A METHOD OF SELECTING FOR AUXOTROPHIC MUTANTS OF HeLa CELLS

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Plate 47

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The experiments described here suggest a method of selecting for auxotrophic mutants[1] in cultures of human or other types of cells. They are based mainly on three findings made by others:

(*a*) A variety of cultivated mammalian cells are unable to proliferate in growth media containing aminopterin or amethopterin (1).

(*b*) The growth-inhibitory effects of amethopterin can be completely reversed with a mixture of adenine, thymidine, and glycine (2, 3).

(*c*) Bacteria that are unable to synthesize thymine, either because of a mutation or because of treatment with sulfanilamide, lose viability when in a thymine-free medium that otherwise contains all factors necessary for growth. In contrast, if the thymine-free medium also lacks one other factor ordinarily necessary for growth, the bacteria remain viable (4). This preferential survival of thymine-starved cells that are also deprived of an additional growth factor should permit their selection from large populations of thymine-starved cells that do not ordinarily require the additional growth factor.

The general plan of this work was to create a thymine deficiency in HeLa cells (5) with the use of aminopterin and to compare the survival of cells in a growth-supporting medium with that of cells in a medium deficient in a single essential nutrient.

Methods and Materials

Media.—Stock cultures were maintained with Eagle's medium (7) supplemented with 5 per cent undialyzed human serum. All experimental media contained, instead, 5 per cent human serum that was dialyzed 24 hours against cold running tap water and 4 hours against cold running distilled water. Except when indicated, media contained 2×10^{-3} M L-glutamine. When glutamine was replaced with 2×10^{-2} M monosodium *L*-glutamate (Experiments 5 and 6) the concentration of NaCl was reduced by the same amount. In Experiment 4 certain media contained 10^{-4} M L-citrulline in place of arginine.

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[1] An auxotrophic mutant is considered here to be a genetically altered cell having at least one more nutritional requirement than the cell type from which it was derived.

Aminopterin (AP), obtained from Lederle Laboratories, was sterilized by filtration and stored frozen.

Cell Strains.—HeLa I-11: a clonal population that can initiate and maintain growth indefinitely when transferred from glutamine-containing medium to medium in which glutamic acid replaces glutamine.

HeLa S3-1: a clonal population isolated at random from HeLa S3 (6) by Dr. Royce Lockart. It is unable to initiate growth when transferred to medium in which glutamic acid replaces glutamine. Experiments 1 to 5 were done with this strain alone.

Growth Experiments.—Cells were grown adherent to glass. Inocula for experimental cultures were prepared by removing cells from the glass of stock cultures with a versene solution (8). Replicate aliquots (about 5×10^4 cells each) of the resultant cell suspension were inoculated into T15 culture flasks. After 2 days, permitting about a twofold increase in cell protein, experimental media were added. Fresh experimental media were provided on days 2, 4, and 5, and then every 2nd day when experiments lasted longer than 6 days. When cell populations were less than about 5×10^5 per T15 the cultures received enough CO_2 at each feeding to adjust the pH to about 7.4. No CO_2 was added at higher cell densities. At intervals, duplicate flasks of each experimental series were removed for growth determinations.

Experiments 2 to 5 had the same basic format. The growth of cells in various experimental media was determined during the first portion of the experiment. All experimental media were then removed from the remaining cultures and were replaced with Eagle's medium containing 5 per cent dialyzed human serum. Determinations of the subsequent growth of the cultures gave some measure of the ability of the cells to resume growth after sojourn in the various experimental media employed during the first portion of the experiment.

Measurement of Growth.—Growth was usually expressed in terms of the protein content of the cultures. Protein determinations were made according to Oyama and Eagle (9). Each value cited is the average of duplicate determinations of duplicate flasks. Duplicate flasks agreed within ±10 per cent of the average.

