A QUANTITATIVE COMPARISON OF FORMATION OF SPONTANEOUS AND VIRUS-PRODUCED VIABLE HYBRIDS

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Abstract.—Ultraviolet-inactivated Sendai virus used in conjunction with a selective system reproducibly gives high yields of viable hybrid cell lines. With two different crosses, the frequency of hybrid colonies obtained after virus treatment was found to be 100 times greater than the frequency of spontaneous hybrids, and this increase was found to show little variation between 33 and 1000 hemagglutinating units of virus. No differences have been found between the properties of hybrids obtained after Sendai treatment and those obtained from untreated mixed cultures of parental cells.

The isolation of somatic hybrids has been greatly simplified by the selective system introduced by Littlefield.[1] However, this method cannot be successfully applied to crosses between normal cells, which contain no selective markers. An alternative approach to the isolation of somatic hybrids is through the increase of the frequency of cell fusion. An effective method for achieving cell fusion has been described by Okada[2] and by Harris and Watkins,[3] who have found that cells fuse following treatment with ultraviolet-inactivated Sendai virus. This procedure has been used by Yerganian and Nell[4] for the isolation of viable hybrids between cells of two hamster lines.

The experiments to be described were undertaken with the aim of determining the optimal conditions for obtaining viable hybrids by treatment of parental cells with Sendai virus. (The term "viable hybrids" will be used hereafter to designate hybrid cells capable of undergoing a sufficient number of divisions to give rise to colonies.) In order to quantitate the results, cells were chosen that can be used in a selective system.[1, 5] Virus-produced hybrids were obtained with a high frequency, and no differences have been found between the properties of these and of spontaneously arising hybrids.

Materials and Methods.—Cells: Three types of cells were used: (1) Two sublines of mouse L cells, killed in selective medium: LM(TK$^-$) *cl* 1 D (hereafter referred to as *cl* 1 D), deficient in thymidine kinase, and isolated by Dubbs and Kit;[6] and A_9, deficient in hypoxanthine guanine phosphoribosyl transferase, and isolated by Littlefield.[7] (2) Subclones of parenchymal liver cells (RL†), isolated from cultures of rat liver cells that were prepared by being cloned directly from the liver of a 5-week-old Sprague-Dawley rat. These cells have remained diploid through many generations in culture.[8, 9]

All cultures were grown at 36.5°C in a gas-flow humidified incubator (5–7% CO_2 in air, 98% relative humidity).

Media: The cells were grown in Ham's F12 medium,[10] modified by doubling the concentrations of the amino acids and pyruvate, adding ascorbic acid (15 μg/ml), and changing the concentrations of glucose and inorganic salts (see footnote 11). Additional sodium bicarbonate (1.5 gm/liter), 33 units/ml of sodium penicillin G (Squibb), 2.50 μg/ml of Fungizone (Squibb), and 5% (v/v) of fetal calf serum (selected for high plating efficiency with rat liver cells) were incorporated. To make selective medium (HAT),

the growth medium was modified by deletion of folic acid and addition of aminopterin, hypoxanthine, and thymidine to make final concentrations of $4 \times 10^{-7}M$, $1 \times 10^{-4}M$, and $1.6 \times 10^{-5}M$, respectively. The media were sterilized by passage through GS Millipore filters.

Preparation of ultraviolet-inactivated Sendai virus: Sendai virus, strain ESW5, was kindly provided by Dr. John Watkins. The virus was propagated by injecting 0.01–0.1 hemagglutinating (HA units) (in Hanks' saline solution) into the chorioallantoic cavity of chicken embryos which had been incubated for 11–12 days at 35.5°C. After inoculation, the eggs were incubated for 3–5 days, cooled for 2–3 hr at 4°C, and the virus was collected by removing the chorioallantoic fluid. The fluid was diluted with an equal volume of 0.1 *M* Tris buffer (free base, pH 10), and the mixture was centrifuged (10 min at $10,000 \times g$) to remove undissolved ureates and debris. The supernatant was decanted, adjusted to 0.005 *M* EDTA, and centrifuged to concentrate the virus (60 min at $30,000 \times g$). The opalescent pellets were resuspended in Hanks' solution to give a five- to tenfold concentration. The suspension was adjusted to pH 7.2–7.4 (phenol red) with HCl. The virus was stored for several months at −60°C, after rapid freezing, without loss of infectivity or HA unit titer.

