QUANTITATIVE STUDIES OF THE GROWTH OF MOUSE EMBRYO CELLS IN CULTURE AND THEIR DEVELOPMENT INTO ESTABLISHED LINES

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ABSTRACT

Disaggregated mouse embryo cells, grown in monolayers, underwent a progressive decline in growth rate upon successive transfer, the rapidity of the decline depending, among other things, on the inoculation density. Nevertheless, nearly all cultures developed into established lines within 3 months of culture. The first sign of the emergence of an established line was the ability of the cells to maintain a constant or rising potential growth rate. This occurred while the cultures were morphologically unchanged. The growth rate continued to increase until it equaled or exceeded that of the original culture. The early established cells showed an increasing metabolic autonomy, as indicated by decreasing dependence on cell-to-cell feeding. It is suggested that the process of establishment involves an alteration in cell permeability properties. Chromosome studies indicated that the cells responsible for the upturn in growth rate were diploid, but later the population shifted to the tetraploid range, often very rapidly. Still later, marker chromosomes appeared. Different lines acquired different properties, depending on the culture conditions employed; one line developed which is extremely sensitive to contact inhibition.

INTRODUCTION

When mammalian cells are placed in culture they grow rapidly, often at a rate substantially exceeding that in the intact animal. However, this growth does not continue indefinitely. Most frequently, after a variable interval, for reasons as yet unclear, the cells die (1, 2). In some cases, usually thought to be uncommon, changes in the cell population are observed to occur, culminating in the development of an established line having a variety of properties which distinguish it from the strain of origin (2). One of these properties is the ability to produce tumors when injected into suitable hosts (3–5), so that the mechanism by which the normal cell is converted into an established cell may have a bearing on the problem of carcinogenesis.

In the following experiments the growth properties of mouse embryonic fibroblasts were closely studied from the time they were placed in culture, and especially during their conversion to established lines. The changes in growth properties were related to chromosomal and morphological changes, and certain criteria were set up for the established condition.

MATERIALS AND METHODS

Media

The medium used was Dulbecco's modification of Eagle's medium (6), containing an approximately fourfold higher concentration of the amino acids

and vitamins described, plus serine and glycine, and 10 per cent calf serum (Colorado Serum Co., Denver). This medium has a high bicarbonate concentration, and the pH was kept at 7.2 by equilibration with 10 per cent CO_2 in air. In the earlier experiments 10 per cent tryptose phosphate was supplemented. All cultures were maintained in 50 mm diameter plastic Petri dishes and were transferred by trypsinization in phosphate-buffered saline (PBS) (7).

Primary Cultures

Cultures of 17 to 19 day old Swiss mouse embryos were prepared by fine mincing of the whole embryos and disaggregating with 0.25 per cent trypsin. The trypsin was removed by centrifugation and the cells were resuspended in medium. The cells were counted and plated at 3×10^6 cells per plate. After 2 to 3 days, confluent monolayers formed. The growth experiments to be described begin with the first transfer thereafter.

Subculture Schedules

All cultures were put on a rigid transfer schedule, being transferred either every 3 days or every 6 days, and inoculated always at the same cell density. The medium was changed on the 1st day for the 3-day transfer regime, and on the 1st, 3rd, and 5th days for the 6-day transfer regime.

At each transfer, duplicate cultures were washed once with dilute trypsin or PBS, and then trypsinized in 2 ml of 0.1 per cent trypsin. After the cells had detached, in 10 to 15 minutes, aliquots of the suspension were taken from each plate for counting in a hemocytometer chamber under phase microscopy. At least 250 cells, and in most cases 500 to 1000 cells, were counted. Appropriate aliquots were then taken for inoculation. Similar counts were made on duplicate cultures 24 hours after inoculation.

The growth in the interval between transfers was calculated by dividing the cell number at the end of the growth interval by the number at 24 hours after inoculation (N/N_0). With fast growing cultures N_0 exceeded the number of cells inoculated, while in slowly growing cultures it was slightly less than the number of cells inoculated, in the range between 80 per cent and 90 per cent. As any growth during the first 24 hours after inoculation is neglected in all calculations of the total number of generations through which a cell population has grown, these are minimal estimates and might, in some cases, be as much as 20 per cent low.

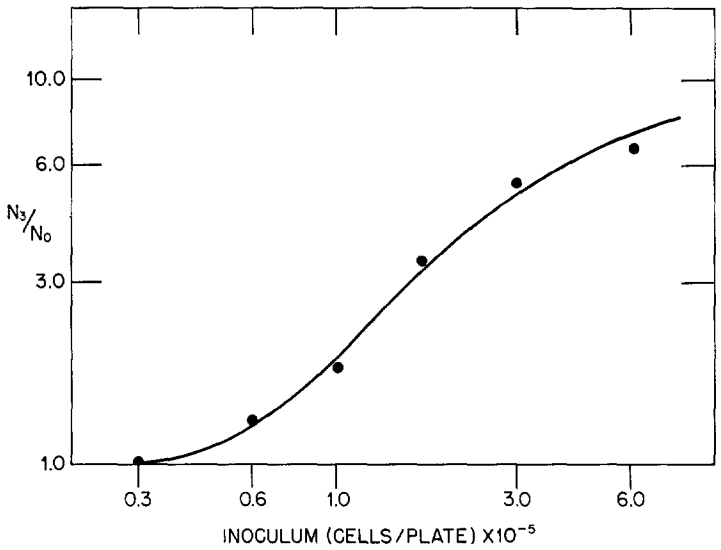

FIGURE 1

Growth of secondary cultures of embryonic fibroblasts at different inoculation densities.

In all experiments, growth over a 2- or 3-day interval is denoted as N_2/N_0 or N_3/N_0, respectively.

Growth of Cells under Agar

Agar casts were prepared by pouring a 4 per cent agar solution in diluted growth medium into Petri dishes and allowing it to harden. The casts were then transferred with a spatula onto monolayers on the 1st day after transfer, and liquid medium was added to the Petri dishes above the agar. Growth of the cells continued under the agar for the subsequent

5 days. The liquid medium was changed at the usual times. On the day the cells were transferred, the agar casts were removed and the cells treated as described above.

Chromosome Preparations

Actively growing cultures were arrested in metaphase for 4 to 6 hours with vincaleukoblastine (Velban) at a concentration of 0.1 μg/ml. Cells were then treated according to a modification of the procedure of Hastings *et al.* (8). Preparations were stained with 2 per cent aceto-orcein. The number of chromosomes was estimated in each metaphase seen, and assigned to a diploid or non-diploid category. This was done without selection of cells in order to avoid prejudice created because the metaphases with higher chromosome numbers are more difficult to count exactly. Exact counts were performed wherever possible, and Table II is based on both kinds of data.