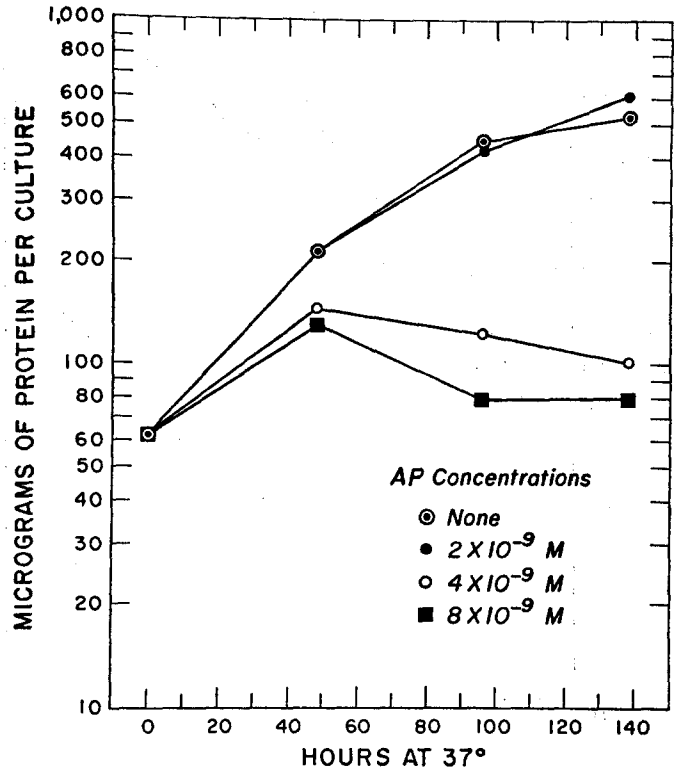
In some cases the numbers of cells in the cultures were determined by removing the cells from the glass with versene and counting them with a hemocytometer. At least 400 cells from each flask were counted and duplicate flasks usually agreed within ±20 per cent of their average count.

RESULTS

Experiment 1. Growth of HeLa Cells as a Function of AP Concentration.—The object of this experiment was to determine the minimum concentration at which AP would inhibit cell growth in Eagle's medium. Text-fig. 1 shows that at AP concentrations of 2×10^{-9} M and lower (not shown) there was no detectable inhibition of growth. At AP concentrations of 4×10^{-9} M and higher there was an initial doubling in the protein content of the cultures followed by a slow decline. The curves for concentrations of 8×10^{-9} M and higher (not shown) are indistinguishable from each other. The minimum inhibitory concentration found here (4×10^{-9} M) agrees well with that previously reported (1).

Experiment 2. The Ability of Cells to Resume Growth after Exposure to AP.—This experiment was an attempt to determine if the growth-inhibitory effect of AP, described in Experiment 1, is a transient one or if it leads to a permanent loss of growth ability. Text-fig. 2 shows that after 133 hours of exposure to AP in Eagle's medium, HeLa cells failed to show any measurable ability to grow

during a 190 hour recovery period in AP-free medium. A 40 hour exposure to AP failed to affect the ability of the cells to grow after its removal while an 87 hour exposure prevented a measurable increase in the protein content of the cultures for approximately 100 hours after the removal of AP.



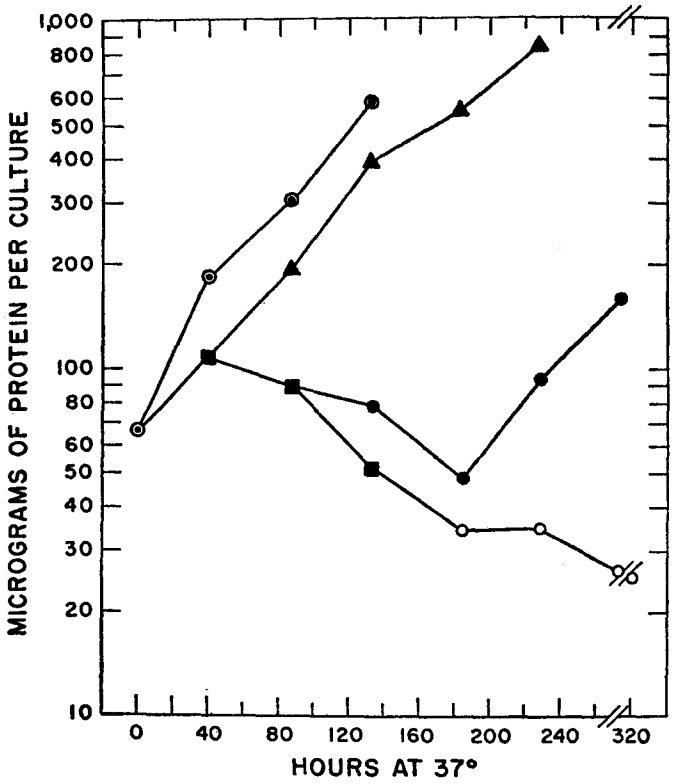
TEXT-FIG. 1. (Experiment 1) Growth of HeLa cells as a function of the concentration of AP. Replicate cultures of HeLa S3-1 were grown in Eagle's medium containing AP at the indicated concentrations. Growth was determined by protein determinations on duplicate flasks.

