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Value of Mammalian Cell Culture as a Biochemical Tool

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Until some 20 years ago the techniques of cultivating mammalian cells in vitro remained almost exclusively research projects. To be sure, Harrison (as far back as 1908) and others had demonstrated that animal cells could be maintained for days and months in vitro, and Strangeways and Fell had made the same demonstration for organ culture some 40 years ago. However, the cumbersome technology, the poor definition of physical and chemical requirements, and a general lack of quantitative methods discouraged all but the most painstaking, devoted, and determined investigators. This picture began to change in the 1940's when G. O. Gey and Wilton Earle and their colleagues showed that cells from a variety of tissues could be successfully maintained in vitro through use of rather simple media and careful attention to certain principles. Now, in 1968, these techniques have been so well defined and the complex media and equipment are so readily available from commercial supply houses that many biochemists, microbiologists, pharmacologists, and other biological scientists use mammalian cell culture as a biochemical tool. Their observations on various phenomena have been summarized in hundreds of publications in scientific journals, and it seems timely to consider the value of mammalian cell culture as a tool. What are its strong points and what are its disadvantages? What are the pitfalls that can be avoided? Where will the use of this tool lead us? What new areas may be exploited in the near future?

There are several applications of mammalian cell culture other than those related directly to virology and to cancer research. These include (i) study of mechanisms of cytotoxicity and correlation of the cytotoxicity of drugs with other pharmacological attributes; (ii) study of the biogenesis of hormones and other "vital" products at the cellular level; (iii) determination of nutritional requirements of mammalian cells from "specialized" tissues or cells grown under unusual stresses; and (iv) study of host-parasite relationships at the cellular level. Cell culture has also been used in studying the life cycle of the cell, radiation effects, and nuclear-cytoplasmic interactions, topics which are outside the scope of this article.

Among the various animal cell types that have been cultivated for long or short periods in vitro are epithelial cells, connective cells, cartilage and bone cells, nerve cells, muscle cells, reticular cells from bone marrow, lymph nodes and spleen, and leukocytes from the blood, all of which may be derived from the embryo or the adult. Depending on their origin, some of these cells can be cultivated continuously, whereas others multiply slowly or not at all. For example, leukocyte cultures are, for the most part, short-lived cultures in which there is cell proliferation without a net increase of the cell population. The changes in morphology and function of leukocytes in such cultures is associated with significant biochemical phenomena. This article is confined to an evaluation of the biochemical potential of established cell lines—that is, cells which have been in tissue culture for many generations. These cell lines, although sometimes of heterogeneous population, are readily available to biochemists and other laboratory scientists from a variety of sources, including the American Type Culture Collection and several commercial biological supply houses.

Mechanisms of Cytotoxic Action

In the past 12 years, since Eagle and Foley (*1*) summarized their observations on the cytotoxic action of carcinolytic agents in tissue culture, the action of thousands of chemical compounds and fermentation concentrates has been

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examined in these mammalian cell systems (*2*). Although the original goal of finding chemical substances more toxic to neoplastic cells than to normal cells has not been achieved, a number of relatively interesting agents have been selected.

In their first studies of the vitamin requirements of mammalian cells in culture, Eagle (*3*) showed that 0.03 microgram of aminopterin per milliliter was sufficient to produce cytological changes in the KB cells (*4*). This observation was used as the basis for a bioassay for cytotoxic compounds, and numerous techniques were devised (*5, 6*) for determining the toxicity of candidate antitumor compounds. A similarity in the response of the various cells to a given inhibitor, irrespective of the source of the cell line (normal or neoplastic) was evident in studies in which compounds of widely varying chemical structure were used (*7*). Foley and Eagle (*8*) concluded that all mammalian cells in vitro, irrespective of the tissue of origin, responded similarly to a given inhibitor when cell protein formation was the parameter measured.

