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*THE INOSINIC ACID PYROPHOSPHORYLASE ACTIVITY OF MOUSE FIBROBLASTS PARTIALLY RESISTANT TO 8-AZAGUANINE**

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Resistance to the analogue 8-azaguanine has generally been found to correlate with loss of guanylic acid-inosinic acid (IMP) pyrophosphorylase activity in several biological systems[1] including 3 mammalian cell lines.[2-4] From 2 of these lines mutants selected for even a small degree of resistance contained very little or no pyrophosphorylase activity.[2, 4] From the other cell line a partially resistant mutant was also isolated in which no change in pyrophosphorylase activity occurred, as well as another mutant (resistant to 8-azaguanosine) which "displayed about one-half of this activity."[3] To investigate further the mechanism of partial resistance we have now isolated several lines of mouse fibroblasts (L cells) which show a small degree of resistance to 8-azaguanine, and have found them to contain 25–44 per cent of the IMP pyrophosphorylase activity of wild-type L cells.

Experimental.—Materials: 8-Azaguanine was obtained from the Francis Earle Laboratories. It was homogeneous on chromatography in isopropanol:H_2O:concentrated HCl (657:176:167).[5] Hypoxanthine-8-C^{14} and 5-phosphorylribose 1-pyrophosphate were obtained from the California Corporation for Biochemical Research, and aminopterin (4-aminopteroylglutamic acid) from the Lederle Laboratories.

Culture methods: An inoculum of L cells was kindly supplied by Dr. L. Siminovitch in 1960, at which time the chromosome number was reported to be approximately 60. This cell line, designated "wild-type," has been maintained in suspension culture in Eagle's "spinner" medium plus 10% calf serum as previously described.[6] It is free of pleuropneumonia-like organisms. Clonal lines were obtained by isolating individual colonies[7] and have been stored at −70°C in medium containing 10% glycerol when not in use.[8]

Determinations of drug resistance or mutant frequency were performed by inoculating an appropriate number of cells (2×10^2 to 5×10^6) from a suspension culture into replicate 60 mm

plastic Petri dishes containing spinner medium supplemented with 10% calf serum, 0.013 *M* $NaHCO_3$, and 0.001 *M* sodium pyruvate, plus the desired concentration of 8-azaguanine. The cultures were equilibrated with 5% CO_2 in air and incubated for 10–20 days at 37°C. When the first grossly visible colonies were noted, the cultures were fixed and stained, and the colonies enumerated (Bactronic Colony Counter, New Brunswick Scientific Co.). For each determination, 3–6 replicate cultures were used, and the colonies were found to be randomly distributed by the chi square test.[9]

Two features of this method deserve mention. To maintain the 8-azaguanine concentration, it was necessary to feed cultures containing more than 10,000 cells, and this was done on the second day and twice thereafter at 2–3-day intervals. Further feeding did not affect the final number of colonies. Without feeding, too numerous colonies or confluent growth occurred, presumably because of incorporation or catabolism of 8-azaguanine, or release of competing purines by disintegrating cells.

The plating efficiency of the cell lines described below ranged from about 10% (AG 3) to 40% (wild-type). However, 2–4 times higher values occurred in reconstruction experiments in which these cells were plated in the presence of 10^6 drug-inhibited cells. Because of this uncertainty, the mutant frequencies below are calculated on the basis of the number of cells inoculated without correction for plating efficiency.

Preparation of cell extracts: About 1.6×10^7 cells were collected by centrifugation, washed with 0.14 *M* NaCl solution at 4°C, suspended in 2 ml of 0.05 *M* phosphate buffer at 4°C and pH 7.5, and disrupted by 3 cycles of rapid freezing and thawing. The resulting suspension was centrifuged at 105,000 × *g* at 4°C for 1 hr. The supernatant fluid was collected, analyzed for protein content,[10] and stored at −70°C. Under these conditions IMP pyrophosphorylase activity was stable for at least 2 months.