To inactivate the virus, suspensions (40 ml in a 150-mm dish) were exposed, with continuous agitation for 15 min, to a GE germicidal lamp (G15T8) in a reflecting fixture placed 10 cm from the dish. The irradiated suspensions were again concentrated by centrifugation and resuspended in F12 (without serum) to yield hemagglutinating titers of about 2000 HA unit/ml. The inactivated virus was frozen rapidly and stored at −60°C. Virus suspensions were titrated against a final concentration of 0.1% chicken erythrocytes.

Cell fusion: The procedure used was patterned directly after the methods of Okada[2] and Harris and Watkins.[3] Suspensions of parental cells were prepared (trypsin and collagenase in the presence of 2% chicken serum[12]), washed, resuspended, and counted. The two suspensions were mixed in a test tube at the desired ratio to yield a total of 10^7 cells. The mixed cell suspension was centrifuged and the supernatant removed as completely as possible. The pellet was resuspended quickly in 0.3 ml of cooled inactivated Sendai virus in F12 without serum. The cells were kept in suspension for 10 min at 2–4°C on a reciprocating shaker bath to permit virus adsorption and cell aggregation.[2] The tube was transferred to 37°C and incubated while being shaken for 30–60 min to permit cell fusion.[3, 13]

The fused cells and aggregates[14] were layered on a column (1×12 cm) of medium with 25–30% fetal calf serum and allowed to settle at room temperature for 1–2 hr to separate the cells from excess virus. The uppermost 7–10 ml of the settling column were removed and the deposited cells gently resuspended. The cellular units were counted, diluted, and plated in growth medium. After 24 hrs the medium was replaced by HAT. Thereafter, the cultures were maintained in selective medium and renewed every 3 days. After 10–14 days, the plates were fixed and stained, and the colonies counted.

In one experiment this procedure was modified as follows: one parental cell type was treated with virus, and aggregation was interrupted after the tube was shaken at 2–4°C. Diluted suspensions were seeded onto a monolayer of the untreated parent.

Results.—Selective medium, HAT, kills both A_9 and *cl* 1 D, while hybrids between cells of these two lines are able to survive in this medium by virtue of complementation for the described deficiencies. Hybrids between cells of either one of these lines and normal cells are viable because normal cells have neither deficiency and can complement either deficient parent.[5] Revertants (to HAT resistance) of *cl* 1 D and A_9 have not been observed in these experiments. Consequently all colonies which grow up from mixed cultures of *cl* 1 D and A_9 in HAT are hybrid colonies, while in the cross of RL × *cl* 1 D, colonies

of liver cells as well as of hybrid cells are formed. Hybrid colonies can be recognized by morphology and hybrid cells can be identified by karyology.

Only hybrid colonies that contained at least 100 cells were counted in these experiments. These counts disregard the viable heterokaryons,[3] which are numerous after virus treatment and most of which do not divide to produce colonies of hybrid cells. The colonies of hybrid cells produced after virus treatment, and which occur in 100-fold excess of the spontaneous frequency, are termed "virus-produced hybrids."

In each experiment, control cultures were prepared to determine the spontaneous hybridization frequency. These crosses were performed by inoculating a mixture of parental cells. In order to determine whether the procedure described above for cell fusion has any influence on the frequency of hybrid formation in the absence of virus, control suspensions were incubated as described but in serum-free F12 containing no virus. The numbers of hybrid colonies obtained by these two procedures were the same.

Dilution of parental cells before plating: To locate the densities of parental cells at which spontaneous hybrids no longer appear, mixtures of A_9 and *cl* 1 D (parental cell ratio 1:1) were plated with increasing dilution. Figure 1 shows

