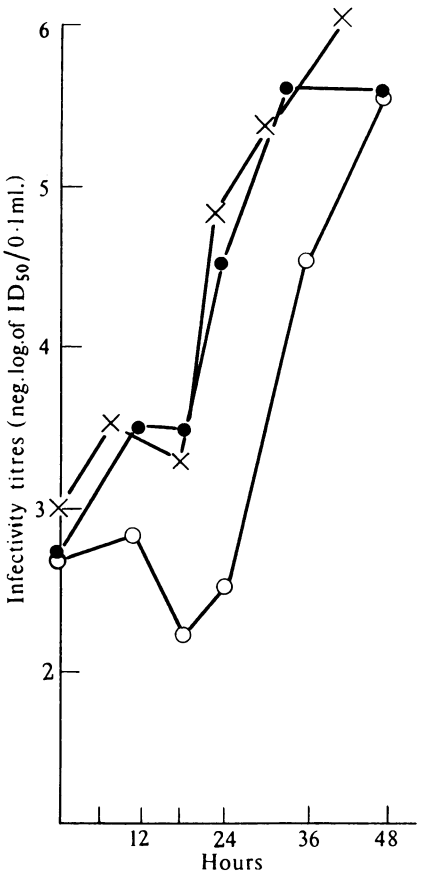



FIG. 1.—Growth curves of the 3 strains of SE polyoma during the first 48 hr. period following inoculation.

Parent strain ●——●
$C^+ H^-$ ×——×
$C^- H^+$ ○——○

solution and 0·5 ml. of virus inoculated. After 1 hr. of adsorption at 37°, the inoculum was drawn out and the tissue washed ×3 with prewarmed Hanks' solution and 10 ml. of Eagle's 5 per cent horse serum added. Infectivity titres of the seed virus and of virus left following adsorption were determined. Two bottles were trypsinized at the beginning of each experiment and the average cell count was used for calculating the multiplicity of infection. Most of the growth curves were carried out with multiples of about 1–2 $TCD_{50}$ per one cell. One bottle each was withdrawn from the incubator at the desired time and the fluids

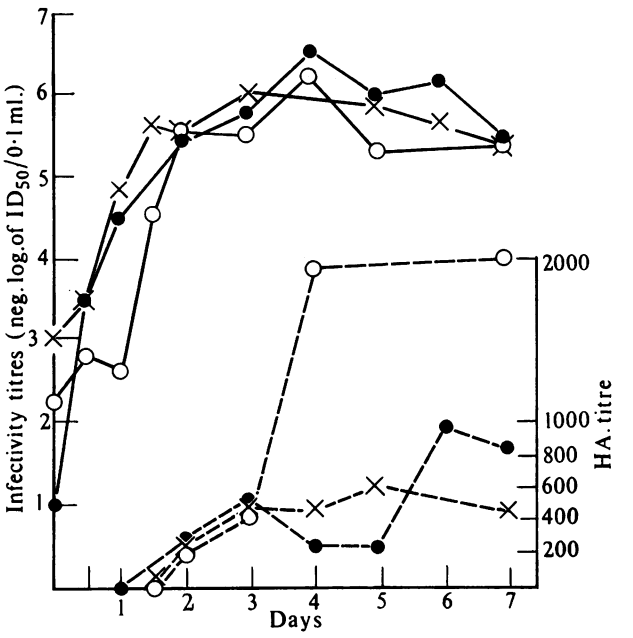



FIG. 2.—Infectivity and HA titres of the 3 strains of SE polyoma during a 7-day period of growth as measured by titration of the fluids.

| | Infectivity | HA titre |
|---|---|---|
| Parent strain | ●——● | ●- - - -● |
| $C^+ H^-$ | ×——× | ×- - - -× |
| $C^- H^+$ | ○——○ | ○- - - -○ |

titrated for HA and infectivity, and the cells counted. In some experiments, HA and infectivity titrations of the virus in the cells were also carried out. The sheet of cells was washed ×3 with Hanks' solution and then trypsinized and the live cells counted. The cells were disrupted by freezing (in a mixture of dry $CO_2$ and alcohol) and thawing 7 times; and the virus released from the cells was titrated.