RESULTS

The Growth Rate of Normal Mouse Embryo Cells upon Successive Transfer

Growth in culture of a variety of mammalian cell types, both normal and established, has been known to be prevented when the cell density falls below a certain critical level (9, 10). This is thought to be due to the loss of labile substrates or intermediates by leakage from the cells and is compensated at high cell densities or on irradiated feeder cells by mutual cell feeding (11, 12). This behavior is shown in the growth of disaggregated mouse embryo cells. In addition, there is a range of cell concentrations where growth occurs but the rate of growth depends upon the inoculation size. Fig. 1 shows the results of a representative experiment to demonstrate this effect on the short term growth of normal secondary cultures. Cells from a healthy subconfluent primary culture were plated at varying dilutions on replicate plates. At 24 hours duplicate samples were counted. The medium was changed on the remaining plates, and the cells were allowed to grow for a further 3 days and were then trypsinized and counted. Growth during the 3-day interval, N_3/N_0, is plotted against inoculation density in Fig. 1. The results show that below a cell concentration of 3×10^4 there is no net growth in the population.[1] At somewhat higher densities, growth is a function of the cell density and rises to a maximum at 6×10^5 cells per plate. At concentrations higher than 6×10^5 cells per plate, growth is again reduced owing to the effect of crowding and the relatively slow rate of growth of normal cells out of the plane of the monolayer.

When cells are transferred successively in culture, the growth rate and ultimate fate of the cultures depend, among other things, on the inoculation density. To show the result of repeated transfer of normal cells at slightly suboptimal densities, cells from a confluent primary culture were plated at densities of 1×10^5, 3×10^5, and 6×10^5, grown for 3 days, counted, and transferred, always at the original cell density. Fig. 2 shows the results of such an experiment. The cells carried at 1×10^5, a markedly suboptimal density, grew through several transfers, but at a decreasing rate, until after 8 generations they had lost all ability to divide *in vitro* and eventually died. The cells transferred at 3×10^5 and 6×10^5 (the latter representing the density which permitted maximal growth in the short term experiments) grew considerably better and had doubling times of 60 hours and 30 hours, respectively, at a time when those maintained at 1×10^5 had ceased to divide. The growth rate of cells at higher densities, however, did decline later to a very low level. When the growth rate of these cultures during each transfer is plotted as a function of the number of generations *in vitro* (Fig. 3), it becomes much more obvious that not only the cells inoculated at 1×10^5 but also those at the other cell densities show a decline in their growth rates beginning virtually as soon as they are put into culture. The rate of this decline, however, is much slower for cells maintained at the higher densities. Between 10 and 20 generations after being put into culture the doubling time for all these cultures exceeded 70 hours.

At concentrations of 12×10^5 cells per plate (Fig. 4, curve C), the initial growth rate of the secondary cultures is somewhat lower than that at 6×10^5 cells per plate because of cell crowding

[1] A small minority of these cells can grow at densities below 3×10^4 cells per plate and would form colonies in sufficient number to permit the calculation of a plating efficiency, which would, of course, be quite low. These cells would constitute too small a proportion of the total population to be detected as an over-all increase in cell number during a 3-day interval. The low plating efficiency reflects the difficulty the cells have in growing in the absence of cell-to-cell feeding and would therefore be a test of their ability to function as independent organisms rather than of their intrinsic ability to grow.

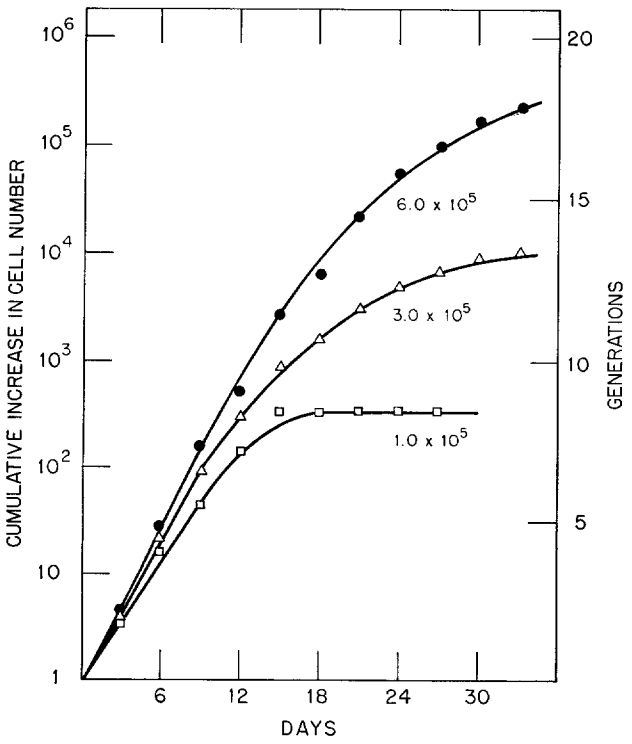

FIGURE 2

Growth of embryonic fibroblasts upon successive transfer at different inoculation densities.

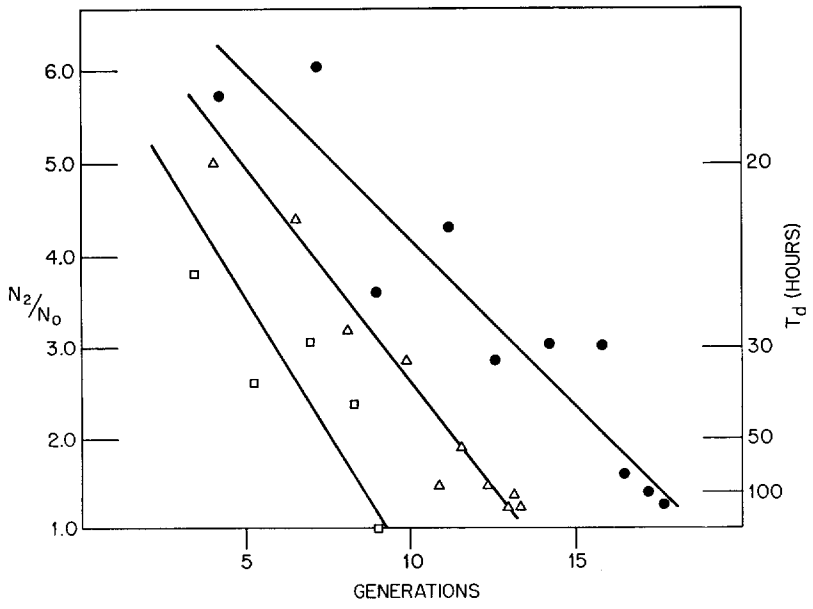

FIGURE 3

Decline in growth rate upon successive transfer at different inoculation densities. Solid circles, inoculum 6 × 10^5 cells; triangles, inoculum 3 × 10^5 cells; squares, inoculum 1 × 10^5 cells. T_d, doubling time.

before the end of the growth interval, but the rate of decline on successive transfer is also slower. Nevertheless the doubling time of this culture, too, rises to over 70 hours by 18 generations. Increasing the cell concentration affects the slope of the decline of the growth rate, but no cell density tested enabled these cells to maintain their growth potential during this period. Since the maximum number of cells per plate attainable (the saturation density) is 5 to 6 $\times$ 10^6 for secondary cultures and even less for later subcultures, clearly it is not possible to raise the inoculation density much higher and still permit appreciable cell division.