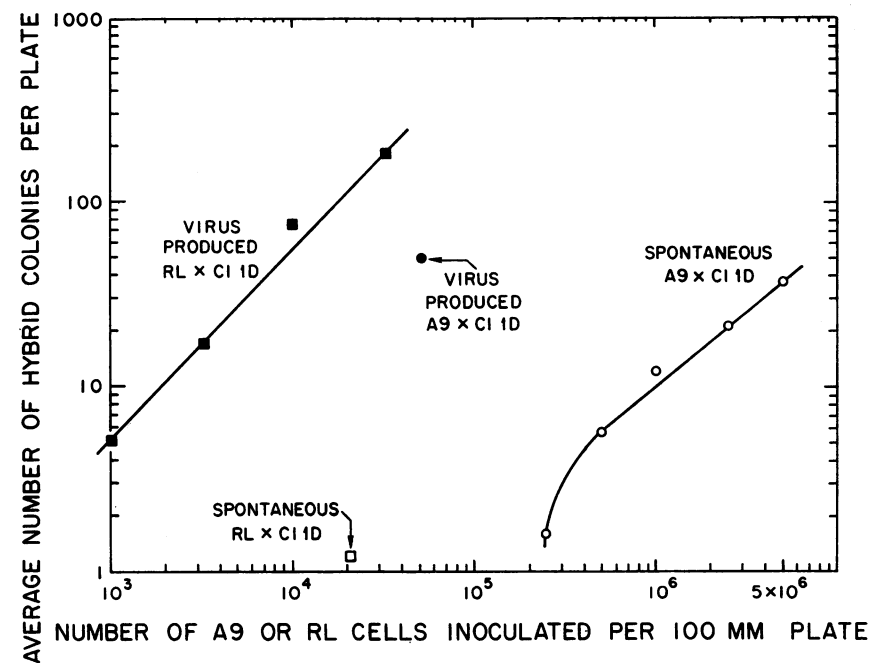

FIG. 1.—Numbers of hybrid colonies obtained at various inocula of parental cells, from virus-treated (*filled symbols*) and untreated (*open symbols*) mixtures. *Open circles*, A9 × *cl* 1 D, parental cell ratio 1:1; *filled circle*, virus-produced A9 × *cl* 1 D, parental cell ratio: *open square*, spontaneous RL × *cl* 1 D, parental cell ratio, 1 RL for every 200 *cl* 1 D cells; *filled squares*, virus-produced RL × *cl* 1 D, parental cell ratio, 1 RL for every 10 *cl* 1 D cells.

The points on this and subsequent figures were obtained by determining the average number of hybrid colonies on 5–10 Petri dishes.

that there is a direct proportionality between the number of spontaneous hybrids and the number of parental cells inoculated. However, at low inocula there is a great decline in the frequency of hybrid colonies, and at densities of 5×10^4 each of A_9 and *cl* 1 D per 100-mm Petri plate, no hybrid colonies have been found upon examination of more than 100 plates.

Crosses in which virus was used were made by mixing parental cells and treating them with Sendai virus before the plates were inoculated. Cell counts of the virus-treated mixtures showed that fewer "cellular units" (many of which were multinucleated) were recovered than had been added to the mixture. These mixtures of parental cells and fused "units" were diluted and plated, either according to the number of "cellular units" counted or according to the number of cells originally added.

Hybrid colonies are so numerous after virus treatment that it is necessary to dilute the parental cell mixtures to obtain discrete colonies. The number of hybrid colonies obtained from a virus-treated mixture of RL × *cl* 1 D (in which 1 RL cell was used for every 10 *cl* 1 D cells) was found to be directly proportional to the number of cells inoculated (Fig. 1). The fraction of cells that form hybrid colonies can be calculated (Table 1). Because virus-produced

TABLE 1. *Hybrid colony frequencies obtained with and without virus treatment.*

A9 × *cl* 1 D			RL × *cl* 1 D		
Ratio of parental cells A9:*cl* 1 D	Virus concentration (HA unit)	Number of hybrid colonies per 10^4 cells of A9	Ratio of parental cells RL:*cl* 1 D	Virus concentration (HA unit)	Number of hybrid colonies per 10^4 cells of RL
1:1	0	0.07	1:200	0	0.6
1:1	33	5.8			
1:1	67	6.7			
1:1	124	8.3	1:200	124	76
1:1	500	9.2	1:200	500	96
1:1	1,000	9.3	1:200	1,000	107
			1:1	1,000	31
			1:10	1,000	52
1:500	1,000	13.0	1:100	124	64
1:5000	1,000	18.0	1:1000	124	90
1:50,000	1,000	15.0	1:10,000	124	180

Since hybrid colony frequencies are defined as the fraction of parental cells that produce hybrid colonies, it should be pointed out that the calculated frequency does not correspond to the fusion frequency, since many of the diheterokaryons probably do not form colonies.

hybrids appear in plates inoculated with very few cells (10^3 or fewer), it is clear that effective contacts are formed before the cells are diluted and that continued high-cell density, which is required for spontaneous hybrid formation, is no longer needed after Sendai virus fusion.