This bioassay method was judged sufficiently sensitive and specific to be used as a screening procedure for antitumor compounds, and programs, organized and supported by the Cancer Chemotherapy National Service Center of the National Cancer Institute, for selection of useful antitumor agents have been based on use of this mammalian cell culture response as the primary screen. It is evident that there is remarkably good correlation between inhibitory activity in mammalian cell assays—for example, inhibition of cell protein formation—and antitumor activity. Typical data on the sensitivity to antitumor compounds of eight tumor systems and of mammalian (for example, KB) cell cultures are summarized in Table 1. It is obvious that the compounds which were the most active in the experimental screening for antitumor compounds were also the most cytotoxic.

In somewhat similar fashion Smith *et al.* (*9*) critically examined the mammalian-cell cytotoxicity tests as a method of predicting the toxicity of a series of chemical structures in tests of acute toxicity in the mouse. Some of their data are summarized in Table 2. They concluded that, in general, "a relationship was observed between acute animal toxicity and mammalian cell cytotoxicity *in vitro* at the 1% level or better." The correlation was not closely predictive, as several compounds highly toxic to mammalian cells were relatively nontoxic to the mouse, and vice versa. In addition, it should be noted that cytotoxic activity in vitro (as measured by inhibition of formation of mammalian cell protein in KB cells) was not predictive of myelotoxicity in man. Thus, while 5-fluorouracil, 5-fluoro-2′-deoxyuridine, mitomycin C, and dactinomycin at useful therapeutic levels severely depress the formation of bone marrow, streptovitacin A and the chemically related cycloheximide, which are also cytotoxic, do not. The limiting toxicologic manifestations with streptovitacin A were vomiting and hypotension, while cycloheximide caused intense vomiting. Thus, agents with high cytotoxicity in vitro do not, a priori, depress formation of bone marrow in man. In retrospect one might conclude that, since biosynthesis of protein (of undefined characteristics) is used as the end point in the cytotoxicity tests, it might be possible to improve the correlation if some cellular function or specific cell component was used as marker for the cytotoxicity assays. On the other hand, some of the lack of positive correlation may be due to a simple failure of the agents to reach the cells.

One reaches a similar conclusion on inspecting the data on the possible relationship between the toxicity to mammalian cells of some corticosteroids in tissue culture and the anti-inflammatory activity of these compounds, as shown in the cotton-pellet implantation test in the rat. Some of the data selected from the literature are summarized in Table 3. It is obvious that the more potent anti-inflammatory agents are very toxic to the mammalian cells in tissue culture. However, the quantitative aspects (for example, increase in potency) of the relationships between structure and activity do not hold as closely as one would wish, and selection of possible anti-inflammatory compounds solely on the basis of mammalian-cell toxicity (as measured in vitro) is probably hazardous. This may be due to steroid interconversions in the rat during the cotton-pellet implantation test, or perhaps to lack of penetration of the steroid into the mammalian cells in the tissue culture studies.

Even more equivocal results were obtained by Yale and Kalkstein (*10*), who noted that modifications in chemical structure led to increased cytotoxicity in substituted 2,3-dihydro-4(1*H*)-quinazolinones. They had hoped that these modifications would introduce antitumor activity into a compound which initially (before modification) was essentially "pharmacologically inert." Unfortunately this proved not to be the case; at least in laboratory tests, the compounds with the higher cytotoxicity had no more antitumor potency than the parent structure, and the modified structures had no more pharmacological activity than the original structures, at least in the common laboratory tests.

Table 1. Sensitivity of experimental tumors and of cell cultures to 14 antitumor compounds.

