TABLE II. Comparison of Nitrous Acid Method and Nitroprusside Method for Determination of Rat Liver Glutathione Reductase Activity.

	Glutathione reductase activity μmoles GSH/mg protein				
	Incubation time (min)				
Analytical method	1	2	3	4	5
Nitrous acid	.25 ± .02*	.55 ± .03	.77 ± .03	.93 ± .03	.94 ± .05
Nitroprusside	.28 ± .01	.56 ± .03	.85 ± .04	.96 ± .04	1.05 ± .03

Conditions: GSSG, 5.0 μmoles; TPN, 3.0 μmoles; EDTA, 10 μmoles; G-6-P, 10 μmoles; G-6-P dehydrogenase, 0.4 Kornberg unit; liver supernatant, 1.0 ml; total volume, 3.0 ml; 38°C. Liver homogenized in 0.25 M sucrose, centrifuged at 18,000 *g*, 10 min.
* Mean and standard error.

supernatant containing less than 2.5 μmoles GSH was mixed with 1 ml of 1 N HCl and 0.25 ml of 0.01 M $NaNO_2$; the volume was adjusted to 3.0 ml with water; the mixture was heated 15 minutes at 50°C and the optical density determined at 334 mμ. The concentration was determined by comparison with a GSH standard curve.

Summary. A reaction between sodium nitrite and reduced glutathione (GSH) yielded a product having an ultraviolet absorption maximum at 334 mμ. No ultraviolet absorbing product was formed when oxidized glutathione was reacted with sodium nitrite. Optimal conditions and stoichiometry of the reaction were described. The reaction was not specific for GSH; ultraviolet absorbing products were formed with a variety of sulfhydryl compounds. The reaction was used to determine glutathione reductase activity of rat liver, and yielded data which compared favorably with that obtained by an accepted method.

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Density Gradient Electrophoresis Studies on Hemagglutination Plaque Formation and Tumor Induction of SE Polyoma Virus.* (28224)

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Hemagglutination (HA), plaque formation (PF) as well as tumor induction (TI) are employed for titration of SE polyoma virus. Because of the relative simplicity, speed and precision of hemagglutination and of tissue culture methods it is important to know whether these 2 parameters measure the same particles which induce tumors. So far it has not been demonstrated whether these 3 biologic attributes of polyoma virus are associated with one or more kinds of physical particles.

Hemagglutination, tumor induction and tissue culture activity were not dissociated by heat treatment, ether treatment and first plaque isolations with the use of limiting dilu-

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tions. The value of the last method for the cloning of polyoma virus has been questioned by Dawe and Law(8). Growing attention has recently been given in other virus-cell systems to contaminating viruses and to the difficulties of their elimination (Friesen and Rubin(11)).

In view of the various manifestations of polyoma virus the involvement of mutants or of different viruses has been taken into consideration (Stoker and Macpherson(16)). Correlation of the biologic activities of polyoma virus with physical particles was examined with more refined methods. Differential migration methods such as density gradient electrophoresis and sedimentation are useful for this purpose. The results obtained by zone electrophoresis in a density gradient are presented here.

This method had been used successfully for purification of SE polyoma HA from HA inhibitors (Cramer and Stewart(5)).

Materials and methods. Virus. Polyoma strain 11189 obtained from Nat. Cancer Inst. and having an HA titer of 1/128/ml was used. This strain was known to give about 100% early tumor formation in mice and hamsters. When assayed by the standard plaque assay procedure plaques of the large type were found (2-5 mm diameter). Virus stocks were made on 3-day-old secondary mouse embryo monolayer cultures. They were inoculated with a virus titer sufficient to infect most cells, and grown in Medium 199 with addition of 10% horse serum. The supernatant was collected after an incubation of 14 days at 37°C.

Preparation of agent. The tissue culture fluids were freed from coarse material by one centrifugation at 6000 *g* for 10 minutes. The supernatant was dialyzed overnight in the cold against the buffer used for electrophoresis. An appropriate concentration of sucrose in buffer was added to fit the density conditions for introduction into the electrophoresis column.

