6-MP. On the other hand, if 6-MP or one of its metabolites from pathways other than that involving xanthine oxidase were the teratogenic agent, addition of HPP would increase the severity of the anomalies or increase the effectiveness of 6-MP at a lower dosage. Both were the case. When HPP at 120 mg/kg was added to the teratogenic dosage of 6-MP, fetal death occurred shortly after administration, as demonstrated by small fetal size and extensive resorption. Fetal anomalies obtained with 6-MP (15 mg/kg) in combination with HPP (30 mg/kg) were similar to those obtained with 6-MP (60 mg/kg) alone. In the latter experiment placental weights were significantly lower than those obtained with 6-MP at 15 mg/kg alone ($p<0.05$), providing additional evidence of the relationship between placental growth inhibition and congenital malformations. Further studies are being carried on to ascertain the nature of this relationship.

Summary. In addition to producing anomalies in rats, 6-mercaptopurine and thioguanine cause a decrease in placental weight. Other purine derivatives which produce no anomalies have no effect on placental weight. These findings suggest that a relationship exists between malformations and placental growth inhibition. Administration of 4-hydroxypyrazolo-(3,4-d)-pyrimidine (a xanthine oxidase inhibitor) with non-teratogenic dosages of 6-mercaptopurine results in anomalies and decreased placental weight. Furthermore, combination of the former drug with teratogenic dosages of the latter causes fetal death.

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Received March 20, 1964. P.S.E.B.M., 1964, v116.

Serum Albumin Supplemented Medium for Long Term Cultivation of Mammalian Fibroblast Strains.* (29346)

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Though many improvements have been made in cell culture technique in the past years, it is still not possible serially to cultivate mammalian cell strains† unchanged for very long periods; either they eventually die out, or they change their properties and develop into established cell lines. In commonly employed media (such as Eagle's medium(1) or some modification thereof) supplemented with 10% serum, human fibroblasts die out after from 50-70 cell generations(2,3); Syrian hamster fibroblasts under the same conditions have a much shorter lifetime(4); mouse fibroblasts decline considerably in growth rate by 20-30 cell generations but then give rise to established lines (5,6).

One respect in which nearly all cell culture media differ from the fluid environment of cells in the animal is in their much lower protein concentration. Most culture media contain 3-12 mg protein per ml (5-20% serum) while plasma contains about 65 mg/ml and lymph or extracellular fluid about 30 mg/ml. Though certain cell lines have been evolved that are able to grow in serum-free medium(7,8), and cell strains may be propagated in protein-free medium to a limited extent, both grow much better in

* Aided by grants from U. S. Public Health Service.

† Terminology of Hayflick aund Moorhead(2).

the presence of serum protein(9). Specific active protein fractions have been described (10-12) and various postulates have been presented for the role of protein in supporting the proliferation of cultured cells(13,14). Beneficial effects of serum albumin on cell growth have also been reported in a variety of systems by Ham(15,16), Moskowitz *et al* (17) and Moore *et al*(18). In some cases these effects appear to be due to small molecules adsorbed to the albumin(15,16); in other cases the albumin is believed to exert a "protective" effect, perhaps against toxic substances in the culture medium(17).

The present experiments demonstrate that addition of serum albumin in high concentration to the standard medium containing 10% calf serum profoundly alters the fate of hamster fibroblast strains, enabling them to grow more rapidly and through many more cell generations. While it does not increase the growth rate of human diploid fibroblast strains, it does make it possible for them to grow through more generations in culture. With neither species did it appear to increase the probability of emergence of established cell lines.