Assay of IMP pyrophosphorylase:[11] The incubation mixture (total volume 45 μl) contained about 3 μg of protein; 110 μg of crystalline bovine albumin; 8 mμm of hypoxanthine-8-C^{14} (7.6 μc per μm); 50 mμm of 5-phosphorylribose 1-pyrophosphate; 270 mμm of $MgCl_2$; and 180 mμm of tris(hydroxymethyl)aminomethane at pH 7.4. After incubation at room temperature for 2 hr, 30 μl of the incubation mixture were transferred to ion exchange paper (Reeve Angel paper, grade SB2, loaded with Amberlite IRA-400 resin-Cl^- form). One-tenth *N* formic acid was allowed to ascend through the origin spot, the paper was dried, and the origin spot was cut out, mounted (Tracerlab, Inc.; E-7B rings and disks), and assayed for radioactivity (Nuclear-Chicago model D47 thin end-window counter) to within 5% error. Under these conditions, phosphorylated derivatives of hypoxanthine-8-C^{14} are held at the origin and are present in an amount proportional to enzyme concentration. The reaction was linear for at least 2 hr. An increased concentration of hypoxanthine-8-C^{14} or 5-phosphorylribose 1-pyrophosphate did not increase the rate of the reaction. Essentially no radioactivity was bound at the origin spot when 5-phosphorylribose 1-pyrophosphate was omitted. The specific activity of IMP pyrophosphorylase referred to below represents the counts per minute of phosphorylated derivatives of hypoxanthine-8-C^{14} per μg of cell extract protein per 2 hr at room temperature.

Results.—Clones resistant to 3–4 μg of 8-azaguanine per ml: When wild-type L cells were incubated in the presence of 3–4 μg of 8-azaguanine per ml, the frequency of resistant cells averaged 5×10^{-7} in 6 determinations over a 2-year period (2, 3, 5, 5, 5, and 8×10^{-7}). That such cells were persistently resistant was suggested by the isolation consecutively of 7 colonies, all of which were again resistant to 4 μg of 8-azaguanine per ml after approximately 20 generations in the absence of the analogue. One such colony (AG 3) was recloned and has been grown for over 2 years in the absence of 8-azaguanine, without losing its resistance. In Figure 1 is shown the plating efficiency of wild-type cells and AG 3 cells at increasing concentrations of 8-azaguanine. The AG 3 cells were about 100 times more resistant than the wild-type cells.

It has been shown previously that resistance to 8-azaguanine generally correlates with a marked decrease or absence of IMP pyrophosphorylase activity,[1–4] and

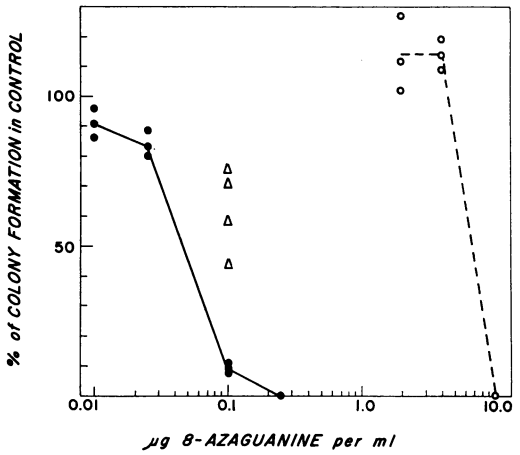

FIG. 1.—Plating efficiency of wild-type (●), AG 46-3 (△), and AG 3 (○) cells at various concentrations of 8-azaguanine (see *Experimental*).

there was no detectable activity in soluble extracts of AG 3 cells (Table 1). Presumably for this reason, AG 3 cells were unable to utilize exogenous hypoxanthine. Thus, there was no incorporation of hypoxanthine-8-C^{14} into the nucleic acids of intact AG 3 cells during 1 hr at 37°C in 2 experiments during which wild-type cells incorporated 370 and 620 cpm.[12]

Clones isolated at a lower concentration of 8-azaguanine: In order to study intermediate degrees of resistance, wild-type cells were incubated in Petri dishes with 0.3 and 1.0 μg of 8-azaguanine per ml. These cultures were not fed, and in retrospect it is unlikely that the level of 0.3 μg per ml was maintained (see *Experimental*). However, several surviving colonies were isolated and enlarged from both the 0.3 and 1.0 μg per ml cultures.