The Process of Establishment

The results obtained from a long term study of the cultures just described and other embryo cultures of independent origin are shown in Figs. 4 and 5. For each of these cultures, transferred at 3-day intervals, it can be seen that after a variable time, from 15 to 30 generations, or from 45 to 75 days after the beginning of culture, the growth rate began to rise again and soon reached a value similar to that at the beginning, with a T_d of from 14 to 24 hours. These cultures may now be said to be established lines; some of them have been carried in culture for over a hundred generations with either a constant or a rising growth rate and have never shown any indication of dying out. Out of a total of 11 secondary cultures, 9 have led to the production of established lines. It appears that higher inoculation size favors establishment, but it will be noted that one experiment maintaining cells at only 3 $\times$ 10^5 also led to establishment (Fig. 4,

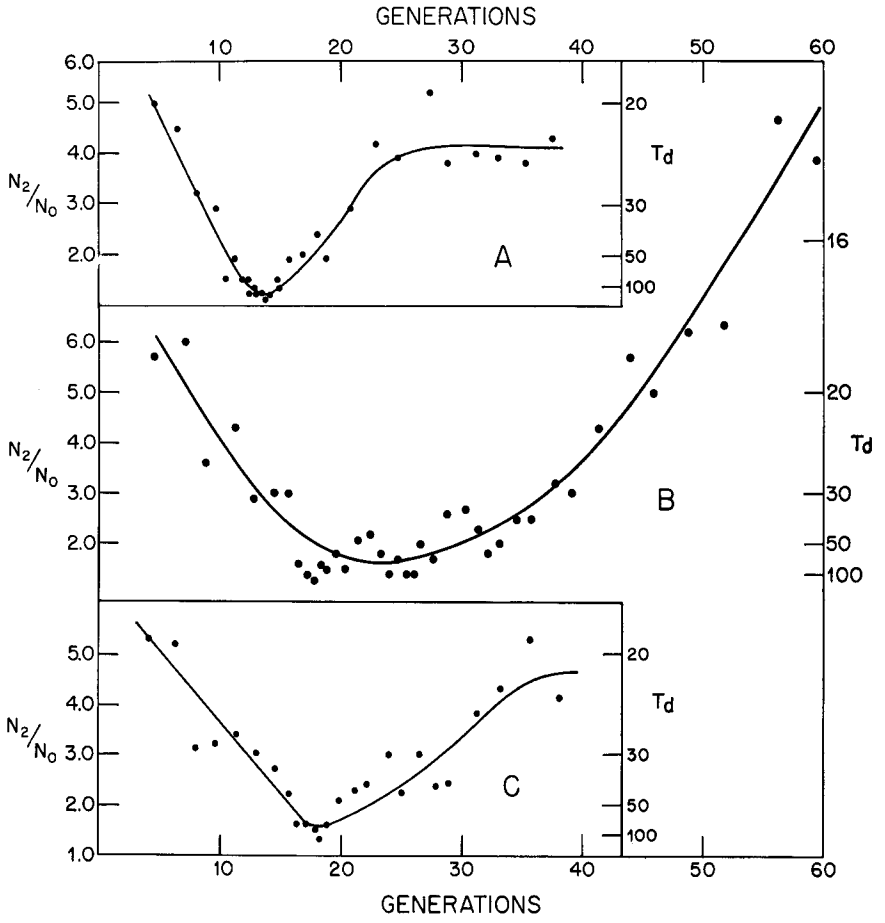

FIGURE 4

Growth rates of fibroblasts upon successive transfer. A, 3T3 (3-day transfer, inoculum 3 $\times$ 10^5 cells); B, 3T6; C, 3T12. T_d, doubling time.

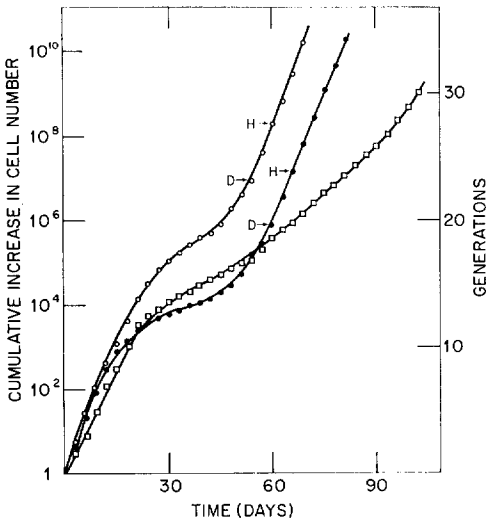

FIGURE 5

Formation of established lines from cells on 3-day transfer regime. Open circles, 3T12; solid circles, 3T3; squares, 3T12A. *D*, population essentially diploid; *H*, population essentially heteroploid.

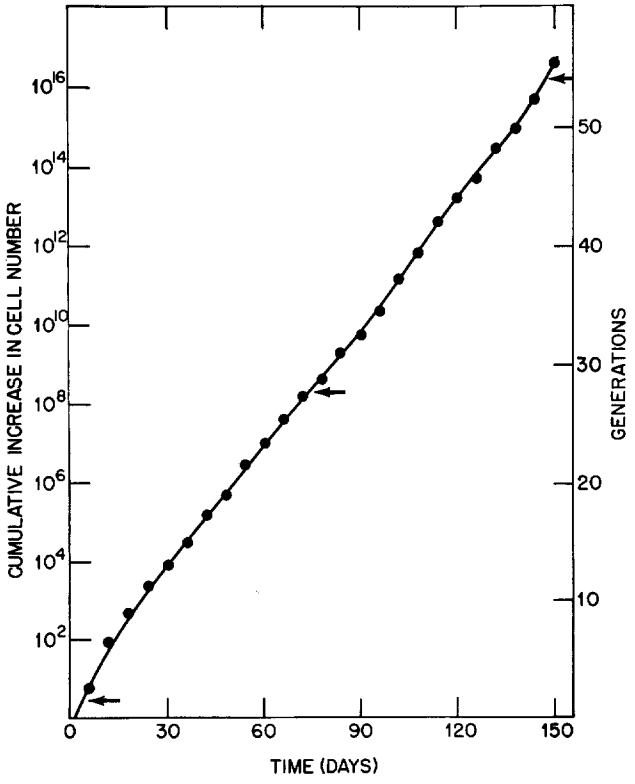

FIGURE 6

Growth of 6T6 upon successive transfer. Arrows indicate times of potential growth rate measurements (see text and Table I).

curve A). In some cases the upward turn in growth rate occurred sharply (Fig. 4, curves A and C) and in others more slowly (curve B).