Methods. The preparation of the primary cultures of Syrian hamster embryo cells and adult human skin fibroblasts (strain A) has been described(4,3). Cell cultures were maintained in 50 mm plastic Petri dishes in the Dulbecco and Vogt modification of Eagle's medium(1), which contains approximately 4-fold higher concentrations of glucose, amino acids and vitamins, and a higher bicarbonate concentration (3.7%). The medium is equilibrated with 10% CO_2 in order to give a pH of 7.2. This medium supplemented with 10% calf serum (Colorado Serum Co.) will be referred to as the "standard medium." The same medium, supplemented with a total of 30% calf serum will be called the "high serum medium" and the standard medium plus serum albumin (20 mg/ml), "albumin medium." The albumin, 3 times crystallized (commercial product of the Armour Pharmaceutical Co.) probably contains a variety of tightly bound small molecules(18).

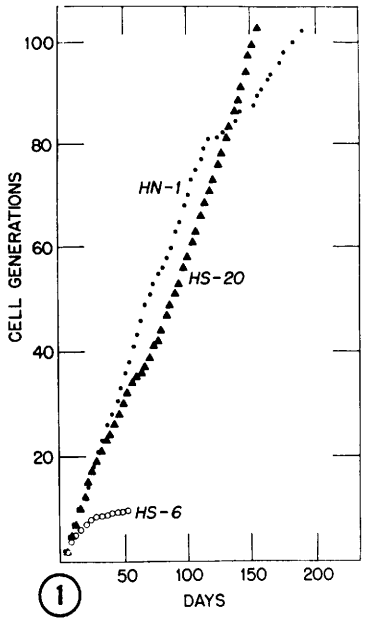
In some experiments, cultures were transferred by simple dilution, usually 1:2, 1:4 or 1:8. At the following transfer, one of the cultures was selected for further dilution. In such experiments the number of cell generations through which the population has grown is known but not the growth rate at any time (Fig. 1). In other experiments, at each transfer the cells were counted and a known number of cells used in the new inoculum. Under these conditions both the number of cell generations and the growth rate of the cells during any given transfer interval are known (Fig. 2-4). In both types of experiment the number of generations which occurred in the primary culture is not known and the figures show the number of generations beginning with the secondary culture.

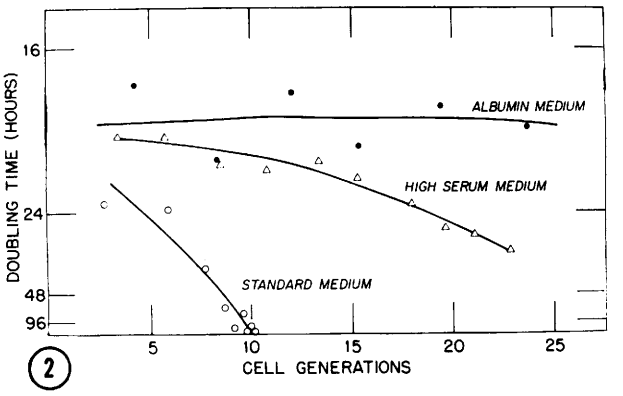
Results. Effect of serum albumin on hamster embryo cells. Hamster embryo fibroblasts maintained on the standard medium and transferred every 4 days at an inoculation density of 6×10^5 cells per plate were usually not able to grow through more than 10 cell generations(4). Fig. 1 shows such a growth curve for one of these strains, HS-6 in which, after 14 transfers, growth ceased, the cells having completed less than 10 generations. Cultures of similar origin (HS-20 and HN-1) carried in the albumin medium could usually be transferred at dilutions of 1:4 or greater twice a week; HN-1 reached 100 cell generations in 160 days (47 transfers), while HS-20 required a somewhat longer time (190 days and 57 transfers). In both cases the cells were still growing well when the experiment was terminated.