In Table 1 are shown the specific activities of IMP pyrophosphorylase in all the cell lines isolated from the 0.3 and 1.0 μg per ml cultures. The activities fell roughly into 3 groups. Two cell lines (AG 55 and AG 63) selected at 1.0 μg per ml, and 1 (AG 43) selected at 0.3 μg per ml., contained very little activity, resembling AG 3 in this respect. Three lines, (AG 19, 23, and 24), selected at 0.3 μg per ml, contained activity similar to that in wild-type cells. The 2 remaining lines selected at 0.3 μg per ml (AG 41 and AG 46) contained 44 and 35 per cent of the activity of wild-type cells. When examined, the specific activities of these lines have remained approximately constant in a number of preparations during several weeks to months of growth in the absence of the analogue.

TABLE 1

SPECIFIC ACTIVITIES OF IMP PYROPHOSPHORYLASE IN EXTRACTS OF CELL LINES

Cell line	8-Azaguanine* μg/ml	Specific Activity† Low	Intermediate	High
AG 3	2.0	0, 1, 0		
AG 55	1.0	6, 0, 6		
AG 63	1.0	1, 0, 0		
AG 80M	1.0	3		
AG 43	0.3	14, 4		
AG 41	0.3		126, 173, 147, 132	
AG 46	0.3		100, 130	
AG 19	0.3			329
AG 23	0.3			369
AG 24	0.3			239, 260
AG 70M	0.1		98, 99, 123	
AG 55R	—		82	
AG 63R	—		124	
Wild-type	—			369, 320, 291

* Represents the concentration of 8-azaguanine at which the cell line was selected.
† Represents cpm of phosphorylated derivatives of hypoxanthine-8-C^{14} per μg of cell extract protein (see *Experimental*). Each value is the average of duplicate determinations on different cell extracts obtained weeks to months apart.

It seems likely that the IMP pyrophosphorylase activities *per cell* would also fall into 3 groups, as do the activities per μg of protein shown in Table 1. For most of the cell lines, the average amount of protein extracted by freezing and thawing was 97 $\mu\mu$g per cell, with a random distribution of values around the mean ($0.067 < P_{n=9} < 0.091$). For wild-type cells this represented about 40 per cent of the total cell protein. A few extracts were obtained from cells growing slowly in the presence of a partially inhibitory concentration of 8-azaguanine; the average amount of protein extracted was 139 $\mu\mu$g per cell, and, when examined, the cells appeared larger than usual. The specific activity of pyrophosphorylase was similar to that found during more rapid growth in the absence of 8-azaguanine.

Extracts of wild-type, AG 3, AG 43, AG 46, and AG 55 cells were assayed alone and in combination with one another (Table 2). Since in all cases the combined activity was approximately additive, there was no evidence for the presence of an inhibitor or activator in these preparations.

TABLE 2

IMP PYROPHOSPHORYLASE ACTIVITY OF COMBINATIONS OF CELL EXTRACTS

Expt.	Cell extract(s)	Cpm*
1	Wild-type	447
	AG 3	0
	AG 43	14
	Wild-type and AG 3	426
	Wild-type and AG 43	495
2	Wild-type	483
	AG 3	0
	AG 46	186
	AG 55	1
	Wild type and AG 46	675
	Wild-type and AG 55	464
	AG 3 and AG 46	194

* Represents the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature (see *Experimental*) by 10 μl of each extract alone or in combination with 10 μl of another extract.