In this experiment the ability of the cells to recover from AP treatment was crudely expressed in terms of protein determinations, which give little information about the number of cells that were actually growing. The experiments cited below show that this measure is a usable rough index of the number of viable cells in a culture.

Observation of cultures treated with AP for 5 or more days reveals a few patches of proliferating cells. If these originate from single cells they represent 10^{-5} to 10^{-6} of the original population. Many of these survivors are mutants that are resistant to AP. With the exception of such cells, this

experiment shows that AP does not merely inhibit the growth of HeLa cells, but eventually destroys their ability to grow.

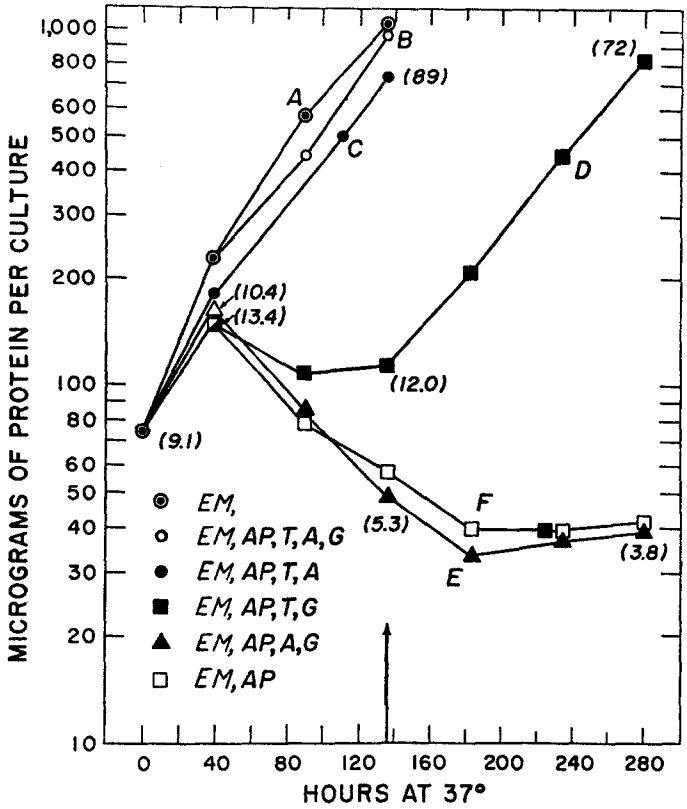
Experiment 3. Reversal of AP-Action and Evidence for Thymineless Death.— Hakala (2, 3) showed that a mixture of thymidine, adenine, and glycine could



TEXT-FIG. 2. (Experiment 2) The ability of cells to resume growth after exposure to AP. HeLa S3-1 was cultivated in an AP-free medium (◉) or in medium containing 10^{-8} M AP (■). After 40 hours (▲), 87 hours (●), and 133 hours (○) of exposure to AP, sets of cultures were given AP-free Eagle's medium in order to permit the growth of surviving cells. Growth is expressed in terms of the protein content of the cultures.

overcome the growth-inhibiting effect of amethopterin. Text-fig. 3 (curves A and B) shows that these compounds also reverse the effect of AP on HeLa cells. Moreover, a comparison of curves E and F shows that it is the thymine deficiency created by AP that leads to its characteristic effects; *i.e.*, an initial doubling in the protein content of the cultures followed by a slow decline and inability to resume growth after the removal of AP. In contrast, HeLa cells in a medium containing AP, adenine, and thymidine, but no glycine, (curve C) grew almost as well as untreated control cultures. When adenine alone was

omitted in the presence of AP (curve D) there was an initial doubling in protein followed by a slight decline. Removal of AP after 136 hours allowed growth to



TEXT-FIG. 3. (Experiment 3) The reversal of AP action and evidence for thymineless death. HeLa S3-1 was cultivated in the media indicated in the figure. *EM*, Eagle's medium. *AP*, aminopterin, 10^{-8} M. *T*, thymidine, 3×10^{-5} M. *A*, adenine, 3×10^{-5} M. *G*, glycine, 10^{-} M. Starting at 134 hours (arrow) all remaining cultures were fed AP-free Eagle's medium in order to determine the residual growth ability of cells treated with the experimental media used during the first 134 hours of the experiment.

Growth is expressed in terms of the protein content of the cultures or in terms of cell number. The numbers in parentheses represent the number of cells ($\times 10^{-4}$) per culture at the indicated points.

resume without a lag, indicating that most of the cells had retained the ability to grow.