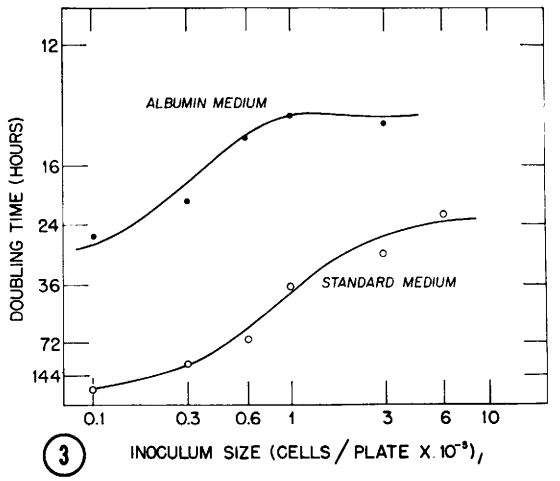
In parallel cultures, successively transferred at constant inoculation density during the first 25 transfers, quantitative determinations of growth rate were performed at each transfer. These results are plotted in Fig. 2, and show that cells on the albumin medium grew more rapidly after the first transfer, maintaining a doubling time of from 17 to 22 hours throughout the first 24 cell generations. The same cells on the standard medium initially doubled in about 24 hours, but the growth rate fell rapidly on successive transfer. High serum medium had effects comparable to those of the albumin medium for a short time, but was not as effective

for the long term cultivation of the hamster cells. With all media including the albumin medium, reducing the cell density much below 3×10^5 calls per plate resulted in rapid and irreversible loss of growth potential.

One of the properties which emerges in fibroblasts as they evolve into established lines is increasing ability to grow at low inoculation density(6). Fig. 3 shows the doubling time over a single growth interval between transfers of secondary cultures of hamster embryo cells at various inoculation densi-







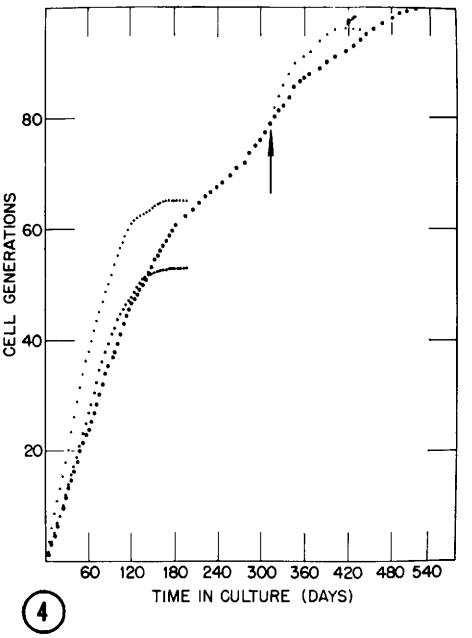


FIG. 1. Growth of hamster embryonic fibroblasts on standard medium (○) and albumin medium (▲ and ●). Each point represents one transfer.

FIG. 2. Growth rate of secondary hamster fibroblasts on successive transfer at constant inoculation density (3×10^5 cells/plate).

FIG. 3. Relation between growth rate and inoculation density of hamster fibroblasts on standard medium and albumin medium.

FIG. 4. Growth of human fibroblasts on standard medium (○), high serum medium (△) and albumin medium (●). Arrow indicates start of a subculture of albumin medium grown cells on high serum medium. Each point represents one transfer.

ties. At 6×10^5 cells per plate on the standard medium the cells have a 24 hour doubling time, at 6×10^4 cells per plate a 72 hour doubling time, while at 1×10^4 cells per plate there is virtually no net increase in cell number. The results with cells on albumin supplemented medium showed greater variability between experiments, but the result shown in Fig. 3 is representative. The cells double in 17 hours at densities greater than 6×10^4 cells/plate, and in about 24 hours at 1×10^4 cells/plate, a cell density that permits virtually no growth in the standard medium. At still lower densities, the cells showed no growth even in the albumin medium, so that the plating efficiency is not appreciably better than on the standard medium.

Cells which had grown for 100 cell generations on albumin medium were retested on that medium and grew about as well at all inoculation densities as they did initially. When tested on the standard medium, cells which had grown through 100 generations in the albumin medium grew more slowly than did the secondary cultures. It appears therefore, that the hamster fibroblasts may be grown through many cell generations on the albumin medium without improvement in their ability to grow at low inoculation density either in the presence or absence of added albumin.