The resistance to 8-azaguanine of AG 55 and AG 63 cells was high and similar to that of AG 3. The resistance of AG 41 and AG 46 cells was slight, but greater than wild-type cells at 0.1 μg of 8-azaguanine per ml (Fig. 1). It was comparable to the first step of resistance to 8-azaguanine in mouse lymphoblasts.[4]

When 5×10^2 AG 41 or AG 46 cells were exposed to a concentration of 8-azaguanine greater than 0.3 μg per ml, no cells survived. Therefore, it was not likely that these cell lines were mixtures of wild-type and highly resistant cells. To exclude this possibility more conclusively, AG 46 was recloned once (AG 46-3) and again (AG 46-3-6 and AG 46-3-7). The pyrophosphorylase activity of AG 46 was 35 per cent, of AG 46-3 was 27 per cent, of AG 46-3-6 was 30 per cent, and of AG 46-3-7 was 39 per cent of the activity of wild-type cells. These results suggested that each cell in the AG 46 culture contained an intermediate pyrophosphorylase activity.

Selection of partially and highly resistant populations of cells: Wild-type cells were incubated in suspension culture with 0.1 μg of 8-azaguanine per ml to determine if this concentration was sufficient to select cells with an intermediate pyrophosphorylase activity. By day 18 resistant cells were apparent (Fig. 2), and these contained 33 per cent of the enzyme activity of wild-type cells (AG 70M, Table 1). That the culture did not consist of a mixture of wild-type and highly resistant cells was indicated by the fact that cell multiplication ceased when the concentration of 8-azaguanine was increased to 1.0 μg per ml on day 30. By day 38, highly resistant cells were apparent, which contained 1 per cent of the enzyme activity of wild-type cells (AG 80M, Table 1).

The generation times of the cells resistant to 0.1 and 1.0 μg of 8-azaguanine per

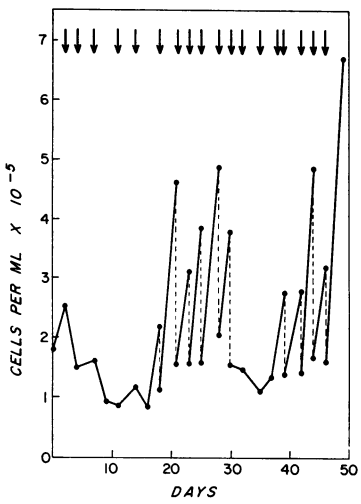

FIG. 2.—Selection of cells partially and highly resistant to 8-azaguanine. Wild-type cells were incubated in suspension culture with 0.1 μg of 8-azaguanine per ml from day 0 to day 30. After day 30 the concentration was increased to 1.0 μg per ml. Feeding with fresh warm medium was carried out regularly (arrows) either by centrifugation and resuspension or by dilution (dashed lines).

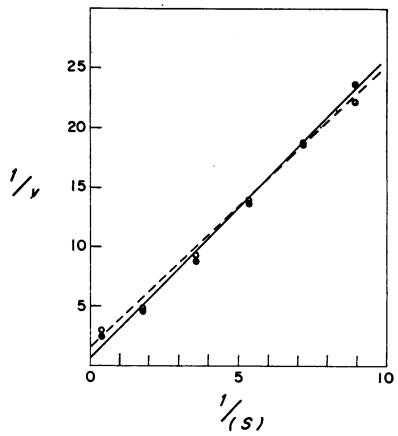

FIG. 3.—Lineweaver-Burk plot to determine the Michaelis constants of IMP pyrophosphorylase in extracts of wild-type and AG 46-3 cells. (S) represents 10^5 times the concentration of hypoxanthine-8-C^{14} in moles/liter, and v represents 10^{-3} times the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature by 10 μl of cell extract (see *Experimental*). 5-Phosphorylribose 1-pyrophosphate was not rate-limiting at any concentration of hypoxanthine-8-C^{14} used. For clarity, only the average of triplicate determinations is plotted for each hypoxanthine-8-C^{14} concentration with wild-type (●—●) or AG 46-3 (○---○) cell extracts. The regression lines were calculated from all the determinations by the method of least squares. The standard errors of the estimates of slopes and intercepts indicated no significant difference between the wild-type and AG 46-3 extracts.