Infectivity titres ($ID_{50}$) were calculated differently for each strain according to its CPE and HA characteristics. The CPE and HA end-points of the parent strain were approximately the same, while with the $C^- H^+$ and $C^+ H^-$ strains, the highest titres either by HA or CPE of each strain respectively were used for the $ID_{50}$ calculations. The growth rates of the 3 strains during the first 48 hr. are

shown in Fig. 1. It can be seen that with all 3 strains, virus starts to appear at 6–12 hr. after inoculation, and following a slight decline in titre, there is a further logarithmic rise up to 48 hr. Strain $C^- H^+$ seems to exhibit a more prolonged latent period than the other 2 strains. In Fig. 2, growth curves of the 3 strains during a prolonged period of time are presented. Here, the infectivity titres of the 3 strains are compared with their respective HA titres. It can be seen that maximum infectivity titres are reached on the 3rd day and afterwards level off

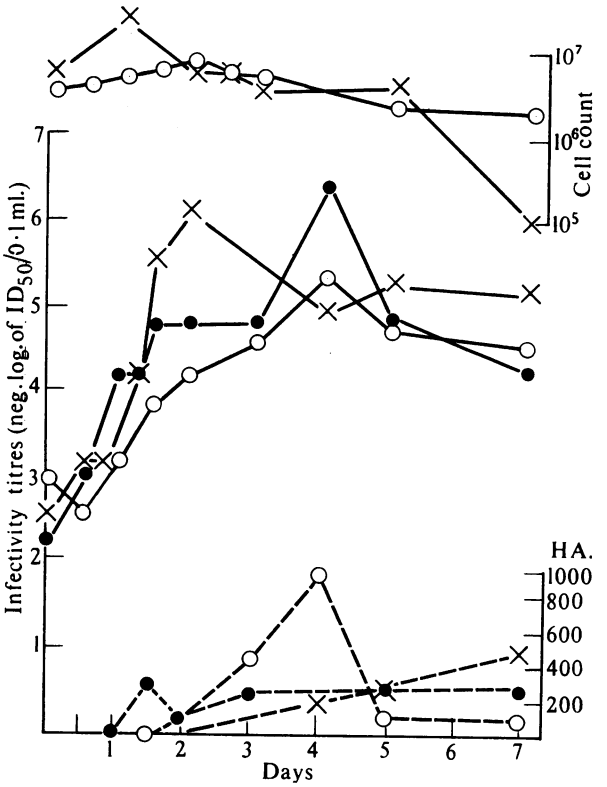
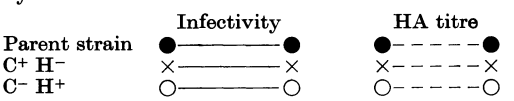



FIG. 3.—Infectivity and HA titres of the 3 strains of SE polyoma during a 7-day period of growth as measured by titration of virus inside the cells.




up to the 7th day. Essentially the infectivity growth curves run parallel in all 3 strains. The 3 strains differ, however, very markedly in their HA titres. Strain $C^- H^+$ stands out by its high HA titre reaching values of 1 in 2000 and higher from the 4th day onwards. The other 2 strains show markedly lower HA titres, especially for strain $C^+ H^-$.

Fig. 3 gives the infectivity and HA titres of the 3 strains in the cells, and the cell counts during a period of 7 days. It can be seen that with $C^+ H^-$ strain, virus accumulates gradually in the cells and reaches its peak some time before it

TABLE IV.—*Effect of pH of Medium on HA Titres of the Three SE Polyoma Virus Strains*

| Strain type | Strain No. | pH of medium | HA titre/0·1 ml. |
|---|---|---|---|
| $C^+ H^-$ | 7380 | 7·6 | 40 |
| | 7404 | 6·8 | 0 |
| $C^- H^+$ | 7387 | 7·6 | 2560 |
| | 7409 | 6·8 | 40 |
| Parent SE polyoma | 7370 | 7·6 | 240 |
| | 7393 | 6·8 | 0 |

does in the other 2 strains. With all 3 strains, the HA titres in the cells are lower than in the fluids. The $C^- H^+$ strain shows an early rise and an abrupt fall in HA corresponding to the rise in HA in the fluid. In the upper part of Fig. 3, the cell counts are shown, and it may be seen that on the 7th day the cells, in which the $C^+ H^-$ strain was growing, were mostly degenerated, while with the $C^- H^+$ strain the cell count declines more slowly. With the latter strain, we were able, by changing the medium every 2–3 days, to keep the cell culture almost intact for 50 days and the cells continued to release virus with a titre of $10^{-5.5}$–$10^{-6.5}$ per 0·1 ml. when measured at every medium change.