In this experiment the protein determinations were supplemented with cell counts at selected times. The omission of glycine (curve C) permitted a tenfold increase in cell number that corresponds to the increased protein content of the cultures. When adenine was omitted (curve D) the cell number increased

50 per cent during the first 48 hours and then remained almost constant until AP was removed. Most of these cells were viable as indicated by the sixfold increase in cell number during the recovery period. Omission of thymidine (curve E) permitted an initial 15 per cent increase in cell number and the subsequent decline in cell number continued after the removal of AP.

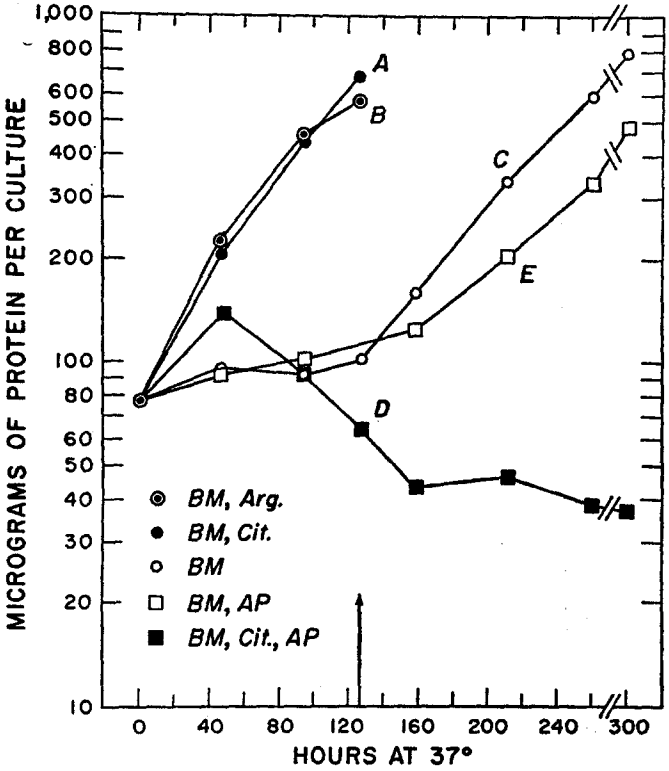
The persistence of countable cells on the glass explains in part the residual protein found during the recovery period of such experiments. Microscopic examination of the cells reveals a characteristic sequence of morphological changes in the thymine-starved cells that is not observed in untreated control cells (Fig. 2 *a*). They swell initially (Fig. 2 *b*), as suggested by the noncommensurate increases in cell protein and cell number. They then become distorted (Fig. 2 c) and detach from the glass. After AP is removed some of the cells swell still further (see Experiment 5) so that protein measurements become the resultant of loss due to detaching cells and continuing protein synthesis by some of the cells that remain.

In every experiment that we have performed with AP a large increase in protein without a corresponding increase in cell numbers has been accompanied by extensive cell loss. The following experiments show that cells tend to survive AP treatment when this disparate increase is prevented by a nutritional insufficiency of the medium.

Experiment 4. The Effect of AP on Cells Deprived of Arginine.—HeLa cells require arginine for growth (10). Citrulline supports growth as well as does arginine (11), indicating that the cells can effect the enzymatic conversion of citrulline to arginine. In this experiment the ability of cells to resume growth after exposure to AP in a medium in which citrulline was the sole source of arginine was compared with that of cells that were treated with AP in the absence of both citrulline and arginine. Comparison of curves C and E (Text-fig. 4) shows that cells completely deprived of arginine, whether AP was present (E) or absent (C), behaved similarly; they grew very little, failed to swell, and resumed growth when AP was removed and arginine provided. This indicates that cells that are unable to grow because of an amino acid deficiency are not subject to the lethal effects of AP. In contrast, cells treated with AP in a medium containing citrulline as arginine source (curve D) underwent typical swelling and distortion, and failed to resume growth when AP was removed. Presumably, mutants unable to use citrulline as an arginine source would tend to survive sojourn in medium containing citrulline and AP.