Chromosome studies of secondary cultures showed cells to have the normal complement of 44 chromosomes. By 100 generations, however, the cells were hyperdiploid with a mode of 46 and there was a substantial number of cells with 45 chromosomes. These studies indicate that the cells were genetically altered during their culture life, though the change in karyotype was not accompanied by detectable alterations in their physiological properties.

Effect of serum albumin on human fibroblast strains. While the growth-enhancing effect of serum albumin is readily apparent on hamster fibroblasts, it is not so readily seen with human fibroblasts. However, albumin does have a substantial effect on the long term cultivation of human cells.

It has been clearly shown by Hayflick and Moorhead(2) that the growth potential of human fibroblasts is limited under the usual conditions of culture. Under our conditions such cultures are able to divide for 50-70 generations and when high serum medium is used an additional 5-10 generations may occur(3).

The growth curves obtained from cells carried at constant inoculation density (3×10^5 cells/plate) in standard and high serum medium are shown in Fig. 4. The cells completed 53 and 65 generations respectively. On the albumin medium the same cell strain grew somewhat more slowly during the first few months, but eventually overtook the cells growing on the standard medium. Growth has continued for over 100 cell generations (80 transfers and 18 months in culture). At the present time the cells are growing very slowly. Serum albumin supplement therefore enables the cells to grow through many more cell generations than they would otherwise be able to, apparently without leading to the development of established lines. Since the growth rate is initially reduced in the albumin medium, it appears that the cultural conditions which permit most rapid growth of a cell strain may not be those which best preserve its growth potential.

At various times during the experiment, cells growing on one medium were transferred to another. Cells maintained for 81 generations on albumin medium, when put on the high serum (Fig. 4) or the standard medium enjoyed, at least for a few transfers, a considerably enhanced rate of growth, but died out after completing fewer generations than the cells left on the albumin medium. Therefore a residual growth potential preserved during growth on the albumin medium can still be expressed on other media. However, the cells transferred to high serum medium died out after completing fewer generations than those left on the albumin medium. Cells which have grown through 50-60 generations in medium with 10% or 30% calf serum, and have virtually stopped growing, do not resume growth when transferred to the albumin medium.

Chromosome preparations on the albumin maintained cells after 95 generations in cul-

ture showed a classical diploid karyotype but with some increase in frequency of tetraploids (6.9%). The spontaneous chromosome alterations that characterize the terminal phase of human fibroblasts in culture(20,21) are not yet present in these cells at their 95th generation. The same cell strain, however, demonstrated the typical terminal aneuploid degeneration after 50-70 generations on the standard medium(22). It is concluded therefore that these chromosomal alterations need not occur after any fixed number of cell generations in culture.

Summary. Serially transferred hamster or human fibroblasts maintained on synthetic medium containing 10% calf serum could be carried through about 10 and about 50-70 cell generations respectively, before growth ceased. Addition of crystallized serum albumin to the medium to a concentration of 20 mg/ml affected the growth potential so that in both cases over 100 cell generations could be obtained. The human fibroblasts after 95 cell generations showed no chromosomal abnormalities while in the hamster fibroblasts some deviation from the diploid number did occur. In neither case was there evidence of the evolution of improved growth properties that characterize established cell lines.

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Received March 20, 1963. P.S.E.B.M., 1964, v116.

Urea Cycle Adaptations in Intact and Adrenalectomized Rats.* (29347)

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In studies on protein catabolism, especially the final stages as urea synthesis, the work of Ratner(1-3), and that of Cohen's group (4-6) in elucidating the individual steps of the urea cycle and in providing relatively simple methods for assaying these enzymes

* This study was supported in part by U.S.P.H.S. Research Grant AM-04732.