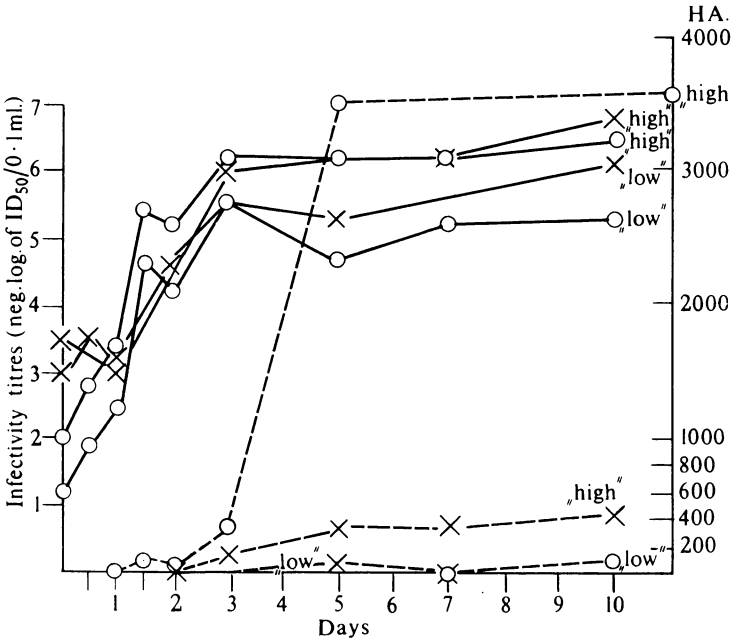



FIG. 4.—Influence of "low" and "high" bicarbonate concentrations on the infectivity and HA titres of the 2 plaque variants, as measured by titration of the fluids.

| | Infectivity | HA titre |
|---|---|---|
| $C^+ H^-$ | ×——×——× | ×– – –×– – –× |
| $C^- H^+$ | ○——○——○ | ○– – –○– – –○ |

*Effect of pH and $NaHCO_3$ on the CPE and HA titres of the $C^-$ $H^+$ strains*

It was observed early in our work that the CPE and HA titres of the 2 strains were influenced by pH and bicarbonate concentration of the medium as was also reported recently by Eddy and Stewart (1959). In order to test the effects of changes in pH and bicarbonate concentrations, modifications were introduced in Eagle's medium so as to obtain media with differing pH and bicarbonate concentrations. The pH ranged from 6·8 to 7·6. Differences in bicarbonate concentrations were obtained by varying the amounts of bicarbonate added. To

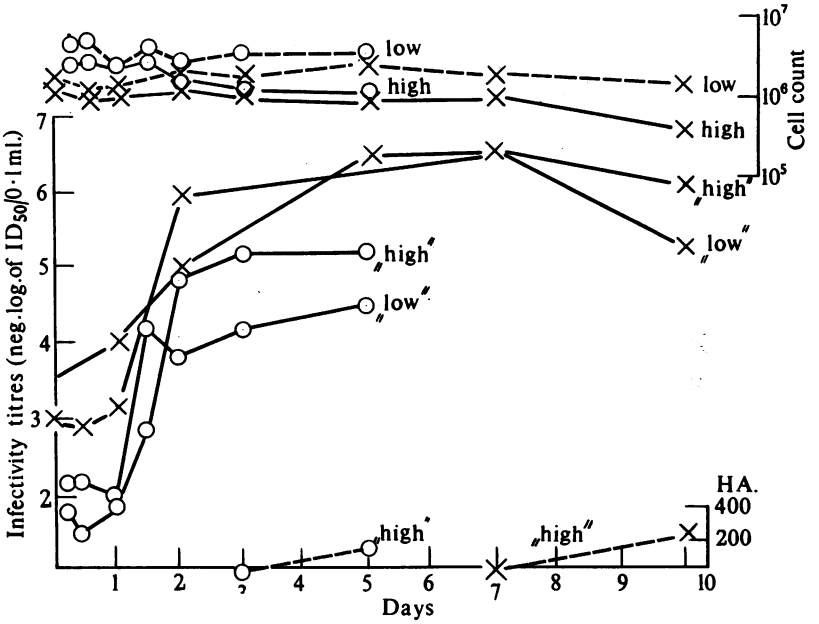



FIG. 5.—Influence of "low" and "high" bicarbonate concentrations on the infectivity and HA titres of the 2 plaque variants as measured by titration of virus inside the cells.

| | Infectivity | HA titre |
|---|---|---|
| $C^+$ $H^-$ | ×——× | ×- - - -× |
| $C^-$ $H^+$ | ○——○ | ○- - - -○ |

1 l. Eagle's 0·55 g. $NaHCO_3$ was added and called "low"—and to 1 l. Eagle's 2·75 g. $NaCHO_3$ was added and called "high" bicarbonate concentrations.