ml were approximately 43 and 37 hr (in contrast to 24 hr for wild-type cells). By extrapolation of these values it was estimated that in the inoculum of wild-type cells the frequency of cells resistant to 0.1 μg per ml was about 1.5×10^{-3}, and of cells resistant to 1.0 μg per ml was about 1×10^{-5}. The frequency of highly resistant cells in wild-type cultures was also determined in experiments with Petri dishes mentioned earlier to be about 5×10^{-7}. However, because of low plating efficiency this value doubtless underestimates the true frequency, and may be 2.5–10 times larger (see *Experimental*) and therefore closer to the value of 1×10^{-5} above. In any case these frequencies, while approximate, are consistent with the concept that highly resistant cells arise from cells which are partially resistant.

Properties of the enzyme: To determine if the IMP pyrophosphorylase present in intermediate amounts in AG 46-3 cells was similar to that present in wild-type cells, quadruplicate aliquots of extracts of AG 46-3 and wild-type cells were heated at 87°C for 10 min, and the residual enzymatic activity compared to that in control aliquots. The average drop in activity of AG 46-3 extracts upon heating was 72

TABLE 3

HEAT INACTIVATION OF IMP PYROPHOSPHORYLASE IN CELL EXTRACTS

	cpm*					
	Wild-type		AG 46-3		AG 41	
Expt.	Control	Heated	Control	Heated	Control	Heated
1	395	143	308	97	—	—
	448	141	323	98	—	—
	514	163	313	92	—	—
	409	156	350	82	—	—
Mean	442	151	324	92	—	—
% decrease	—	66	—	72	—	—
2	309	122	—	—	261	109
	258	123	—	—	302	99
	376	121	—	—	241	99
	319	127	—	—	251	87
Mean	315	123	—	—	264	99
% decrease	—	61	—	—	—	63

* Represents the cpm of hypoxanthine-8-C^{14} converted to phosphorylated derivatives in 2 hr at room temperature (see *Experimental*). Quadruplicate determinations were done. With AG 46-3 and AG 41 more cell extract protein was used than with the wild-type extract.

per cent and that of wild-type extracts was 66 per cent (Table 3). These results were not significantly different ($0.4 < p_{n=6} < 0.5$). This type of experiment was also carried out with AG 41 (Table 3); in this case the average drop in activity upon heating of the AG 41 extracts was 63 per cent, while that of the wild-type extracts was 61 per cent.

The Michaelis constants of the pyrophosphorylase present in AG 46-3 and that in wild-type cells were determined by the method of Lineweaver and Burk to be 2.9×10^{-4} *M* and 7.4×10^{-4} *M*, respectively (Fig. 3). These values also were not significantly different ($0.2 < p_{n=36} < 0.3$). Together with the heat inactivation studies, they suggest that the enzyme in AG 46-3 cells is the same as that in wild-type cells.

Reversion: In media containing 4×10^{-7} *M* aminopterin supplemented with 3×10^{-6} *M* glycine, 1.6×10^{-5} *M* thymidine, and 1×10^{-4} *M* hypoxanthine, cell growth occurs only if the exogenous hypoxanthine is utilized.[3, 13] Since AG 3 cells are unable to do this, no colonies were formed in this medium by 5×10^{6} AG 3 cells, while the plating efficiency of wild-type cells was not decreased. AG 41 and AG 46 cells survived as well as wild-type cells, and it was not possible to separate these cells from wild-type cells at a low concentration of hypoxanthine.