The effect of dilution of virus on hybrid colony frequency: Serial dilutions of stock virus (2000 HA unit/ml) were used for treatment of parental cell mixtures. In Figure 2, the numbers of hybrid colonies obtained after treatment with different titers of virus are expressed in two ways: as the number of hybrid colonies per cell of one parent initially present, and as the number of hybrid colonies formed per "cellular unit" present after virus treatment. In both

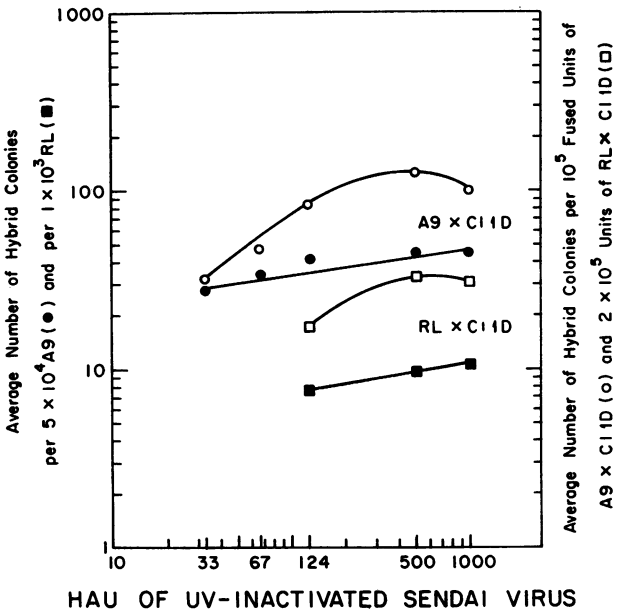

FIG. 2.—Numbers of hybrid colonies of A9 × *cl* 1 D and RL × *cl* 1 D formed after treatment with different titers (HA unit) of ultraviolet-inactivated Sendai virus. The data are expressed in two ways: as yield of hybrid colonies per A9 or RL initially present, i.e. the number present before virus treatment (left-hand ordinate), and as yield of hybrid colonies per fused "cellular unit" present after virus treatment (right-hand ordinate). In the A9 × *cl* 1 D cross, equal numbers of parental cells were used; in the RL × *cl* 1 D cross, 1 RL cell was used for every 200 *cl* 1 D cells.

crosses, the "hybrid colony frequency" (calculated as the number of hybrid colonies formed per 10^4 of the less numerous parent, Table 1) shows very little difference after treatment with various virus concentrations. This result suggests that the large numbers of heterokaryons which are formed at high virus concentrations do not contribute to the yield of hybrid colonies. However, since the formation of large numbers of multinucleated cells clearly results in a decreased number of cellular units present after fusion, the frequency of hybrid colonies per "cellular unit" shows a progressive increase between 33 and 500 HAU, while the calculated "hybrid colony frequency" remains nearly constant. The similar values of hybrid colony frequency obtained at different virus concentrations show that low titers of virus, which cause much less total fusion than high titers, are just as effective as high titers in producing the cellular units that give rise to hybrid colonies.

Effect of parental cell ratio on hybrid colony frequency: The curves of Figure 3 show that the frequency with which hybrid colonies appear is proportional to the dilution of the limiting parent from both virus-treated and untreated mixtures. Moreover, the frequency of hybrid colonies obtained after Sendai treatment is the same whether both parental cells are exposed to virus before plating or whether virus is first adsorbed to one parent and the suspension