Table IV shows the effects of 2 pH concentrations on HA titres of the 2 strains. It will be observed that very low or no haemagglutinins were formed in acid pH, while various HA titres were obtained for the different strains in alkaline pH. The influence of pH was most conspicuous with the $C^-$ $H^+$ strain, in which maximal HA titres were obtained at alkaline pH and very low at acid pH.

In order to test the influence of the 2 bicarbonate concentrations on the infectivity and HA titres of the $C^-$ $H^+$ and $C^+$ $H^-$ strains, growth curves were made in the usual manner employing "low" and "high" bicarbonate concentrations. $ID_{50}$ and HA titres were determined for both the supernatant and cells.

The results of these experiments are shown in Fig. 4 and 5. It will be observed

that there is a difference of about one log in the infectivity titres of each of the 2 strains when grown in media of " high " and " low " bicarbonate concentration. The most striking effect of bicarbonate concentration was in the HA titres. This is especially evident for the $C^- H^+$ strain in which " high " bicarbonate concentrations resulted in a great rise in HA titres whereas " low " bicarbonate suppressed almost completely production of haemagglutinins. The HA titres of the $C^+ H^-$ strain were low at " high " bicarbonate concentration and nearly none were formed in medium containing " low " bicarbonate. Although it is realized that this may have been affected by the differences in infectivity titres at " low " and " high " bicarbonate concentrations, it cannot wholly account for such a wide divergence in the HA titres. Fig. 5 shows the influence of " low " and " high " bicarbonate concentrations on the infectivity and haemagglutinin contents of the cells. These results are correlated with the respective cell counts. No or very low HA titres can be detected inside the cells, at both bicarbonate concentrations of the 2 strains, in contrast to the large amounts of haemagglutinins released into the fluids when the " high " bicarbonate concentration was used (Fig. 4). The bicarbonate concentration seemed to have a great influence on the cell counts ; with both strains, low bicarbonate concentrations resulted in better maintenance and longer survival of the cells.

*Animal tests*

When the tissue culture fluids of the 2 strains were inoculated in one-day-old mice or in infant hamsters, a difference in the tumour-inducing capacity of the 2 strains was observed. This was especially evident in regard to the percentage of animals developing tumours or dying as well as in the time of appearance of the tumours after inoculation and their site. The difference in response to inoculation with the 2 strains was more conspicuous in mice than in hamsters. Table V demonstrates condensed data of our experiments. For comparison, results of animal inoculations of the parent SE polyoma strain are included.

It can be seen from Table V that strain $C^- H^+$ induced a significantly higher percentage of tumours than the $C^+ H^-$ strain. This was evident in many experiments carried out with the 2 strains. Following inoculation of the $C^+ H^-$ strain, tumour development was retarded up to 5–6 months after inoculation. The $C^- H^+$ strains induced tumour formation regularly in up to 100 per cent of the animals. Most of these tumours appeared up to 4 months following inoculation as indicated in Table V. The behaviour of the parent SE polyoma virus seemed to be closer to the $C^- H^+$ strain in this respect. Mice dying of unknown reason were not included in the calculations since it was not always possible to determine the exact cause of death. In addition to the difference in the tumour inducing capacity, the $C^- H^+$ strain showed a high percentage of animals with haemorrhages in the lungs and with paralysis of the hind legs due sometimes to visible tumours in the spinal cord. On the other hand, the $C^+ H^-$ strain was characterized by limited tumour formations mostly of the mammary and lymph glands.