Experiment 5. The Effect of AP on Cells Deprived of Glutamine.—The glutamine requirement of HeLa I-11 cells can be satisfied with glutamic acid (12). These cells die in a medium containing glutamic acid and AP but survive AP treatment when both glutamine and glutamic acid are absent. The behavior of HeLa S3-1 is in contrast to this, as described in Text-fig. 5. Curve B shows that this strain does not initiate growth in a glutamic acid-containing medium that supports good growth of HeLa I-11. In such a glutamine-free medium, HeLa

S3-1 cells behaved similarly whether AP was present (curve C) or absent (curve B). The cultures declined in both protein content and cell number, but resumed growth when AP was removed and glutamine provided; *i.e.*, they behaved like cultures of HeLa I-11 that are deprived of both glutamine and



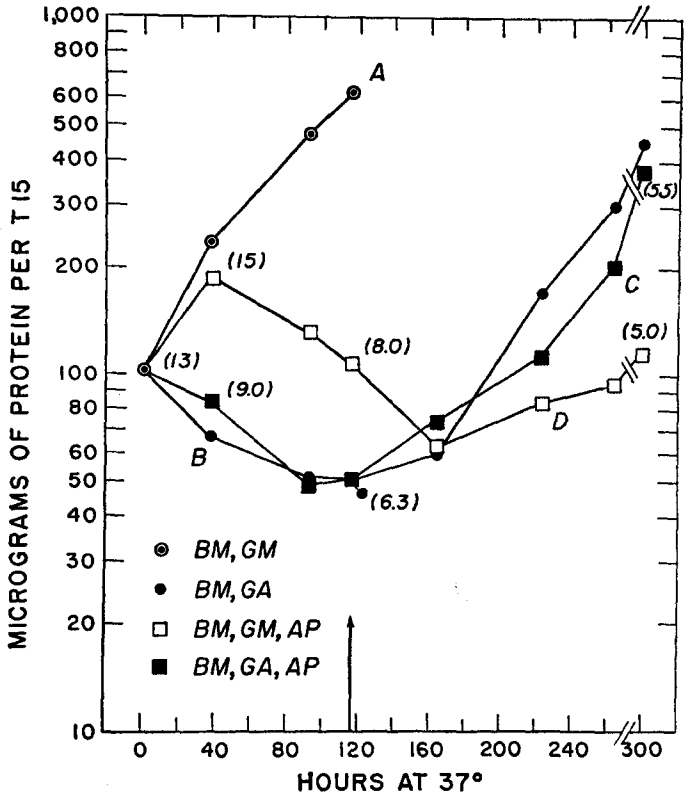
TEXT-FIG. 4. (Experiment 4) The effect of AP on cells deprived of arginine. HeLa S3-1 was cultivated in the media indicated in the figure. *BM*, Eagle's medium minus arginine. *Arg.*, arginine, 10^{-4} M. *Cit.*, citrulline, 10^{-4} M. *AP*, aminopterin, 10^{-8} M. Starting at 127 hours (arrow) all remaining cultures were fed complete Eagle's medium without supplements in order to assess the residual growth ability of the cells. Growth is expressed in terms of the protein content of the cultures.

glutamic acid. Curve A shows that HeLa S3-1 grew at about the same rate as HeLa I-11 in medium containing glutamine and, when this medium contained AP (curve D), the cells showed the usual effects of AP. In this case, the cells were exposed to AP for only 120 hours, permitting more cells than usual to persist. These did not increase in number but did synthesize protein, the average protein content of the cells at the end of the experiment being about tenfold greater than at the beginning.

This result permitted us to test the usefulness of thymine starvation in

selecting for infrequent auxotrophs (HeLa S3-1 type) in a large population of non-auxotrophic (HeLa I-11 type) cells.

Experiment 6. Selection for Glutamine-Requiring Cells.—Petri dishes (60 mm. diameter) were inoculated with a mixture of 5×10^4 HeLa I-11 and 2.5



TEXT-FIG. 5. (Experiment 5) The effect of AP on cells deprived of glutamine. HeLa S3-1 was cultivated in the media indicated in the figure. *BM*, Eagle's medium minus glutamine. *GM*, glutamine, 2×10^{-3} M. *GA*, monosodium glutamate, 2×10^{-2} M. *AP*, aminopterin, 10^{-6} M. Starting at 116 hours (arrow) all remaining cultures were given complete Eagle's medium without supplements in order to assess the residual growth ability of the cells. Growth is expressed in terms of the protein content of the cultures or in terms of cell number. The numbers in parentheses represent the number of cells ($\times 10^{-4}$) per culture at the indicated points.