Compound	Positive inhibition of tumors*	Cell culture toxicity [ID_{50} (μg/ml)]†
Diethylstilbesterol	0/8	>10.0
Urethan	0/8	>10.0
Potassium arsenite	1/8	0.1
Myleran	1/8	1.0
Hydrocortisone	1/8	7.0
6-Mercaptopurine	2/8	0.07
Vinblastine	2/8	.1
Methotrexate	3/8	.01
Dactinomycin	3/8	.004
5-Fluorouracil	3/8	.8
N-Deacetylthiocolchicine	2/8	.001
2′-Deoxy-5-fluorouridine	5/8	.008
Mitomycin C	5/8	.025
Cyclophosphamide	8/8	.01

* The ratios indicate that, of eight tumors, none were inhibited when the animal host was treated. The tumors used included melanoma B16, carcinoma C1025, adenocarcinoma EO771, Ridgeway osteogenic sarcoma, sarcoma T241, Walker carcinosarcoma 256, Ehrlich ascites tumor, and Mecca lymphosarcoma [data from Tarnowski *et al.* (*32*)]. † ID_{50} = amount needed to reduce growth of KB cell cultures to approximately half that observed in an untreated control [data selected from several publications (*6, 9*)].

Table 2. Comparison of cell culture toxicity and acute toxicity in mice. [Data from Smith *et al.* (9)]

Compound	Cell culture toxicity [ID_{50} (μg/ml)]*	Acute toxicity in mice [LD_{50} (mg/kg)]†
Dactinomycin	0.004	0.7
5-Fluoro-2′-deoxyuridine	.008	~650
Echinomycin A	.01	0.4
Mitomycin C	.025	8
Streptovitacin A	.035	6.5
Cycloheximide	.1	100
6-Mercaptopurine	.15	200
5-Fluorouracil	.8	188
Chartreusin	1.3	300
Sodium azide	6	65
Amicetin	7	>1000
Chlortetracycline	50	650
Celesticetin	230	300
Fluoroacetic acid	>500	10

*ID_{50} = concentration required to limit cell protein formation in KB cells to 50 percent that observed with the unsupplemented cell culture. † Dose administered intraperitoneally.

Table 3. Data showing possible relationships between cytotoxicity and anti-inflammatory activity of corticosteroids.

Steroid	Cytotoxicity test*	Anti-inflammatory activity†
Hydrocortisone	1	1
Cortisone	0.2	0.5 (0.3 to 0.8)
Prednisone	.8	1.0 (0.6 to 1.5)
Prednisolone	7	2.7 (1.7 to 4.2)
9α-Fluoro-16α-hydroxyhydrocortisone	7	2.6 (1.6 to 4.0)
9α-Fluoro-16α-hydroxyprednisolone-16α-,17α-acetonide	70	48.5 (31.2 to 75.7)
9α-Chloro-11β-chloro-pregnadiene-16α-methyl-17α-ol-21-acetate	10	1.7
9α-Chloro-11β-chloro-pregnadiene-16α-methyl-17α-butyrate-21-acetate	100	27
6α-,9α-Difluoro-16α-hydroxyprednisolone-16α-,17α-acetonide	70	103 (62.5 to 170)
9α-Fluoroprednisolone	70	17.7 (9.4 to 34.6)
6α-Chloro-9α-fluoro-16α hydroxyprednisolone-16α-,17α-acetonide-21-acetate	7000	446 (273 to 730)

*Reciprocal of the ratio of the concentration of the test steroid needed to give 50-percent inhibition of growth of Earle's L cells to the concentration of hydrocortisone needed to give equivalent inhibition of growth [data in part from Perlman *et al.* (*33*)]. † Reciprocal of the ratio of the concentration of the test steroid needed to give remission of the inflammatory response in the cotton-pellet test to the concentration of hydrocortisone required for this remission [data in part from Lerner *et al.* (*34*)].