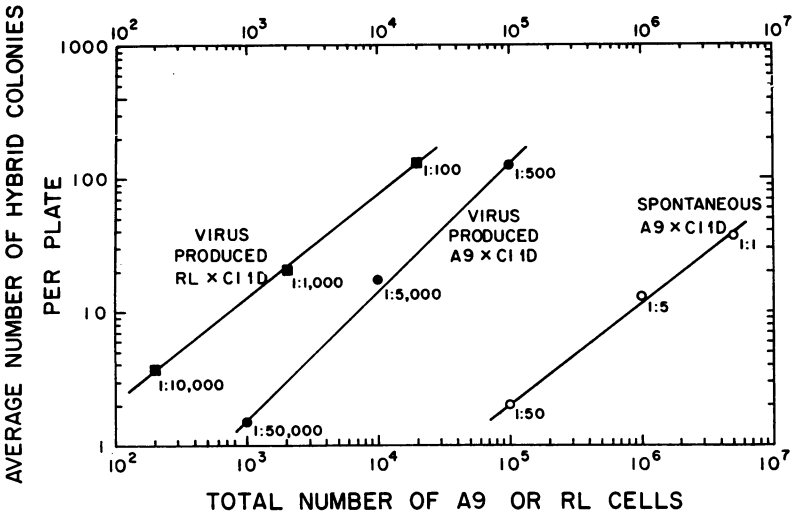

FIG. 3.—The effect of variations of parental cell ratios on the number of colonies of spontaneous and virus-produced hybrids. (○) Spontaneous hybrid colonies and (●) hybrid colonies of A9 × *cl* 1 D obtained after virus treatment. At each inoculum (indicated on the abscissa) suspensions of virus-treated or untreated A9 cells were added to a monolayer of 2×10^6 *cl* 1 D cells. (■) Hybrid colonies of RL × *cl* 1 D were obtained by mixing 1×10^5, 1×10^4, and 1×10^3 RL cells with 1×10^7 *cl* 1 D cells before virus treatment, to give parental cells ratios of 1:100, 1:1000, and 1:10,000.

plated on a monolayer of the second parent. Davidson[15] has found that exposure to virus of a small number of cells attached to a plate, followed by a brief rinse and addition of the second parent, also produces a significant increase in the frequency of hybrid colonies.

In virus-treated mixtures of RL × *cl* 1 D, the efficiency of hybridization is not identical throughout the range of parental cell ratios tested (see Table 1). The hybrid colony frequency shows a maximum when greatly unequal ratios of parental cells are used, and decreases as the ratio approaches 1. This unexplained feature of hybridization experiments has also been noted in spontaneous crosses: Davidson and Ephrussi[16] have studied the variations in efficiency of hybridization of diploid mouse cells and A_9, and similar variations have been noted[17] in crosses between the mouse line 3T3[18] and *cl* 1 D.

Efficiency of hybridization and of fusion: The total number of units (including both mononucleate parental cells and fused units) that are present after virus treatment compared to the number of parental cells initially added permits calculation of the total recovery (of cells and units) after treatment with various titers of virus. The data in Table 2 show that the manipulations described for cell fusion allow nearly complete recovery of the parental cells (see control, 0 HA unit), and that the number of cellular units (including single cells and fused units) declines progressively as virus titer increases. The decline in total number of units occurs in parallel with an increasing frequency of large fused cells (cf. fusion index of Okada[19]). By this criterion, more fusion occurs in a mixture of RL and *cl* 1 D than in a mixture of A_9 and *cl* 1 D. This

TABLE 2. *Comparative recovery of units* after treatment with various titers of virus.*

HA unit	Per Cent Recovery	
	A9 × *cl* 1 D (ratio 1:1)	RL × *cl* 1 D (ratio 1:10)
0 (Control)	95	97
33	80	68
67	70	—
124	50	36
500	37	17
1,000	45	11

* Discrete units were counted after virus treatment. The values given were obtained by counting the total numbers of units in mixtures which included variable proportions of mononucleate parental cells and multinucleated fused units. The reduction in total number of units recovered reflects the extent of fusion that has occurred in the mixture.

fact could account both for the greater hybrid colony frequency observed for RL × *cl* 1 D (180 in 10^4 or 1/55) than for A_9 × *cl* 1 D (20 in 10^4 or 1/500) and for the reduction in efficiency of hybridization found with RL × *cl* 1 D at high proportions of RL cells. At high titers of virus or in the presence of many easily fused RL cells, it is likely that large heterokaryons form and effectively remove the limiting numbers of RL cells from the mixture.

Properties of virus-produced and spontaneous hybrids: The hybrid colonies from virus-treated cultures can be propagated continuously, as has been shown for spontaneously arising hybrids.[20] Four lines of virus-produced RL × *cl* 1 D hybrids have been cultured for more than eight months with an average generation time of one day.[21] Virus-produced hybrids between *cl* 1 D and a rat hepatoma cell line (H4IIEC3)[22] have been grown for more than 60 cell generations.[23] Virus-produced hybrids between the mouse cell line 3T3 and a human cell line VA-2[24] exhibit the preferential loss of human chromosomes already described for spontaneously arising human-mouse hybrids,[25] and they contain the SV40 T-antigen characteristic of the virus-transformed human parent.[26] Karyological analysis of ten hybrid colonies obtained from Sendai-treated mixtures compared to six colonies from untreated mixtures (A_9 × *cl* 1 D) has shown no difference in chromosome number or range between the hybrids obtained by the two methods.[21]

Discussion.—Treatment with ultraviolet-inactivated Sendai virus has been found to be a widely applicable and highly efficient means of increasing the frequency with which somatic cells form viable hybrids. The properties known to be characteristic of spontaneously arising hybrids have also been found in virus-produced hybrids. The approximately 100-fold increased hybridization frequency observed after virus treatment should permit investigation of factors influencing the growth potential of fused cells, which have been observed to be more numerous than viable hybrid colonies.