$\times 10^2$ HeLa S3-1 cells per dish. Control dishes contained only HeLa I-11. These were grown for 2 days in a medium containing glutamine to permit the formation of small clones of HeLa S3-1. On the 3rd day this medium was replaced with one containing glutamic acid in place of glutamine. One day was allowed for the HeLa I-11 cells to start growing in this medium and then AP (2×10^{-6} M) was added. At this time the cultures contained 2.5×10^5 cells

and about 2.5×10^2 small clones of HeLa S3-1 per dish. The medium was replaced every day for 6 days and on the 7th day AP was removed and glutamine was provided. Surviving cells were allowed to proliferate in this medium for 12 days. At this time unseeded control dishes appeared as in Fig. 1 *A* while plates seeded with HeLa S3-1 appeared as in Fig. 1 *B*. The photographs in Fig. 1 are of plates in a second experiment of this kind in which about 500 HeLa S3-1 cells per dish were used. Every seeded plate had many large clumps of cells visible to the naked eye. Twenty-two clumps were picked and propagated into large populations (about 10^6 cells) in medium containing glutamine. Small clumps of viable cells were detected with the microscope in unseeded control plates. Fifteen of these were picked and propagated in glutamine. The 37 populations were then tested for their ability to grow in medium free of glutamine but containing glutamic acid. Nineteen of the cultures derived from seeded plates were unable to grow, one was able to grow, and two were unscorable. Fourteen of the populations derived from the unseeded control plates were able to grow and one could not. The nature of this single glutamine-requiring population (HeLa I-11a) was studied further.

When poliovirus Type I is plated on cell monolayers it forms 5 times as many plaques (average diameter 5 mm.) on HeLa I-11 as it does on HeLa S3-1 (average plaque diameter 2 mm.) (J. Darnell, personal communication). HeLa I-11a responded as did HeLa I-11 when tested in this way, indicating that it probably represented a new isolation of a glutamine-requiring strain rather than a chance contamination of the control plate with HeLa S3-1. Each of 58 clones picked at random derived from HeLa I-11 have been tested and shown to be able to initiate growth in glutamic acid medium.

DISCUSSION

Two situations have been used here to study the effects of aminopterin on the growth of HeLa cells. The first situation (Experiments 3 to 5) involves a comparison of the behavior of a single cell strain (HeLa S3-1) in aminopterin-containing media that either contain all the nutritional factors necessary for growth of the cells or, in contrast, are deficient in a single essential nutritional factor. In complete growth medium, aminopterin evokes a characteristic set of changes: cessation of cellular multiplication, doubling of the average protein content of the cells, cellular distortion and detachment of most of the cells from the glass of the culture vessel. Few of the cells that remain can resume proliferation when aminopterin is removed. Cells of the same strain in a deficient medium, while failing to increase in number, also fail to increase their protein content, and tend to remain attached to the glass. Most of these cells can multiply when aminopterin is removed and the missing growth factor is supplied.

The second situation (Experiments 5 and 6) compares two different cell

strains in the same medium. HeLa I-11 can grow when transferred from glutamine-containing medium to one in which glutamic acid replaces glutamine and it is subject to the lethal action of aminopterin in such medium. In this same medium, HeLa S3-1 is unable to initiate growth and tends to survive aminopterin treatment. When an artificially mixed population, containing about one per thousand HeLa S3-1, is treated with aminopterin in a glutamine-free medium containing glutamic acid, almost all the HeLa I-11 cells are killed. Removal of the aminopterin and provision of glutamine then permit the HeLa S3-1 cells to resume proliferation so that they can be recovered as discrete colonies with high efficiency. This, and the isolation of a new glutamine-requiring strain from HeLa I-11, demonstrates the practicality of using aminopterin in selecting for auxotrophic mutants of HeLa cells.

Our experiments show that aminopterin creates growth requirements for thymine and adenine. They also show that it is mainly the thymine deficiency that leads to cell death in a complete growth medium. In this respect, and in the disparate increase in protein relative to cell number, the effects of aminopterin on HeLa cells resemble those of thymine deficiency in bacteria (4). In contrast, the aminopterin-induced purine deficiency permits a high survival of HeLa cells under our experimental conditions.

The creation by aminopterin of requirements for thymine and a purine accords, on the one hand, with the participation of reduced folic acid coenzymes in their synthesis, and, on the other, with the reported inhibition of folic acid reduction by aminopterin (13, 14). It is unexpected, therefore, to find that aminopterin-treated cultures can increase tenfold in both protein content and cell number in the absence of added glycine or serine. The cellular reserves of these amino acids could account for no more than a 30 per cent increase in protein (15) and it is possible that they are derived in appreciable amounts from sources not involving folic acid coenzymes. These ambiguities make it desirable to use an agent more specific than aminopterin for inducing thymine deficiency. Preliminary experiments by ourselves, Dr. L. A. Herzenberg, and by Dr. N. P. Salzman (personal communication) indicate that 5-fluorouracil deoxyriboside will be suitable with the HeLa cells as it is with other animal cells (16).