These results emphasize some of the limitations in using cytotoxicity, with either inhibition of protein synthesis or increase in cell numbers as the end point, in screening for pharmacological activity. Cells growing in tissue culture are usually undifferentiated and contain all the enzymes, and produce all the products, needed for cell replication, whereas cells growing in the animal may be dependent upon each other for nutrients, for enzymes, and for other products. Thus, rather than expend effort to screen compounds on the basis of inhibition of growth alone, we should examine the effects of pharmacologically active materials on the enzyme profile of the cell, the biosynthesis of certain important metabolic intermediate compounds in the cell, and the composition of the cell membrane and other structures. A number of reports (*11*) show that the supplementation of cell culture media with corticosteroids or the addition of corticosteroids to cells growing in suspension culture induces or represses enzyme synthesis. Other reports (*12*) show that addition of cytotoxic agents induced change in genetic makeup of the cell, as shown by change in chromosome numbers. Cytotoxicity screening in the future will take advantage of the past experience and concentrate on selection of compounds with specific inhibition sites rather than on growth in general terms.

This change in end point will require a more complete knowledge than we now have of the relative value to the cell of the metabolic systems within the cell. However, the use of mammalian cells in in vitro culture for such studies will continue, since such laboratory test systems provide the opportunity to work with cells which are all of the same physiological state and age (and, presumably, biochemical composition). Translation of the results of experiments at the cellular level to the whole-animal level will continue to be hazardous; perhaps one way of reducing the hazards is to carry out the laboratory studies with a number of cell lines derived from a variety of tissues.

Biosynthesis of Hormones

One of the more interesting developments in cell culture has been the isolation of cell lines which maintain their ability to produce the hormones found in the tissue of origin. Although there are many reports of the establishment of mammalian cell cultures which have produced hormones, it seems that until recently only traces were produced (*13*), and that after a number of subcultures these cells lost their ability to synthesize the hormone products. For example, Thompson *et al.* (*14*) reported that serial cultivation of cells obtained from a human pituitary was carried out and that the cells, when grown in media containing human, equine, or bovine serum, produced somatotropin, corticotropin, and gonadotropin. However, Reusser *et al.* (*15*), working with cell cultures derived from three human pituitary glands, did not obtain production of human growth hormone (as detectable by the highly sensitive fluorescent antibody technique or by an immunochemical assay of the Ouchterlony type). On the basis of their observations, Reusser concluded that human pituitary cells grown in tissue culture do not, under the culture conditions described, retain their functional properties for production of growth hormone.

A more successful series of experiments, designed to isolate hormone-producing cells, was recently reported by Sato and Yasumura (*16*). They had concluded that selective overgrowth is the most serious obstacle to the culturing of functionally differentiated cells. Starting with this hypothesis, they tried to reproduce in mammalian cell culture the enrichment techniques used in microbiology for isolation of "specialized bacteria." In Sato's method, functionally differentiated, transplantable tumors are put into mammalian cell culture. After a brief period the cells that have survived this treatment are injected into host animals, and new tumors are obtained. These culture-derived tumors have enhanced ability to grow and function in mammalian cell culture (*16*). The first passage through the mammalian cell culture selects hardy cell variants which withstand the culture conditions. The passage through the mice eliminates the connective tissue elements which sometimes overgrow the "specialized" cell types in cell culture; since these elements do not contribute to the growth of the tumor, they are effectively lost. By the time the cells have been through multiple passages in mammalian cell culture and in animals, a very specific part of the population is selected.

This procedure was successfully used to select certain types of cells, including (i) cells from the adrenal cortex, with ability to produce 20α-hydroxyprogesterone, progesterone, 11β-,20α-dihydroxyprogesterone, and 11-keto-20α-hydroxyprogesterone (*17, 18*); (ii) cells from a rat pituitary tumor which synthesize ACTH, growth hormone, and prolactin (*18*); and (iii) melanin-producing cells from a teratoma (*19*). Sato's mammalian cell cultures have been maintained for several years and have not lost the ability to synthesize these hormones. The availability of these mammalian cell cultures should make possible uncomplicated studies of the mechanisms involved in the biogenesis of these hormones.