Under similar conditions, most AG 55 and AG 63 cells, like AG 3 cells, did not survive. However, in both AG 55 and AG 63 cultures, unlike AG 3, the rate of reversion was high, i.e., approximately 10^{-2} and 3×10^{-3}, respectively. It seemed likely that the presence of revertant cells caused the slight pyrophosphorylase activity of AG 55 (Table 1). For both AG 55 and AG 63 a mixture of revertant colonies was grown together over 10 generations in medium containing aminopterin, glycine, thymidine, and hypoxanthine, in order to remove residual highly resistant cells. The pyrophosphorylase activities of these uncloned revertant populations (designated AG 55R and AG 63R) were 25 and 38 per cent, respectively, of the activity of wild-type cells (Table 1). Three clonal derivatives of AG 55R contained 34, 45, and 49 per cent of the activity of wild-type cells. In contrast to these intermediate enzyme levels, Szybalski has described a revertant line of human bone marrow cells which contained "less than one-twentieth" of the IMP pyrophosphorylase activity of wild-type marrow cells.[3]

Attempts to increase enzyme activity: The different IMP pyrophosphorylase activities in these several cell lines could be caused by various degrees of repression of enzyme formation,[14] which aminopterin might be expected to "de-repress." Therefore, pyrophosphorylase activity was determined in several of the cell lines after exposure either to aminopterin, glycine, and thymidine for 16 hr, or to aminopterin, glycine, thymidine, and hypoxanthine for 2–3 days. Under the latter conditions, partially resistant or wild-type cells multiplied almost as well as control untreated cells, whereas the number of AG 3 cells per ml decreased. Essentially no change in pyrophosphorylase activity occurred in any of the cultures under either condition (Table 4). Thus, the enzyme could not be made to appear in AG 3 cells, nor increased in partially resistant or wild-type cells.

TABLE 4

ATTEMPTS TO INCREASE IMP PYROPHOSPHORYLASE ACTIVITY

	Specific Activity*		
Cell line	Control	Aminopterin	Aminopterin and hypoxanthine
AG 3	0	0	0
AG 41	132	—	128
AG 46-3	78	—	69
AG 46-3-6	98	114	—
AG 70M	107	90	—
Wild-type	327	323	352

* See *Experimental.*

Suspension cultures were incubated with 4×10^{-7} *M* aminopterin, 3×10^{-6} *M* glycine, and 1.6×10^{-5} *M* thymidine for 16 hr, or with these 3 compounds and 1×10^{-4} *M* hypoxanthine for 2–3 days. The values for the control specific activities represent one or more preparations.

Discussion.—While genetic studies in tissue culture would preferably be undertaken with diploid cell strains from individuals of known genetic composition,[15, 16] the aneuploid cell lines have such advantages as variety of markers and selection methods, high plating efficiency and mutant frequency, ability to grow in suspension, and recently two putative recombinational techniques.[17, 18]

In the present studies two clonal lines (AG 41 and AG 46) and an uncloned cell population (AG 70M) were selected which showed a small degree of resistance to 8-azaguanine and in which the activity of IMP pyrophosphorylase per μg of protein (and presumably the activity per cell) was reduced to somewhat less than one half of that in wild-type L cells. Pyrophosphorylase activity remained approximately constant in these lines, as well as in wild-type cells, on repeated testing and on attempts to "de-repress" the synthesis of the enzyme. The inactivation upon heating and the Michaelis constants of the enzyme in wild-type and partially resistant cells were not significantly different. These results suggest that such partially resistant cells contain a decreased amount of active enzyme rather than an unchanged amount of enzyme of altered kinetic properties.[19] Finally, the frequencies with which partially and highly resistant cells occurred were consistent with the concept that the partially resistant cell is an intermediate between the wild-type and highly resistant cell.