The second part of Table V contains the comparative results of inoculations of these strains in infant hamsters. Owing to the high susceptibility of infant hamsters to polyoma virus (Eddy *et al.*, 1958c ; Rowe *et. al.*, 1959) titrations of the various strains in hamsters were carried out in order to enable better differentiation. Strain $C^- H^+$ could be readily titrated in hamsters showing a diminishing effect in the animals inoculated with the various dilutions of virus. The per-

TABLE V.—*Tumour Development in New-born Mice and Hamsters Following Inoculation with Three Strains of Polyoma Virus*

| Strain type | Inoculation dilution | Number of animals inoculated | Number of animals developing tumours during (months) 1–4 | 5–6 | Death reason unknown | Percent of tumour development |
|---|---|---|---|---|---|---|
| | | *Mice* | | | | |
| $C^+ H^-$ | Undiluted | 16 | — | — | — | 0* |
| | $10^{-1}$ | 30 | — | 5 | 4 | 19 |
| $C^- H^+$ | Undiluted | 19 | 10 | 1 | 2 | 65 |
| | $10^{-1}$ | 21 | 14 | — | 4 | 82 |
| Parent SE polyoma | Undiluted | 25 | 12 | 6 | — | 72 |
| | $10^{-1}$ | 5 | 5 | — | — | 100 |
| | $10^{-2}$ | 6 | 1 | — | — | 16 |
| | $10^{-3}$ | 6 | 1 | 1 | — | 32 |
| | $10^{-4}$ | 5 | 2 | 1 | — | 60 |
| | $10^{-5}$ | 4 | 1 | 1 | — | 50 |
| | $10^{-6}$ | 7 | — | — | — | 0 |
| | | *Hamsters* | | | | |
| $C^+ H^-$ | Undiluted | 7 | 4 | 2 | — | 85 |
| | $10^{-1}$ | 2 | 1 | — | — | 50 |
| | $10^{-2}$ | 4 | — | 2 | — | 50 |
| | $10^{-3}$ | 3 | — | 1 | — | 33 |
| | $10^{-4}$ | 4 | 1 | — | — | 25 |
| | $10^{-5}$ | 5 | — | — | — | 0 |
| $C^- H^+$ | Undiluted | 9 | 9 | — | — | 100 |
| | $10^{-1}$ | 4 | 4 | — | — | 100 |
| | $10^{-2}$ | 5 | — | 4 | — | 85 |
| | $10^{-3}$ | 4 | 1 | 1 | — | 50 |
| | $10^{-4}$ | 7 | — | 3 | — | 43 |
| | $10^{-5}$ | 6 | — | 1 | — | 18 |
| | $10^{-6}$ | 4 | — | — | — | 0 |
| Parent SE polyoma | Undiluted | 11 | 11 | — | — | 100 |
| | $10^{-1}$ | 5 | 2 | 3 | — | 100 |

* Observation discontinued after 4 months.