Nutritional Requirements of "Specialized Cells"

The first tissue-culture media were biological fluids, chosen to imitate as closely as possible the natural environments of cells. With the development of the science of biochemistry it was possible to identify many of the components of these mixtures and to prepare chemically defined media to replace the biological fluids, peptide mixtures, and vitamins. Almost all chemically defined media were designed for long-term, progressive growth of cells. Because this approach was successful—that is, many kinds of cells would grow fast in similar media—it seemed that all cells had similar growth requirements. In retrospect this is now recognized to have been an artificial situation, since only the rapidly growing cells survived. Moreover, for some cells, combinations of ingredients were as effective as a single nutrient: mixtures of glycine, thymidine, and adenine reduced the requirement for folic acid.

The absolute requirements for cell multiplication have not been completely defined for very many cell lines. Most of the studies with cells grown in a chemically defined medium supplemented with a small amount of a dialyzed serum fraction show a requirement for the following amino acids: arginine; cystine; histidine; leucine; isoleucine; lysine; methionine; phenylalanine; threonine; tryptophan; tyrosine; valine; and glutamine. The deletion of any of these compounds from the culture medium leads rapidly to a complete arrest of both growth and cell division. Amino acids that appear to be nonessential (in media supplemented with the dialyzed serum fraction) include glycine, alanine, serine, aspartic acid, glutamic acid, proline, and hydroxyproline. When no serum, protein hydrolyzate, or peptone was used to supplement the chemically defined medium, a more nearly complete spectrum of amino acids was found necessary for maximum growth of strain NCTC 2071-L cells (*20*). There seem to be no basic differences in the amino acids required for growth of freshly isolated cells and for growth of established cell lines.

Several slight variations in the pattern of amino acid requirements have been described: asparagine is required by the cell lines from the Jensen rat sarcoma and the mouse leukemia L-5178Y (*21*); serine is required for one strain of rabbit fibroblasts (*22*). Although glutamic acid at high concentrations can be used as a substitute for glutamine in the nutrition of HeLa cells (*23*), no similar response occurs directly with Earle's L cells, but, rather, an adaptation period is needed, after which there is increased glutamine synthetase activity (*24*).

The essential role of specific vitamins for cell growth in vitro has also been documented in recent years. In experiments with Earle's L cells and HeLa cells, choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin, and thiamine were shown to be required. The omission of any of these factors led ultimately to death, but deficiency states did not develop immediately. A requirement for vitamin B_{12} was noted when cells were grown in media deficient in deoxycytidine and thymidine (*25*), and it is likely that other requirements will be noted when "nutritionally depleted" cells are used in experimental studies.

Several general interpretations have been made in an effort to explain the anomalous finding that mammalian cell populations in vitro appear to have amino acid requirements differing from those of the rat or other animals. It is possible that in the intact animal some cells produce an excess amount of growth factor or amino acid and supply the needs of adjacent cells. In vitro, the cells that survive the isolation procedure may not have these "extra capabilities" and thus may require amino acids that the animal does not require. Another possibility is that the chemically defined media are "imbalanced" and that additional factors are required to produce a menstruum conducive to the growth of the mammalian cells.

Up to the present, no requirement has been reported for the fat-soluble vitamins, biotin, or lipoic acid. Whether these, too, are not required under the culture conditions or are present in sufficient amounts as trace contaminants in the other ingredients of the media (for example, in the dialyzed serum component) is a matter still to be determined. There are no indications that isolated cell strains can acquire the ability to synthesize vitamins not ordinarily produced, but, at least in the case of inositol, variants independent of the usual requirement have been noted in a series of HeLa cell lines (*26*).