A possible explanation for these results is that the lines with an intermediate amount of pyrophosphorylase are heterozygous for the ability to synthesize active enzyme, analogous to the situation in individuals heterozygous for galactosemia,[20, 21] acatalasia,[16, 22] and orotic aciduria.[23] If so, the transition from partially to highly resistant cell may involve a recombinational process rather than mutation.[24] The

aneuploid nature of the L cell lines does not preclude heterozygosis, since it is not known if the chromosomes on which the IMP pyrophosphorylase are represented are present in excessive number, nor if so, whether or not they are active. Indeed it is suspected that the supernumerary X-chromosomes present in certain disorders do not function.[25]

It is interesting that an intermediate enzyme activity occurred also in the uncloned revertant populations (as well as clonal derivatives thereof) selected from two highly resistant lines. While this suggests that the revertants too may be heterozygous, the situation is likely to be complex, since several reversion mechanisms, some perhaps extrachromosomal, are known to occur in bacteria.[26]

Summary.—Several lines of mouse fibroblasts with a small degree of resistance to 8-azaguanine were isolated. They contained an amount of inosinic acid pyrophosphorylase activity which was intermediate between wild-type and highly resistant cells. This activity was constant on repeated analyses and despite attempts to "de-repress" the synthesis of the enzyme. The heat inactivation and Michaelis constants of the enzyme were the same in wild-type and partially resistant cells. It is suggested that the partially resistant cells may be heterozygous for the ability to synthesize active inosinic acid pyrophosphorylase.

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EFFECT OF VIRUSES ON EARLY MAMMALIAN DEVELOPMENT, I. ACTION OF MENGO ENCEPHALITIS VIRUS ON MOUSE OVA CULTIVATED IN VITRO

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The possible effect of viruses on mammalian eggs has never been explored. The closest studies that have been reported deal with relatively late embryonic stages.[1] This is unfortunate, since a knowledge of the interaction of mammalian eggs and viruses would be valuable for at least three reasons. First, such knowledge would clarify the known relationship between viruses and congenital defects.[2] Secondly, it would elucidate the origin of innate resistance to virus infection, such as is known in certain strains of mice.[3] Thirdly, it would show us whether the specific cellular receptors required by certain viruses for attachment to the host cell are present on the surface of the vitellus, or whether they are formed later in development.

This communication reports the effect of Mengo encephalitis virus on mouse eggs at the 2-cell stage. The virus was found to pass through the zona pellucida surrounding the egg and to block further development *in vitro*.

Materials and Methods.—Randomly bred 6- to 8-week-old Swiss mice were superovulated by the intraperitoneal injection of 5 I.U. of pregnant mare serum gonadotrophin (Gestyl, Organon), followed 43 hr later by 5 I.U. of human chorionic gonadotrophin (Pregnyl, Organon). A mature male was placed with each female at the time of the second injection. This treatment results in ovulation and mating about 12 hr afterwards. Females with vaginal plugs (usually 70–90% of the animals) were killed 34–36 hr later, that is, 10–12 hr after the expected time of the first cleavage division.[4] The 2-cell eggs were flushed from the Fallopian tubes using a syringe with a blunted no. 30 needle. The medium used for flushing out the eggs and also for culturing them was developed by Dr. R. L. Brinster and is to be published elsewhere.[5] This medium consists of modified Krebs-Ringer balanced salt solution, supplemented with sodium lactate and crystalline bovine plasma albumin.

To determine whether the zona pellucida was a barrier to viral entry it was removed with *Streptomyces griseus* protease ("Pronase," Calbiochem Co.).[6] Ova were exposed 5–10 min at room temperature to 0.25% "Pronase" in phosphate buffered saline, containing 1.0% polyvinylpyrrolidone (PVP). The PVP was added to protect the naked blastomeres and to prevent their attachment to the glass. Further details of the action of "Pronase," and other enzymes, on the zona pellucida of the mouse egg are described elsewhere.[7]

The eggs, either naked or with their zonae intact, were placed in drops of the lactate-albumin medium, with or without virus or antiserum. The drops were submerged in mineral oil in a 60 mm Petri dish to permit gas-exchange while preventing evaporation. The culture dishes were incubated at 37°C in an atmosphere of 5% CO_2 in air.

The 37A (heat-stable mutant) of Mengo encephalitis virus, isolated by Brownstein and Graham,[8] was used. The virus was assayed by the plaque technique decribed by these authors.