Experiments 4 and 5 show that aminopterin-treated cells that are also deprived of an essential nutrient tend to survive the period of thymine starvation. The survival of these cells depends in part on the care taken in depleting cellular reserves of the nutrient to be omitted. Appreciable unbalanced growth and cell loss occur when the cells are not washed with and incubated in deficient medium prior to the addition of aminopterin. Survival also depends on the ability of the cells to withstand starvation for substances, other than thymine, that are necessary for growth. This varies with the particular nutrient omitted, glutamine starvation (Experiment 5) usually evoking more rapid

cell loss than starvation for arginine (Experiment 4) or adenine (Experiment 3). The period of exposure to aminopterin, which will, in part, determine the efficiency of selection for auxotrophs, must be adjusted to this limitation, but Experiment 2 shows that most non-deficient cells are lost after a 4 day treatment. Repeated cycles of short term exposure to aminopterin in minimal medium followed by growth of survivors in complete medium without aminopterin should permit a progressive increase in the frequency of auxotrophs to the point at which the testing of individual clones becomes feasible. Experiment 6 shows that a single 6 day cycle of glutamine starvation and aminopterin treatment permits efficient recovery of glutamine-requiring cells. It also shows that in this case not enough cross-feeding occurs in the presence of aminopterin to seriously impair the recovery of these cells.

The morphological difference between unswollen, auxotrophic and swollen, non-auxotrophic cells during thymine starvation might permit early detection of small clones of auxotrophs by systematic microscopic examination of mass cultures. This difference is readily apparent after about 48 hours of thymine starvation when, on the one hand, the deleterious effects of starvation for nutrients other than thymine are minimized and, on the other, there is a high survival of non-auxotrophs if thymine starvation is terminated. The recovery of viable non-auxotrophs in association with auxotrophs might be important in detecting homozygous auxotrophic and prototrophic segregants arising together from heterozygous cells.

Selection for auxotrophs with aminopterin is confused by the occurrence of aminopterin-resistant mutants. However, resistance occurs at several different levels in these cells and most of the resistant mutants selected for at 10^{-8} M aminopterin are sensitive at 5×10^{-8} M. Unbalanced growth and cell loss follow the same course at both concentrations so that the background of resistant cells can be largely eliminated by selection at the higher concentration.

We have been unable to determine cell viability directly by clone formation. The combined effects of starvation, aminopterin treatment, and the procedures for removing the cells from the glass reduce the cloning efficiency almost to zero. Some of these difficulties might be avoided by the use of cell suspensions. Despite this difficulty in our work, in every experiment in which there was a large increase in the protein content of the cultures after the removal of aminopterin there was a corresponding increase in cell number. This, and the efficient recovery of the glutamine-requiring cells in experiment 6, lend validity to the use of thymine starvation in selecting for auxotrophs.

The basis of the glutamine requirement of HeLa S3-1 is not known. It takes up glutamic acid from the medium at almost exactly the same rate as does HeLa I-11. HeLa I-11, grown with glutamine has a low but detectable level of glutamine synthase (17), which rises about fifteenfold during growth in glutamic acid (18 and unpublished data). The behavior of this glutamine-syn-

thesizing enzyme in HeLa S3-1 is now being studied. Reversions to glutamine independence occur in this strain with a frequency of about 10^{-7} in newly recloned populations.

SUMMARY

1. Aminopterin prevents the multiplication of HeLa cells in Eagle's medium. The joint addition of adenine and thymidine removes the inhibition.

2. The aminopterin-induced thymine deficiency specifically results in a cessation of cell division and a doubling in the average protein content of the cells. Continued starvation for thymine results in the inability of cells to proliferate after aminopterin is removed. After 6 days only 10^{-6} to 10^{-5} of the original population proliferates.

3. The omission of a single essential amino acid, such as arginine or glutamine, from the medium during deprivation of thymine prevents a net increase in protein and results in about a 10^5-fold greater cell survival after 6 days, when aminopterin is removed and the missing amino acid is supplied.

4. Glutamine-requiring, auxotrophic mutants of HeLa exhibit a high survival after exposure to aminopterin in a glutamine-free medium. Glutamine-independent HeLa cells show a much lower survival in the same medium. Low frequencies of the auxotrophs can be specifically and efficiently selected for in artificial mixtures of the two cell types by treatment with the glutamine-free medium containing aminopterin.