Density gradient electrophoresis. This technique was introduced by Brakke(1,2). A detailed description of the technique used in this study has been previously given by Cramer *et al.*(4), Polson and Cramer(13), Cramer and Svensson(6).

Veronal buffer at pH 8.6 containing 205 g sodium diethyl barbiturate and 28 g diethylbarbituric acid in 10 liters of distilled water was used. Electrophoresis was allowed to proceed for 2-3 hours at room temperature with a voltage gradient of 3.5 V/cm and a current of 15 ma. Rabbit hemoglobin and phenol red served as reference substances in some experiments. After completion of electrophoresis, the column fractions were obtained by collection of successive samples from an installed capillary tube whose upper end was located at the electrophoretic origin.

Hemagglutination titration. HA titers were determined by the pattern method (Eddy *et al.*, 10) using 0.2 ml of a dilution of each fraction and an aliquot of a 0.30% suspension of guinea pig erythrocytes in Veronal buffer. HA titer was calculated as the reciprocal of the dilution producing partial hemagglutination.

Tissue culture activity. End point method. All fractions were first tested by the end point method in a dilution series of 10^{-1} to 10^{-8} in secondary mouse embryo tissue cultures using 2 cultures per dilution. The cultures were incubated for 14 days and the supernatant was titrated for HA. The infected cultures were scored after the results of the HA titrations. The 50% infectivity end points were calculated according to Reed and Muench(14).

Plaque tests. The fractions found to be active by the end point method were retitrated using plaque tests. Plaque titers were determined by a modification of the methods described by Dulbecco and Freeman(9) and by Winocour and Sachs(18). Serial 10-fold dilutions of the 10^{-2} dilutions of fractions 6, 8, 9, 10, 11, 12, 14 were prepared in dilution liquid of 1% horse serum in Eagle's Basal Medium and plated in quadruplicate in secondary mouse embryo monolayers; all tissues were derived from embryos of a single mouse. The plaque readings were made on the fifteenth day after inoculation.

Tumor production. Oncogenic activity was tested by subcutaneous inoculation into newborn hamsters less than 24 hours old. Each fraction was injected into 5 animals in a 0.3 ml volume of a 1/50 dilution, and they were observed for tumor induction for an 8-month period.

Results. Six independent electrophoretic experiments performed under standardized

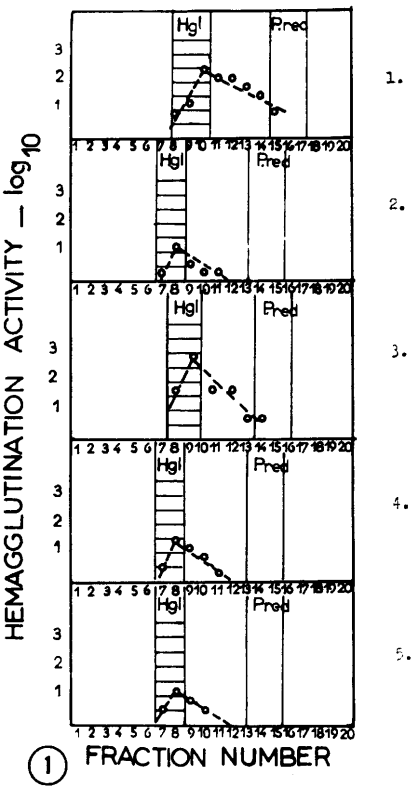
conditions showed that the HA, as judged by maximum activity peaks, always assumed a mobility slightly higher than the rabbit hemoglobin which had been added as a reference substance (Fig. 1). A similar separation pattern was observed in all experiments for the HA with respect to 2 turbid zones of impurities. These zones served as useful references for the electrophoretic mobility in further experiments in which no hemoglobin was added. The electrophoretic mobility of HA was found to be independent of the presence of hemoglobin.

Several experiments were performed to determine the best separation with a minimum of diffusion. It was found that the time of electrophoresis should not exceed 3 hours since the accurate localization of HA activity became more difficult with longer electrophoresis runs because of the diffusion of the components.

The relationship between the viral onco-

TABLE I. Hamster Inoculations.