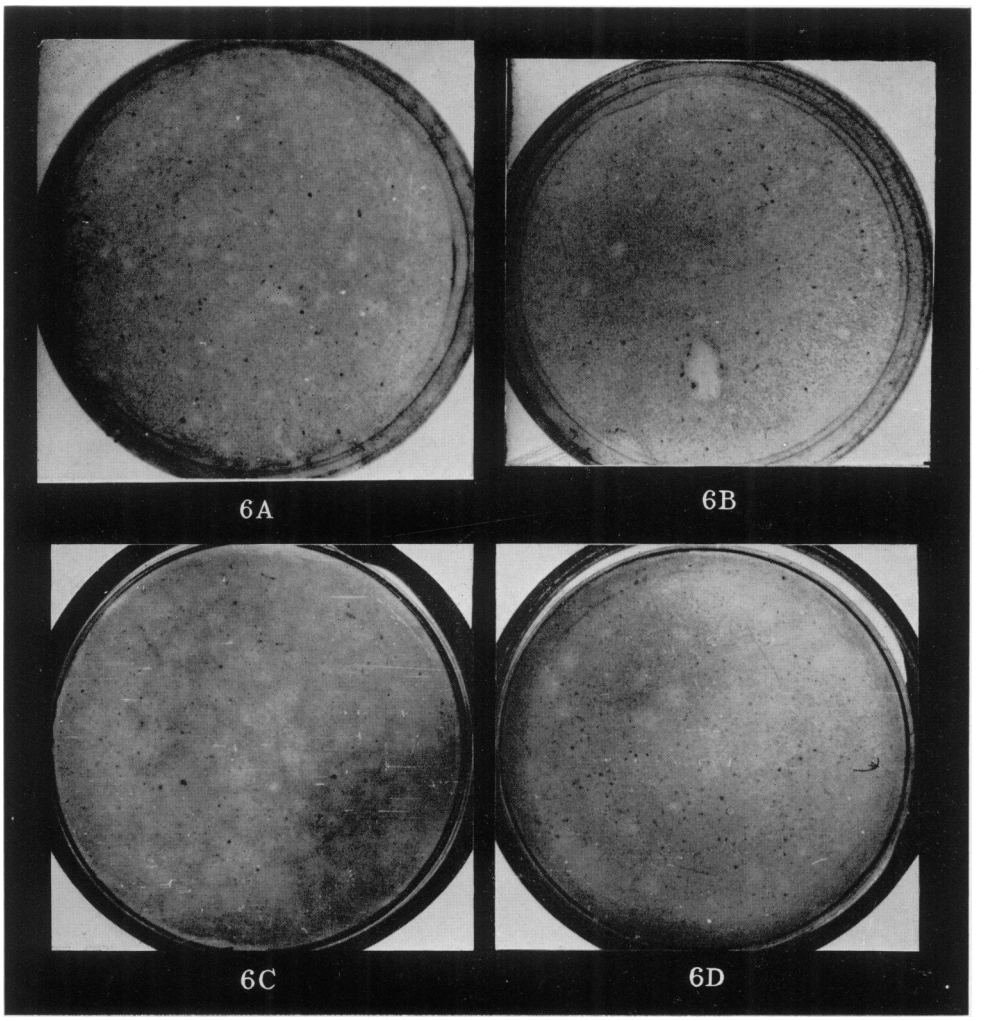
## EXPLANATION OF PLATES

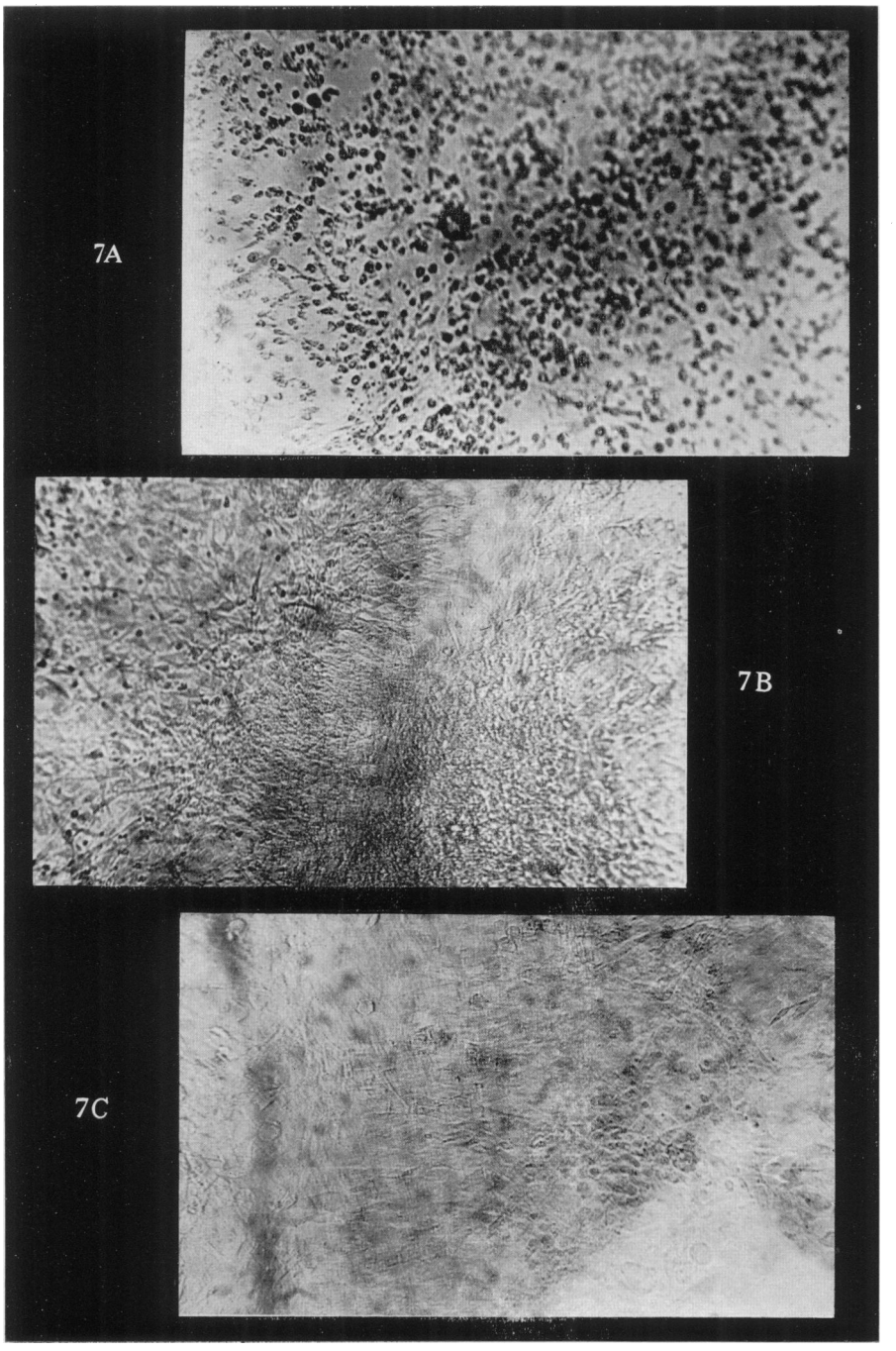
FIG. 6.—Plaques of $C^+ H^-$ and $C^- H^+$ strains in mouse embryo cell culture plates.