If it is assumed that hybrids arise from diheterokaryons, then the greatest hybrid colony frequencies will be observed when the frequency of virus-induced diheterokaryons is at a maximum. Although it seemed likely that changes in the concentration of virus to produce fusion might influence the numbers of hybrid colonies obtained, their frequencies were very similar between 33 and 1000 HA units of virus. The observation that larger numbers of hybrid

colonies are obtained per fused cellular unit at high virus titers suggests that this method may be used to isolate hybrid colonies from crosses for which no selective markers exist. Thus, by reducing the number of mononucleate parental cells relative to the number of fused cells, the fusion process itself may be used in place of a selective system.

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‡ Abbreviations used: RL, rat liver; HAT, hypoxanthine, aminopterin, and thymidine; HA unit, hemagglutinating unit.

[1] Littlefield, J., *Science*, **145**, 709 (1964).

[2] Okada, Y., *Exptl. Cell Res.*, **26**, 98 (1962).

[3] Harris, H., and J. F. Watkins, *Nature*, **205**, 640 (1965).

[4] Yerganian, G., and M. B. Nell, these PROCEEDINGS, **55**, 1066 (1966).

[5] Davidson, R. L., and B. Ephrussi, *Nature*, **205**, 1170 (1965).

[6] Dubbs, D. R., and S. Kit, *Exptl. Cell Res.*, **33**, 19 (1964).

[7] Littlefield, J., *Nature*, **203**, 1142 (1964).

[8] Coon, H. G., *J. Cell Biol.*, **39**, 29a (1968).

[9] Coon, H. G., *Carnegie Institution of Washington Year Book* **67** (1969), p. 419.

[10] Ham, R. G., these PROCEEDINGS, **53**, 288 (1965).

[11] A working solution was made containing eightfold concentrations of amino acids and pyruvate, fourfold concentrations of vitamins, and of all other components except inorganic salts and glucose; the latter were present in the concentrations specified by Ham. One part of working solution was diluted for use with 3 parts modified Hanks' saline solution (see Konigsberg, I. R., *Science*, **140**, 1273 (1963)).

[12] Coon, H. G., these PROCEEDINGS, **55**, 66 (1966).

[13] Okada, Y., F. Murayama, and K. Yamada, *Virology*, **28**, 115 (1966).

[14] It has been found that the rate of fusion is not the same with all cell combinations. For example, a mixture of RL × *cl* 1 D, after incubation at 37°C, usually shows spherical masses containing many nuclei. Mixtures of A9 × *cl* 1 D at this stage may show both fused spherical masses as well as small grapelike clusters of incompletely fused cells.

[15] Davidson, R. L., manuscript in preparation.

[16] Davidson, R. L., and B. Ephrussi, manuscript in preparation.

[17] Weiss, M. C., unpublished experiments.

[18] Todaro, G. J., and H. Green, *J. Cell Biol.*, **17**, 299 (1963).

[19] Okada, Y., and J. Tadokoro, *Exptl. Cell Res.*, **26**, 108 (1962).

[20] Scaletta, L. J., and B. Ephrussi, *Nature*, **207**, 1169 (1965).

[21] Coon, H. G., and M. C. Weiss, *Wistar Inst. Symp. Monogr.*, in press (1969).

[22] Pitot, J. C., C. Peraino, P. A. Morse, and V. R. Potter, *Natl. Cancer Inst. Monograph*, **13**, 229 (1964).

[23] Weiss, M. C., unpublished experiments.

[24] Ponten, J., F. C. Jensen, and H. Koprowski, *J. Cellular Comp. Physiol.*, **61**, 145 (1963).

[25] Weiss, M. C., and H. Green, these PROCEEDINGS, **58**, 1104 (1967).

[26] Weiss, M. C., B. Ephrussi, L. J. Scaletta, these PROCEEDINGS, **59**, 1132 (1968); Weiss, M. C., manuscript in preparation.