Cells maintained on the 6-day transfer regime also gave rise to established cell lines. While in the case of the 3-day transfers the phases of falling and rising growth rate are obvious from the curves described, the phenomenon is not so clearly shown by the cultures maintained on 6-day transfer (Fig. 6). The growth rate of 6T6 declined only slightly following the beginning of culture, the doubling time (T_d) rising from 45 hours to 63 hours at 28 generations, and this difference might

TABLE I

Actual vs. Potential Growth Rate

Strain	No. of generations in culture	Growth rate			
		Actual		Potential*	
		N_2/N_0	T_d(hr.)	N_2/N_0	T_d(hr.)
6T6	2	2.1‡	45	5.1	20
	28	1.7‡	63	1.9	52
	54	2.1‡	45	3.8	25
	116	2.4‡	38	6.3	18
3T12A	2	4.0	24	5.1	19
	23	1.4	99	1.3	128
	35	1.7	63	2.0	48
	51	2.0	48	2.8	33
	70	3.6	26	4.6	22

* Inoculation density, 6×10^5 cells.

‡ Calculated from the total growth over the 5-day interval.

be regarded as of doubtful significance. However, during most of the time these cells were studied, their growth rate was reduced by the cell crowding which occurred during the last half of each transfer interval. At a cell inoculation density of 6×10^5 per plate, the maximum rate of cell growth occurs during the 2nd and 3rd days after inoculation. The growth rate during this interval represents the potential growth rate of which the cells are capable under optimal conditions, and is appreciably higher than the average rate of growth over longer intervals or at higher inoculation densities.

If one examines the potential growth rate of cells taken from the 6T6 line at different times, it is clear (Table I) that while the actual growth rate declined only slightly between the 2nd and 28th generation, the potential growth rate declined markedly, with the T_d rising from 20 hours to 52 hours at 28 generations. At the latter time, the actual and potential rates virtually coincided, so that the cells were growing at close to their maximal rate through the entire 5-day growth interval. By 54 generations (144 days) the potential growth rate was found to have increased considerably (T_d = 25 hours) while the actual growth rate rose relatively little (T_d = 45 hours). It is therefore clear that while the cells on the slower transfer regime might appear to be maintaining an almost constant growth rate, in fact they do lose growth potential, but this is concealed by the transfer regime, which does not allow its expression. The subsequent rise in potential growth rate indicates establishment of the line. The potential and actual growth rates of 3T12A (Fig. 5), a line maintained on a 3-day transfer regime, are also shown in Table I. The pattern of development is the same for the two transfer schedules.

Growth Properties of the Established Lines

Ability to Grow at a Low Inoculation Density

The established cells have a much greater ability to grow at low cell density. Reduced dependence on cell feeding is characteristic of all cell lines tested and serves to separate them as a class from normal cells. Fig. 7 shows a representative example of 3T12A, tested at different times: as a secondary culture, during the phase of slowest potential growth, and then again after establishment at 67 and 107 generations. The cells at 24 generations grew more slowly at all densities than did cells at 2 generations and were similarly unable to grow substantially at inoculation densities of 5×10^4 cells per plate or less. These were as yet unestablished diploid cells. The established cells that subsequently emerged showed a progressively improving capacity to function as independent organisms by acquiring ever greater ability to grow in the absence of cell-to-cell feeding. The 3T12A cells, in their entire *in vitro* life, had never been at a density less than 10^6 cells per plate and were therefore not selected for growth at low density; yet, when tested after 67 generations *in vitro*, they could grow at almost 50 per cent of the maximal rate at 3×10^4 cells per plate, and after 107 generations they could grow maximally at this density.

Maximum Cell Number in Crowded Culture

Secondary mouse cultures allowed to get as crowded as possible reached densities of 5 to 6×10^6 cells per plate, but on subsequent transfers this saturation density would fall to 2 to 3×10^6, only to rise again after establishment. The established cells showed a progressive increase in saturation density; some established lines reached densities of 10^7 cells per plate in cultures maintained for 10 to 14 days with frequent medium changes. The increased saturation density of the cell lines reflects both the increased growth rate and the ability of the cells to grow over one another and form multilayers, the latter suggesting changes in cell surface properties that are known to be associated with malignant properties *in vivo* (13).

A striking exception to this pattern of growth is shown by line 3T3, established by other criteria, but virtually unable to grow once a confluent state is reached. Fig. 8 shows the results of an experiment in which 3T3 and 3T12 cells were plated on replicate plates at 3×10^5 and 12×10^5 cells per

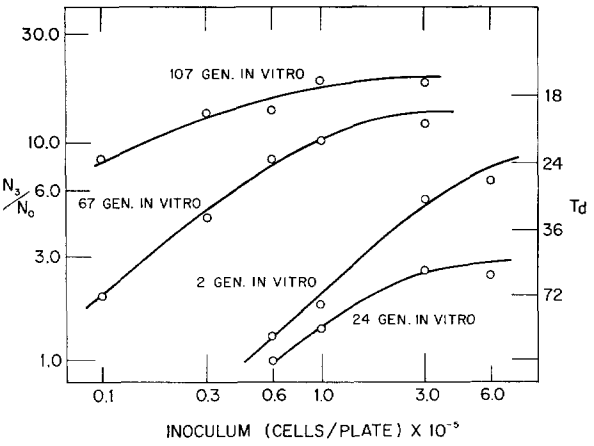

FIGURE 7

Relation between inoculation size and growth rate of mouse fibroblasts at different times in the course of formation of the established cell line 3T12A. T_d, doubling time.