A. Strain $C^+ H^-$, dilution $10^{-3}$.
B. „ $C^+ H^-$, „ $10^{-3.5}$
C. „ $C^- H^+$, „ $10^{-4.5}$
D. „ $C^- H^+$, „ $10^{-5.5}$

FIG. 7.—Differences in cytopathogenic effect between $C^+ H^-$ and $C^- H^+$ strains in mouse embryo cell culture tubes; 11th day after inoculation.

A. Strain $C^+ H^-$ "strong" CPE.
B. Strain $C^- H^+$ "slight" CPE.
C. Uninoculated culture.


6A
6B
6C
6D




Gotlieb-Stematsky and Leventon.

centage of animals developing tumours as a result of inoculation with the $C^+ H^-$ strain was lower at the various dilution levels than with the $C^- H^+$ strain. Also a great difference in the intensity and spread of tumours and haemorrhages was noted between the 2 strains, the most conspicuous being a high percentage of haemorrhages in the livers and lungs of animals inoculated with the $C^- H^+$ strain.

## DISCUSSION

The question whether mouse leukaemia and/or virus-induced mouse tumours are of a single or multiple viral aetiology has been raised in the past by various investigators engaged in experimental work in this field (Graffi, Bielka and Fey, 1956 ; Gross, 1958 ; Friend, 1959 ; Sachs *et al.*, 1959 ; Eddy and Stewart, 1959). The findings that the virus capable of inducing mouse tumours can be grown in cell cultures (Stewart *et al.*, 1957 ; Eddy *et al.*, 1958), opened the possibility of using the plaque technique in an attempt to isolate pure virus lines for further study. By using the plaque technique, we were able to isolate at least 2 distinct plaque variants from the parent SE polyoma virus. In addition to the difference in plaque size, virus lines derived from these 2 distinct plaques were shown to differ markedly in other characteristics like CPE and haemagglutinating activity, and their tumour inducing capacity. Since no detailed histological analysis was attempted in further differentiation of the tumour types caused by these strains and their blood and bone-marrow pictures, their identity and relation to the other strains causing mouse leukaemia cannot be determined. It was evident, however, that these 2 strains differ in their tumour inducing capacity and organ-preference in mice and hamsters. The widespread occurrence of SE polyoma virus in normal mice (Rowe, Hartley, Brodsky, Huebner and Law, 1958 ; Rowe, Hartley, Estes and Huebner, 1959) raises the possibility that one of these strains originated from our mouse cell cultures. This cannot be ruled out altogether since we succeeded by repeated subcultures to isolate a virus closely related to SE polyoma from our mouse colony. In addition, it was observed that our uninoculated mice have varying HI titres against SE polyoma virus. Against this assumption stands out the fact that we were able to repeat several times the isolation from plaques of these 2 virus lines. The characteristics of the 2 virus strains, $C^- H^+$ and $C^+ H^-$, were maintained after inoculation in animals. It was also possible to isolate strains of varying CPE and HA characteristics directly from animals inoculated with the parent SE polyoma virus without the plate method.

The most important feature which deserves special mention seems to be the differences observed in the tumour-inducing capacity of the 2 variants, $C^- H^+$, and $C^+ H^-$. The difference in their capacity to induce tumours in both mice and hamsters varied significantly. This character seemed to be linked with the haemagglutinating capacity of the strain ; high HA titres were always correlated with a high percentage of animals with tumours, whereas low HA titres—with few or no tumours.