In all these studies on the nutritional requirements of mammalian cells in vitro, emphasis was placed on the growth of the cells and little attention was paid to their properties or composition. Perhaps new requirements will be noted if enzyme biosynthesis or nucleic acid composition of the cell is taken as the parameter measured, rather than just cell multiplication or protein formation. We now have remarkably sensitive methods for measuring enzyme activity and establishing enzyme identity, and perhaps studies at the cellular level will determine what nutritional factors are needed for synthesis of the enzymes which produce growth hormone, insulin, corticosteroids, and specific antibodies. Knowledge of these requirements may lead to new chemotherapeutic use of "old" compounds or to the development of new ones. We may expect that nutritional studies at the cellular level will show special requirements for rejuvenation of aging cell populations, for "repair" of physically or chemically injured cells, and for the combating of intracellular infections by microorganisms or viruses.

Host-Parasite Relationships

Mammalian cell culture techniques have proved to be an invaluable aid in the study of intracellular parasitism by certain microorganisms. Particular attention has been given to *Mycobacterium tuberculosis* (*27*), brucellae (*28*), salmonellae (*29*), *Stephanurus dentatus* (*30*), and *Trichomonas vaginalis* (*31*). It is not known with any certainty whether the susceptibility to certain diseases depends upon the establishment of the etiological agent intracellularly, and culture of cells from naturally resistant and susceptible tissues presents an opportunity to examine this question. This system—that is, the infectious agent growing in a "pure mammalian cell culture"—provides the biochemist with the opportunity for a controlled operation which is not easily obtained in infected animals.

Perhaps a cautionary note should be included in any discussion of the usefulness of mammalian cell culture techniques. There are many textbooks which adequately describe the techniques needed for quick isolation of mammalian cell lines from a variety of tissues and for maintenance of these cells once they are growing in vitro. The chief hazards for the biochemist are contamination of the cells with bacteria, fungi, or mycoplasmae and the possible mixing of cells from one cell line to another. The microbial infections can

usually be cured through judicious use of antibiotics, and the mixing of cell lines can sometimes be resolved by immunochemical testing or the acquisition of new cultures. Once these hazards are appreciated, the biochemist and others interested in mammalian cell culture have a new tool at hand for the exploitation of new areas of research.

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A British Perspective on American Science Policy

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Science, in its issue of 12 January, devoted a News and Comment item to what it calls "the most comprehensive look at American Science Policy ever taken by outside observers"—a report which was recently issued by OECD (the Organisation for Economic Cooperation and Development, the successor to the Marshall Plan in Europe). The heart of this report, *Science* says, is the analyses by four "examiners," and these, it is claimed, are "filled with impressions of weakness in the American system." I was one of the examiners, and was assigned the task of commenting on American science policy in relation to academic science and the universities. I certainly did not intend my analysis to be an attack on American practice. It was in fact aimed far more toward the European members of OECD than toward the Americans (or even the Japanese). The questions and suggestions which seemed to me to arise did not actually get published by OECD and do not emerge clearly in the reports published by *Science*, the New York *Times*, and *Nature*. I should like to give them here, with the following preliminary comments.

I understand *science policy* to mean the complex issues involved in spending some 18 to 20 billion dollars a year of federal money, together with large amounts of state, foundation, and other public funds: How much to health, space, physics, oceanography, and so on?

I certainly did not understand my job to be that of telling Americans how to run their own science. I was reporting to OECD, a body with 21 members, of whom the 20 non-American ones have more to learn from the U.S. in this respect than the U.S. has to learn from them. At the same time, I saw no reason to pretend that the U.S. science policy is perfect, and did not hesitate to ask the Americans questions challenging enough to be interesting. But by picking out only these items from my report, *Science* failed to give a true picture of the balance of the whole.

True, as *Science* says, we were officially in the States for only 14 days. But I started being personally involved in American science policy (as a recipient of a U.S.-financed fellowship) a third of a century ago. I suppose I have visited the States about 30 times, for various periods; in the laboratory of which I am chairman there are at least a dozen people who have postdoctoral experience in U.S. laboratories. The OECD official 2 weeks is not the whole story.

Summary and Questions

(*Questions are addressed to the Americans, suggestions to the Europeans.*)

1) The United States has a well-developed system of organizations concerned with formulating science policy.

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