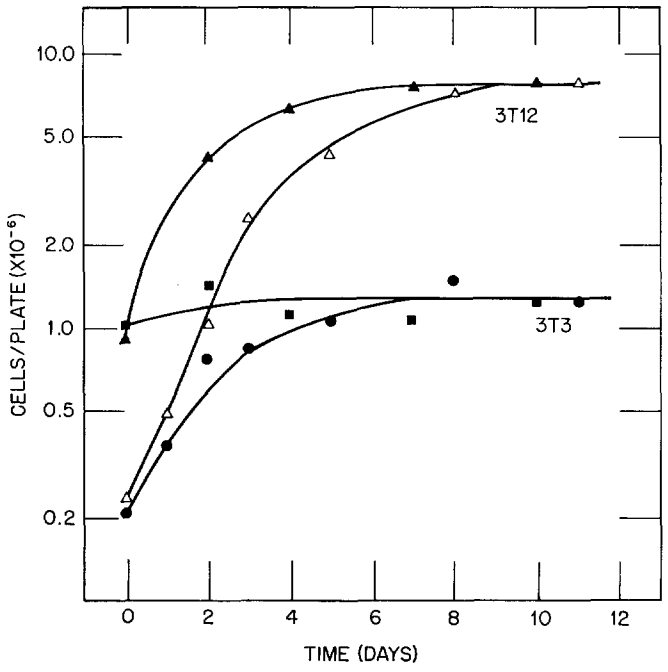

FIGURE 8

Growth of two established lines, 3T12 and 3T3. Solid triangles and squares, inoculum 1.2×10^6 cells; open triangles and solid circles, inoculum 0.3×10^6 cells. The saturation density is independent of the inoculum size for both lines.

plate and the medium changed the following day, and every other day thereafter. Duplicate plates of each line were counted for each time point. 3T3 cells and 3T12 cells when plated at 3×10^5 grew equally well at first, with a doubling time of about 24 hours. When 3T3 reached a density of close to 10^6 cells per plate all growth ceased, while 3T12 continued to grow to a saturation density of about 7×10^6 cells, over 6 times higher than that of 3T3. With larger inocula (12×10^5 cells) the final saturation densities were not changed. In the case of 3T3, over 10^6 cells attached but no growth occurred, as the saturation density was already attained. These cells could be kept with frequent medium change for a month with no increase in cell number; yet they appeared fully viable on replating at low densities. 3T12 inoculated at 12×10^5 cells began to grow at maximal rate, and reached the same density as previously. These differences in the behavior of the two cell lines are not to be accounted for by differences in cell volume. Mean cell diameter of trypsinized suspensions was 16.8 μ for 3T3, and 17.9 μ for 3T12. It appears, therefore, that the growth properties (and morphology, see below) of the emerging established line may be very different according to the culture conditions employed during and after the process of establishment.

Growth of Cells under Agar

The rate of normal embryo cell growth under agar is reduced, since the application of the agar inhibits growth out of the plane of the monolayer (14). Fig. 9 shows the growth curve for cells maintained on a 6-day transfer regime under agar. Their growth rate was reduced throughout the entire period, and especially after 60 days under agar. The application of agar was terminated in some cultures at 60 days, in others at 96 days, and in still others as late as 150 days, by which time cell growth was practically arrested ($N_2/N_0 = 1.3$). In spite of the repression of growth by the agar, the first two of these lines had become established by 150 days of culture, as indicated by study of their

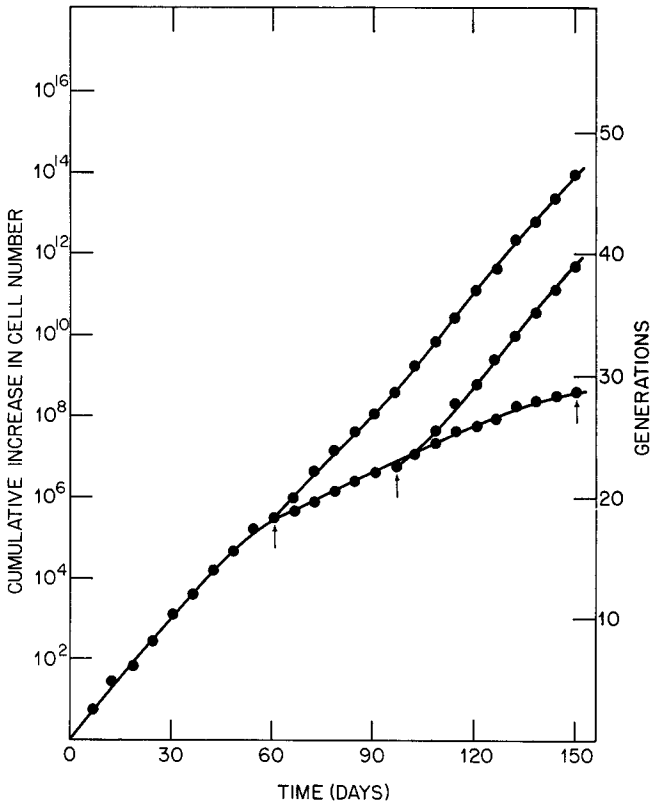

Figure 9

Growth of 6T6 under agar (6-day transfer, inoculum 6×10^5 cells per plate). Time of release of cells from agar inhibition indicated by arrows.

potential growth rate. The cells maintained under agar for the entire interval were not established at this time, but subsequently they too became established.

Morphology and Growth Pattern of the Established Lines

After the upturn in growth rate the cells in most experiments could be seen to become slightly more refractile and less firmly attached to the Petri dish substrate. They tended to appear less fusiform in shape, and in crowded cultures there was less parallel orientation of the cells and more cell interlacing and overgrowth, suggestive of the loss to some degree of contact inhibition (20). Nevertheless layered membrane formation did occur in cultures left without frequent transfer. As the growth rate increased, the cell size, as seen in the counting chamber, became notably smaller. All the changes that occurred were gradual and were not readily apparent until long after the establishment was detected by the upturn in growth rate. In no case did morphological variants arise as a locus of cells differing markedly from the parents.

The cells of line 3T3 differed in appearance from the other lines. In sparse culture, they also looked fibroblastic but grew considerably flatter, appeared finely granular, and were more difficult to trypsinize. In confluent cultures cell borders were obscured and a thin syncytium-like sheet formed with no tendency toward multilayering.

Cells maintained under agar also became very flat and epithelium-like. This morphology persisted for months after the removal of agar, but later gradually changed toward a more fibroblastic type.