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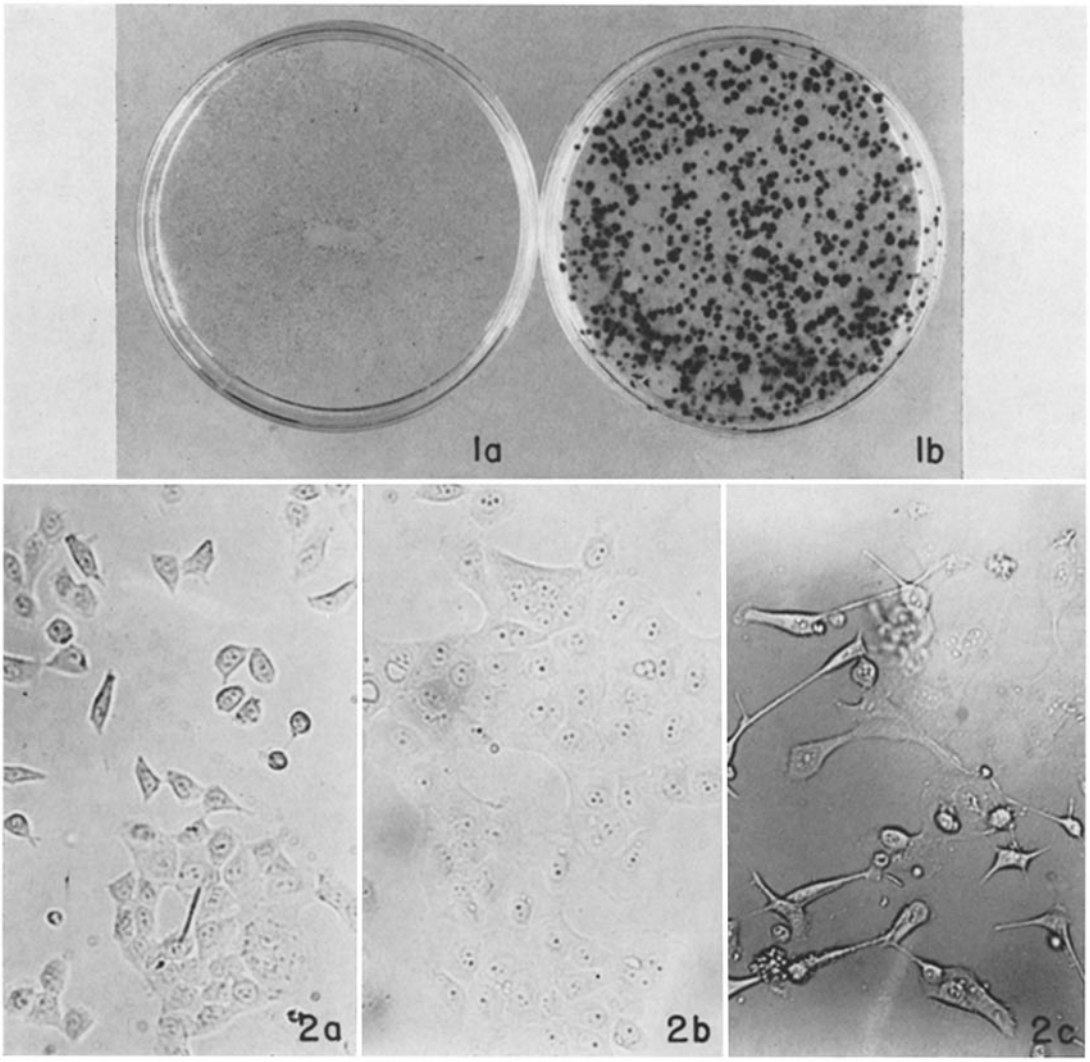
EXPLANATION OF PLATE 47

FIG. 1. Selection for glutamine-requiring cells. See text, Experiment 6 for explanation. (*a*) control plates containing only HeLa I-11. (*b*) similar plates seeded with about one per thousand of HeLa S3-1. Stained with methylene blue.

FIG. 2. The microscopic appearance of HeLa cells in Eagle's medium (*a*) or in Eagle's medium containing 10^{-6} M AP after 44 hours (*b*) and 135 hours (*c*). See text, Experiment 3 for explanation. × 140.



(De Mars and Hooper: Auxotrophic Mutants of HeLa cells)