Data obtained from the growth curves of the 2 strains in the fluids and inside the cells may point to a different mechanism of cell destruction and virus release. The $C^- H^+$ strain is presumably released continuously from the cells and therefore the virus content, as measured by HA, is higher in the fluid than in the cells, and little cell destruction occurs. The behaviour of the $C^+ H^-$ strain is different in

this respect. Virus seems to accumulate early in the cells causing cell destruction during cell burst.

Since the less cytopathogenic strain is also the one that induces a higher percentage of tumours in animals, it remains to be proved whether its behaviour at the cellular level in tissue culture can be translated into what occurs *in vivo*; especially in view of our experimental findings that cell destruction by this strain *in vitro* could be kept at a minimum for a long period of time if changes of medium were carried out at frequent intervals.

The role of bicarbonate concentration and its effects on the growth of the 2 strains is of interest in view of the findings obtained; its role in synthesis of protein and RNA has been recently pointed out by Chang (1959). Whether bicarbonate concentrations have an influence on the tumour inducing capacity of these strains remains to be investigated.

SUMMARY

The isolation of 2 strains of virus from the parent SE polyoma virus and the description of their characteristics is reported. These strains, designated $C^-$ $H^+$ and $C^+$ $H^-$, were isolated from plaques of different size.

The $C^-$ $H^+$ strain was isolated from a large plaque, 3–4 mm. in size, and was characterized by a limited CPE, high HA titre and a high tumour and haemorrhage inducing capacity in new-born mice and hamsters. The $C^+$ $H^-$ strain came from a small plaque, diameter 1–1·5 mm., was highly cytopathogenic, gave low HA titres and was limited in its tumour-inducing activity.

The growth characteristics of the 2 virus strains as compared with the parent strain are presented. It was found that the pH of the medium and especially the bicarbonate concentration have a marked influence on the various characteristics of the 2 strains. The relationship between the *in vitro* and *in vivo* behaviour of the strains is discussed.

We wish to thank Dr. N. Goldblum for his valuable help and encouragement throughout this investigation, and to the Misses M. Hermann, E. Stambulka and Y. Shaya for their able technical assistance.

## REFERENCES

CHANG, R.—(1959) *J. exp. Med.*, **109**, 229.
DULBECCO, R. AND FREEMAN, G.—(1959) *Virology*, **8**, 396.
EAGLE, H.—(1955) *Science*, **122**, 501.
EDDY, B. E., ROWE, W. P., HARTLEY, J. W., STEWART, S. E. AND HUEBNER, R. J.—(1958*a*) *Virology* **6**, 290.
*Idem* AND STEWART, S. E.—(1959) *Amer. J. publ. Hlth*, **49**, 1486.
*Iidem* AND BERKELEY, W.—(1958*b*) *Proc. Soc. exp. Biol., N.Y.*, **98**, 848.
*Idem*, STEWART, S. E., YOUNG, R. AND MIDER, G. B.—(1958*c*) *J. nat. Cancer Inst.*, **20**, 747.
FRIEND, C.—(1959) *J. exp. Med.*, **109**, 217.
GRAFFI, A., BIELKA, H. AND FEY, F.—(1956) *Acta haemat.*, **15**, 145.
GROSS, L.—(1958) *Cancer Res.*, **18**, 371.
REED, L. J. AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
ROWE, W. P., HARTLEY, J. W., BRODSKY, I., HUEBNER, R. J. AND LAW, L. W.—(1958) *Nature*, **182**, 1617.
*Idem*, HARTLEY, J. W., ESTES, J. D., HUEBNER, R. J.—(1959*a*) *J. exp. Med.*, **109**, 379.

*Idem*, HARTLEY, J. W., LAW, L. W. AND HUEBNER, R. J.—(1959*b*) *Ibid.*, **109**, 449.
SACHS, L., FOGEL, M., WINOCOUR, E., HELLER, E., MEDINA, D. AND KRIMM, M.—(1959) *Brit. J. Cancer*, **13**, 251.
STEWART, S. E., EDDY, B. E. AND BORGESE, N. G.—(1958) *J. nat. Cancer Inst.*, **20**, 1223.
*Idem*, EDDY, B. E., GOCHENOUR, A. M., BORGESE, N. G. AND GRUBBS, G. E.—(1957) *Virology*, **3**, 380.
WINOCOUR, E. AND SACHS, L.—(1959) *Ibid.*, **8**, 397.
YOUNGNER, J. S.—(1956) *J. Immunol.*, **76**, 288.