TABLE II

Chromosome Alterations during the Process of Establishment

Cell strain	Transfer	Generation	Diploid cells		N_2/N_0
			Counted	Percentage	
3T3	2	2	93/100	(93)	5.0
	5	10	23/25	(92)	2.9
	10	12	—	—	1.2
	15	14	—	—	1.2
	18	16	—	—	1.9
	20	18	79/100	(79)	2.4
	22	20	3/72	(4)	2.9
	25	24	0/50	(0)	3.8
3T6	5	11	77/81	(95)	4.3
	16	21	46/50	(92)	2.1
	21	25	—	—	1.4
	24	28	50/56	(89)	1.7
	27	31	46/52	(87)	2.3
	30	35	24/50	(48)	2.5
	32	39	4/74	(6)	4.0
3T12	2	2	93/100	(93)	5.3
	5	10	45/50	(90)	3.2
	7	13	67/82	(82)	3.0
	12	18	—	—	1.5
	14	19	—	—	1.6
	16	21	41/50	(82)	2.3
	18	23	89/100	(89)	3.0
	20	26	11/50	(22)	3.0
	22	29	0/67	(0)	3.8

Changes in Chromosome Constitution and the Relation of the Alterations in Karyotype to the Process of Establishment

Levan and Biesele (5) and Rothfels and Parker (15) have demonstrated a relation between changes in karyotype and the development of established mouse lines. Since we were able to detect an established line quite early in its development, it was possible to examine this relation in greater detail.

In Table II it is seen that as early as the second transfer in strains 3T3 and 3T12 there is a significant polyploid element in the population. An average of 8.7 per cent of 527 cells counted in secondary cultures were in the tetraploid range. During the phase of declining growth rate the percentage of tetraploids stays constant or rises only slightly, the population remaining mainly diploid for from 20 generations (3T3) to over 40 generations (6T6). The subsequent increase in the growth rate is accompanied in all cases by a change from a primarily diploid population to one with few, if any, diploid cells. All the lines, after establishment, had the great majority of cells in the hypotetraploid region with considerable variation around a modal number in the seventies. The mode of 6T6 at 45 generations (135 days) was 76, and 3T12 at 34 generations (69 days) had a mode of 78. The early established cells contained no grossly abnormal chromosomes; no metacentrics were seen at this time. At a later time abnormal chromosomes did appear and the number of metacentrics and minute chromosomes progressively increased in the population. 3T12 at 92 generations had in 30 per cent of its cells from one to three metacentrics. A significant minority also had minute chromosomes.

The mode in this case had also shifted from the hypo- to the hypertetraploid region.

Examination of the data indicates that in at least two of the three lines, 3T3 and 3T12, and probably 3T6 as well, the period of rapid change in ploidy occurred *after* the upturn in growth rate. 3T3, for example, after a long period of virtually no growth had, by the 20th transfer, increased its growth rate ($N_2/N_0 = 2.4$); yet the population was still predominantly diploid. Two transfers later, however, well after the start of the upturn in growth rate (Fig. 4, curve A; Fig. 5) the population had become almost entirely heteroploid. Similarly, 3T12 at the 18th transfer (23rd generation), after N_2/N_0 had risen from a low of 1.5 to 3.0, was still 89 per cent diploid. At this time, exact counts on 50 cells in the diploid range showed the great majority of cells to have exactly 40 chromosomes, and the remainder to have the same small variation in chromosome number that is seen in secondary cultures. Two transfers (3 generations) later, the population was largely in the tetraploid range. In 3T3 the change in ploidy occurred between the 18th and the 20th generation, and in 3T6 between the 31st and the 39th generation. This brief period when the mass of the population develops an altered karyotype is not associated with any striking further increase in growth rate.

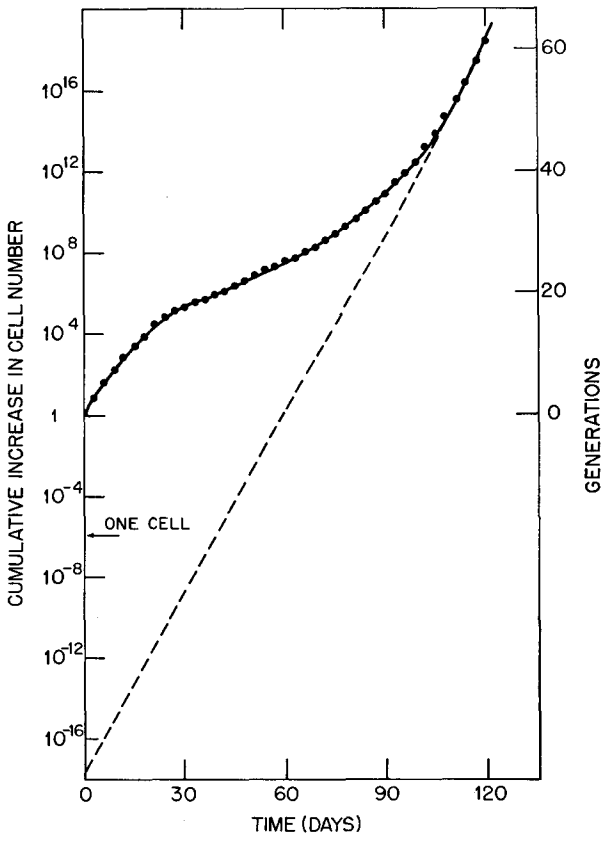

FIGURE 10

The formation of established cell line 3T6.

The change in the cases of 3T3 and 3T12 occurred rapidly enough, within as little as 2 or 3 generations, to make it unlikely that it was the result of selection of a rare heteroploid variant. Rather, it is suggestive of conversion of a large fraction of the population to the heteroploid state.

Origin of the Established Line

The time of appearance of the cell type with improved growth properties under the 3-day transfer

regime varies from roughly 45 to 90 days. This population could represent the outgrowth of a small minority of unaltered cells present in the original population and requiring this period of time to become predominant. Alternatively, they could be cells that acquired new properties during the time *in vitro*, giving them selective advantage. The simple case of the first alternative can be eliminated by extrapolating the final growth curve of 3T6 back to zero time (Fig. 10), whereupon one sees that even if just one cell present in the original population were growing at the same rapid rate from the beginning, it would have taken over the population at a much earlier time.[2]

The growth rates of the established cells, after they have emerged, progressively increase and continue to do so long after the diploid elements are no longer present in the population. 3T6, after 96 days, is almost completely non-diploid. In the subsequent 8 transfers the generation time decreased from 29 to 15 hours and morphological and karyotypic changes also continued to occur. The cells of the long established line are different genetically and metabolically from both the original euploid cells and the early established cells.

DISCUSSION

The development in cell culture of established lines from normal cell populations has in most cases been considered a rather infrequent and unpredictable occurrence (1, 2), perhaps like the development of a "spontaneous" tumor in the animal. The results presented here show that it is possible to produce established lines from mouse embryo cells with a high degree of probability (9 out of 11 cultures initiated), most of them within 3 months of culture.

Established lines and normal cell strains have been distinguished on the basis of a number of criteria by Hayflick and Moorhead (2). Perhaps the most essential of these is the capacity for unlimited growth, possessed by established cell lines, but not by cell strains. This difference, while quite significant as a matter of experience, is difficult to employ practically as a distinguishing criterion. The assumption is usually made that if cells have been in continuous culture for many months or more than a year, they probably constitute an established line (16, 17). Billen and Debrunner (18) have routinely maintained mouse bone marrow cells in serial transfer from 6 months to a year and assumed that they were established. On the other hand, it is possible for cells to go through over 50 generations and still not be established (2, 19).