Fraction	No. with tumors/ No. that could be evaluated	Dead or killed with tumors
1	0/5	—
2	0/5	—
3	0/5	—
4	0/5	—
5	0/5	—
6	0/5	—
7	2/5	2 (late; killed 6 mo, s.c. tumors)
8	0/5	—
9	0/5	—
10	4/4	3 (early; dead 1-2 mo) 1 (late; dead 7 mo, thoracic tumor)
11	1/5	1 (late; killed 4 mo, s.c. tumor)
12	2/5	2 (late; dead 5-6 mo, s.c. tumor)
13	0/5	—
14	1/5	1 (late; dead 4 mo, stomach tumor)
15	1/5	1 (late; dead 5 mo, abd. tumor)
16	1/5	1 (late; dead 5 mo, thoracic tumor)
17	0/5	—
18	0/5	—
19	0/5	—
20	0/5	—
21	0/5	—
22	1/5	1 (late, living at 7 mo with s.c.t. tumor)
23	0/5	—
24	0/5	—
25	0/5	—



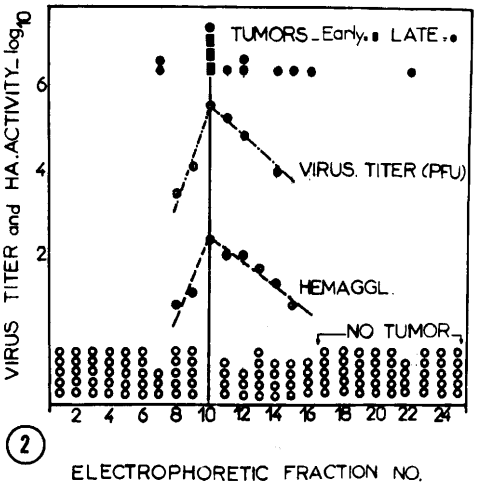
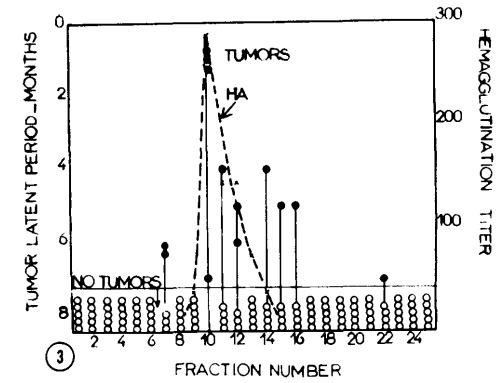


FIG. 1. Electrophoretic mobility of SE polyoma HAmo in relation to the reference substances, rabbit hemoglobin (Hgl) and phenol red (P.red). HA titers indicated by a log scale on ordinate. Consecutive electrophoretic fractions shown on abscissa.

FIG. 2. Zone electrophoresis diagram showing correlation of SEP activities. HA = ⓘ, PF = ⊙ and TI = ●, ■. Titers of HA and PF indicated by a log scale on ordinate. Consecutive electrophoretic fractions shown on abscissa.



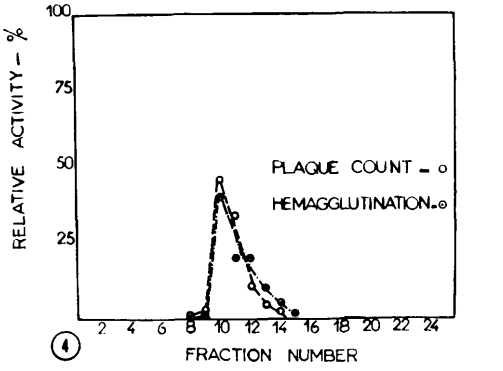


FIG. 3. Zone electrophoresis diagram showing tumor latent periods in relation to HA titers of corresponding fractions. Latent period is plotted from top of ordinate for graphic simplicity.

FIG. 4. Correlation between plaque counts and HA activity found for electrophoretic fractions given as a percentage of total activity found.

genic, cytolytic and HA activities of the electrophoretic fractions was examined in an experiment in which a maximum of HA activity was found in a single fraction. Electrophoresis time was 2 hours and 15 minutes and conditions of electrophoresis were those described above. The volume of the electrophoretic fractions was chosen to be one-half the volume of the suspension in which the virus was introduced into the column, so that a fine resolution could be expected. Each of the parameters: oncogenic activity†, plaque formation‡ and hemagglutination§ was measured in a different laboratory.