The experiments presented here provide a relatively precise criterion for establishment, in terms of growth capacity. In all cases when cells were placed in culture they showed at first a progressively declining potential growth rate, with the doubling time rising to over 60 hours. Thereafter, the ability of the cells to maintain a rising growth rate, *under conditions where the maximum growth rate could be expressed*, constituted the first indication of establishment. This occurred as early as 30 to 45 days of culture in some of the 3-day transfer experiments (Fig. 4) but required a longer time in the 6-day transfer experiments. This may be due, at least in part, to the more rapid rate of growth under the 3-day regime. Cell lines which were found to be established by this criterion never died out subsequently, and always began a series of evolutionary changes expressed in a variety of new properties.

Under the conditions of our experiments, a change involving the development of new properties appears to be necessary in order that the cells continue to grow indefinitely *in vitro*. That this is not simply a population change resulting from selective overgrowth of cell types whose properties were fixed since the beginning of the culture is strongly suggested by the experiment of Fig. 10. New growth properties must have been developed by some cells during the first 15 to 30 generations of culture. Whether a large or only small proportion of the original population was capable of undergoing this change cannot be decided from these experiments.

Following establishment, a further rise in growth rate always occurred, the doubling time reaching values equal to or less than that of the initial cell

[2] It could be argued that the cells which finally take over the population were indeed the unchanged progeny of a small number of cells present from the beginning, but that their growth rate was repressed by the other cells while the latter constituted the majority of the population. Experiments in which the cells of the established 3T6 were mixed with 10^2 or 10^4 times their number of secondary embryo cells showed, however, that there was no inhibition at all of the growth of the 3T6 cells under these conditions.

population, usually by 45 generations of *in vitro* life. In some cases, evolution continued until doubling times of less than 14 hours were reached (line 3T6).

Cell morphology of the early established lines was virtually indistinguishable from that of normal fibroblasts. In crowded cultures (with the exception of 3T3) the cells became progressively more able to form multilayers and interlace with one another. With time, saturation density increased, indicating a progressive loss of the contact inhibition characteristic of normal cells.

The particular conditions under which cells are cultivated influence the properties of the emerging cell type, as seen from examining line 3T3 and, to a lesser degree, those cell lines maintained under agar for many months. The cells of line 3T3 were always plated at a relatively low cell density and were transferred frequently so that they never were allowed to become confluent. There was little or no cell-to-cell contact prior to establishment. The resultant line, established by all other criteria and grossly abnormal in karyotype, remained extremely sensitive to homologous contact inhibition and ceased growing completely as the culture reached confluence. Its final saturation density was less than one-fifth that of the other established lines. Contact inhibition (20) is a property of normal cells lost to some degree both by malignant and by most established cells. The strikingly different behavior of 3T3, as compared with the other established lines, may reflect the peculiar conditions of its establishment. The loss of contact inhibition and, perhaps, the malignant properties of many established lines may be the result of the selective processes usually operating in cell culture and not related to the process of establishment *per se*.

The karyotypes which develop when mouse cells become established in culture have been studied in detail by Levan and Biesele (5), by Rothfels and Parker (15), and by Hsu *et al.* (21), and the changes found in our experiments were similar to those described by these investigators: first a change of chromosome number to the tetraploid and hypotetraploid range, and, later, the gradual development of grossly abnormal chromosomes. However, in our experiments, wherever the relation between growth rate and chromosomal changes was closely examined it was found that the population began to grow more rapidly before the chromosome number shifted. It therefore appears that the drastic karyotypic alterations characteristic of established mouse lines are not essential for establishment, or at least its initial phase. When the shift to heteroploidy did occur it took place very rapidly, during an interval in which the growth rate was not changing very much, suggesting that the shift was not produced by selection of a very small rapidly growing heteroploid minority of cells, but rather by a conversion of a large fraction of the diploid or quasidiploid population.

In the mouse, long established cell lines have been found invariably to be markedly heteroploid (22). In other species, however, such as the hamster (23–25) and the pig (26), establishment need not be followed by such drastic changes in the chromosome constitution. The mouse, it has been suggested, may have an inherently unstable karyotype. To determine whether this instability makes establishment of lines easier than for cells of other species would require systematic efforts to produce established lines in a variety of species.

One of the cellular properties that emerges very soon and very strongly after the initial phase of establishment is the ability to grow at low inoculation density. The relative inability of freshly cultured cells to do so is an important factor in the progressive reduction in their growth rate upon successive transfer (Fig. 2). No improvement takes place unless establishment occurs, whereupon, as the potential growth rate rises, the ability to grow at low density also improves. This represents an increased ability to function as independent organisms (a highly unnatural condition for normal mammalian cells). Eagle and Piez (27) have demonstrated that even established lines, at lowered population density, demonstrate growth factor requirements for substances the cells are capable of making. The reason for this is believed to be that the synthetic processes of the cells are unable to keep up with the leakage of these substances to the medium. However incomplete the autonomy of established cells, it is considerably more developed than that of non-established strains.

Trypsinization, because it increases the leakiness of cells to small molecules (28, 29), may be expected to aggravate this condition. It may be that the process of establishment involves a reduction of the normal leakiness of the cell for metabolites to a level where cell growth can continue, under the given culture conditions. Further improvement in this capacity can result, by mutation-selection or

by some form of adaptation. The end result, at any rate, is a cell which can grow at very low inoculation density and therefore gives a relatively high plating efficiency when tested by conventional methods. Experiments (24) comparing the relative capacity of established mouse lines and normal mouse strains to act as feeders for normal cells suggest that part of the adjustment made by the established cell is, in fact, an increased ability to control the rate of loss of intermediates. (See also reference 30.)

Inability to become established seems to be more common in the case of cells of certain species other than the mouse. Normal human fibroblasts, for example, are thought to undergo establishment very rarely, if at all, and Hayflick and Moorhead (2) have suggested that the finite *in vitro* lifetime of such cells may reflect an aging process expressed at the cellular level, because of the loss of some factor necessary for cell survival, or because its rate of duplication is less than that of the cell. All cell densities used in culture are orders of magnitude below those *in vivo* (27) and it might be that all cell densities technically feasible in culture will be suboptimal with respect to cell-to-cell feeding, and lead to the depletion of substances needed for cell division. In our experiments, the rate of decline of potential growth rate and total number of generations the cells went through before establishment occurred was rather constant for the particular cell type maintained under a particular set of conditions. However, the fact that the inoculation density used, where all other conditions are identical, directly affects the decline of growth rate and the number of generations grown shows that the latter is not a fixed property of these cells but can be greatly modified by culture conditions. Whether the ever increasing generation time displayed by cells which fail to establish in culture reflects a fundamental property of the normal cell, or rather these inherent difficulties in the *in vitro* system, has not been resolved.

If methods were devised to make the cell:medium ratio comparable to that *in vivo*, or to reduce the rate of loss of metabolic intermediates, it might well be possible to maintain diploid cells at their maximal potential growth rate for much longer periods of time, perhaps indefinitely. In this case the modifications of cell properties which lead to establishment, if adaptive in nature, need not occur; if mutational, they need not be selected.

Some of the results described in this paper are in close agreement with those of similar experiments recently reported by Rothfels *et al.* (30). In addition, these investigators showed that under their conditions most established lines developed neoplastic properties over the course of long term serial culture. Various factors involved in cell feeding were also extensively investigated, and differences were found in the capacity of different cell types to act as feeders for normal embryo cells.

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BIBLIOGRAPHY

1. HAFF, R. F., and SWIM, H. E., Serial propagation of 3 strains of rabbit fibroblasts, *Proc. Soc. Exp. Biol. and Med.*, 1956, **93**, 200.
2. HAYFLICK, L., and MOORHEAD, P. S., The serial cultivation of human diploid cell strains, *Exp. Cell Research*, 1961, **25**, 585.
3. EARLE, W. R., and NETTLESHIP, A., Production of malignancy *in vitro*. V. Results of injections of cultures into mice, *J. Nat. Cancer Inst.*, 1943, **4**, 213.
4. GEY, G. O., Cytological and cultural observations on transplantable rat sarcomata produced by inoculation of altered normal cells maintained in continuous culture, *Cancer Research*, 1941, **1**, 737.
5. LEVAN, A., and BIESELE, J. J., Role of chromosomes in cancerogenesis, as studied in serial tissue culture of mammalian cells, *Ann. New York Acad. Sc.*, 1958, **71**, 1022.
6. EAGLE, H., OYAMA, V. I., LEVY, M., and FREEMAN, A. E., Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture, *J. Biol. Chem.*, 1957, **226**, 191.
7. DULBECCO, R., and VOGT, M., Plaque formation and isolation of pure lines with poliomyelitis virus, *J. Exp. Med.*, 1954, **99**, 167.
8. HASTINGS, J., FREEDMAN, S., RENDOW, O., COOPER, H. L., and HIRSCHHORN, K., Culture of human white cells using differential leucocyte separation, *Nature*, 1961, **192**, 1214.
9. SANFORD, K. K., EARLE, W. R., and LIKELY, G. D., The growth *in vitro* of single isolated tissue cells, *J. Nat. Cancer Inst.*, 1948, **9**, 229.
10. EARLE, W. R., SANFORD, K. K., EVANS, V. J.,

WALTZ, H. K., and SHANNON, J. E., Influence of inoculum size on proliferation in tissue cultures, *J. Nat. Cancer Inst.*, 1951, **12,** 133.

11. FISCHER, H. W., and PUCK, T. T., On the functions of x-irradiated "feeder" cells in supporting single mammalian cell growth, *Proc. Nat. Acad. Sc.*, 1956, **42,** 900.
12. MOSER, H., Modern approaches to the study of mammalian cells in culture, *Experientia*, 1960, **16,** 385.
13. VOGT, M., and DULBECCO, R., Virus-cell interaction with a tumor producing virus, *Proc. Nat. Acad. Sc.*, 1960, **46,** 365.
14. GREEN, H., and NILAUSEN, K., Repression of growth of mammalian cells under agar, *Nature*, 1962, **194,** 406.
15. ROTHFELS, K., and PARKER, R. C., The karyotypes of cell lines recently established from normal mouse tissue, *J. Exp. Zool.*, 1959, **142,** 507.
16. CLAUSEN, J. J., and SYVERTON, J. T., Comparative chromosomal study of 31 cultured mammalian cell lines, *J. Nat. Cancer Inst.*, 1962, **28,** 1, 117.
17. FOLEY, G. E., DROLET, B. P., MCCARTHY, R. E., GOULET, K. A., DOKOS, J. M., and FILLER, D. A., Isolation and serial propagation of malignant and normal cells in semi-defined media, *Cancer Research*, 1960, **20,** 930.
18. BILLEN, D., and DEBRUNNER, G. A., Continuously propagating cells derived from the normal mouse bone marrow, *J. Nat. Cancer Inst.*, 1960, **25,** 1127.
19. PUCK, T. T., CIECIURA, S. J., and ROBINSON, A., Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects, *J. Exp. Med.*, 1958, **108,** 945.
20. ABERCROMBIE, M., The bases of the locomotory behavior of fibroblasts, *Exp. Cell Research*, 1961, Suppl. 8, 188.
21. HSU, T. C., BILLEN, D., and LEVAN, A., Mammalian chromosomes *in vitro*. XV. Patterns of transformation, *J. Nat. Cancer Inst.*, 1961, **27,** 515.
22. HSU, T. C., Chromosomal evolution in cell populations, *Internat. Rev. Cytol.*, 1961, **12,** 69.
23. YERGANIAN, G., and LEONARD, M. J., Maintenance of normal *in situ* chromosomal features in long-term tissue cultures, *Science*, 1961, **133,** 1600.
24. MACPHERSON, I., and STOKER, M., Polyoma transformation of hamster cell clones—An investigation of genetic factors affecting cell competence, *Virology*, 1962, **16,** 147.
25. TODARO, G. J., and GREEN, H., unpublished observations.
26. RUDDLE, F. H., Chromosome variation in cell populations derived from pig kidney, *Cancer Research*, 1961, **21,** 885.
27. EAGLE, H., and PIEZ, K., Population dependent requirements by cultured mammalian cells for metabolites they can synthesize, *J. Exp. Med.*, 1962, **116,** 29.
28. PUCK, T. T., MARCUS, P. I., and CIECIURA, S. J., Clonal growth of mammalian cells *in vitro*: Growth characteristics of colonies from single HeLa cells with and without a "feeder" layer, *J. Exp. Med.*, 1956, **103,** 273.
29. LEVINE, S., Effect of manipulation on P^{32} loss from tissue culture cells, *Exp. Cell Research*, 1960, **19,** 220.
30. ROTHFELS, K. H., KUPELWIESER, E. B., and PARKER, R. C., Effects of x-irradiated feeder layers on mitotic activity and development of aneuploidy in mouse embryo cells in *vitro*, *in* Fifth Canadian Cancer Congress (Honey Harbor), New York, Academic Press, Inc., 1963.