The relationship between the 3 activities of the electrophoretic fractions is indicated in Fig. 2, 3, 4. From Fig. 2 it is seen that the 3 activities have a similar general electrophoretic pattern. Moreover, a peak of HA as well as of plaque forming activity was found in the fraction 10 which contained a maximum of oncogenic activity. The peak of HA activity was determined in duplicate.

As far as plaque counts are concerned a statistical treatment has shown that the difference in titer of the electrophoretic fractions is significant. The relation between titer and electrophoretic mobility has been studied by a regression analysis made on fractions 8, 9. 10 and 10, 11, 12, 14. In both cases the slopes of the respective curves have been found to be significantly different from 0 ($p<10^{-4}$); the first being positive and the second negative.

Insofar as tumor induction is concerned. fraction 10 was the only fraction to which all animals responded with tumors. Another indication that a maximum of oncogenic activity was contained in this fraction was shown by the fact that the only tumor formation appearing within 1 to 2 months in 3 animals was found in this fraction. This can be seen in Fig. 3 in which the incubation period of the observed tumors is indicated with relation to the HA activities of each fraction.

In Fig. 4 relative HA and plaque-forming activities are given as a percentage of total activity found. In general an excellent correlation between the 2 activities is found. It seems, however, that a minor electrophoretic inhomogeneity of the HA cannot be excluded. The small oncogenic activity found for fraction 22 might be due to a trailing artifact, or alternately be explained by the fact that particles with higher electrophoretic mobility can still retain some minor oncogenic activity.

In this experiment the recovery of total activity was about 100% for HA activity. The recovery of plaque forming units could not be determined because the sample containing the input virus was lost in transport. For all the electrophoretic fractions the large plaque type (2-5 mm diameter) has been found as for the original virus.

Discussion. The 3 biological activities of SEP do not seem to have been tested quantitatively by the use of differential migration methods such as electrophoresis and sedimentation in density gradients. Kahler *et al.*(12)

† Stewart.

‡ O'Connor.

§ Cramer.

studied the correlation of HA activity and physical particles. They used sedimentation in sucrose density gradients and made the particle counts employing the method of Sharp (15). They found a close correlation between particle counts and HA titer.

Crawford *et al.*(7) have studied SEP in density gradients of cesium chloride and found HA in at least 2 fractions of different buoyant density. A heavier fraction was found to be infectious in tissue culture and contained complete virus particles (virions) when treated by the negative staining method of Brenner and Horne(3), whereas a lighter fraction was found to be noninfectious in tissue culture and contained empty capsides only. Such findings were to be expected from the work of Wildy *et al.*(17) who previously found virions as well as empty capsides in negatively stained preparations of SEP.

The results so far published by Crawford *et al.*(7) do not allow, however, a definite correlation between HA, PF, and TI in the fractions obtained by density gradient sedimentation.

The present work suggests that a maximum of the 3 biological activities of SEP can be correlated with virus particles of the same electrophoretic mobility, as no separation of HA, PF and TI was observed when SEP was submitted to electrophoresis. The failure in separating empty and complete particles by electrophoresis is probably explained by the fact that for both particles the same aspect of the surface and the same type of hemagglutination is found and therefore a similar electrophoretic mobility can be assumed. However the present work was mainly concerned with maximum activities and so far a minor electrophoretic inhomogeneity of HA cannot be excluded.

The fact that HA, PF and TI could not be separated by electrophoresis adds further evidence that the same virus particles might be involved. Studies are in progress to examine the buoyant density of these virus particles, and if it could be shown that all 3 activities reside in the same particle as suggested by the electrophoretic studies, the plaque count assay would be justified for titration of this oncogenic virus.

Summary. The supernatant of a tissue culture infected with polyoma virus was submitted to zone electrophoresis in a density gradient of sucrose. A maximum of hemagglutination, tumor induction and tissue culture activity has been found in the same electrophoretic fraction. This supports the conclusion that the 3 biologic attributes of polyoma virus can be correlated with particles of the same electrophoretic mobility.

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