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## *Review Article*

# Tissue Culture in the Study of the Effects of Drugs

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ALTHOUGH the techniques of cell, tissue, and organ culture have been known and developed over many years, surprisingly little use has been made of them until recently in pharmaceutical research for the direct examination of the effects, beneficial or adverse, on cells, healthy or abnormal, of substances of physiological or medicinal interest. Even now the full potential of the technique for this purpose is not being realized. The applications presently being made of tissue culture techniques are mainly in the fields of cytology, cell biochemistry and metabolism, cell differentiation, growing of viruses in cells, pathology (especially cancer), and the storage of viable material for transplantation. Thus, there is scope for considerable expansion in the application of tissue culture techniques to drug screening and action, where it can be a useful precursor or supplement to whole-animal tests in determining the effects of drugs at cellular level.

The method has several advantages, the most obvious being a reduction in the number of animal tests required, the relative cheapness of the technique, and the fact that less drug is used. Another advantage is that elimination of misleading results due to species difference may be brought about by the use of material of human origin. That species difference exists even at cellular level has been demonstrated (1, 2). Nevertheless, of the 1,461 references on the topic of the effects of drugs on cells retrieved by the Medlars organization recently, only 182 had made use of human cells. This organization is a computerized information retrieval system using tapes made from the references quoted in "Index Medicus." These 1,461 references cover the period mid-1963 to end-1966. Thus, it can be seen that even at the present time advantage is not being taken of the fact that tissue culture allows the use of human material on a scale not possible *in vivo*. There are, in fact, workers who deliberately choose amphibian material because it is poikilothermic. However, the present authors do not see that the provision of warmed surroundings, either in the form of a standard incubator or in the form of a warmed microscope stage, presents an insurmountable obstacle.

The use of cell culture also makes possible the setting up of replicate experiments and controls using identical material. Established cell lines are now easily available from many commercial suppliers. There is also a collection maintained by the American Type Culture Collection, and in Great Britain information on private collections is obtainable through the British Society for Cell Biology (formerly the British Tissue Culture Association).

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Many cell lines are now stored deep-frozen to avoid the work of continuous subculturing and to keep down changes which may be introduced in new generations (3). It has, however, recently been observed that this does not guarantee freedom from variation in the course of time, and this point must be remembered if one is comparing current work with earlier work (4). Also even a few days' freezing and subsequent thawing have been observed (5) to cause cell changes, and even the dimethylsulfoxide usually used in the technique of deep freezing may *per se* produce cell changes (6, 7). Thus, although large quantities of uniform material may be used at any one time, caution must be observed in comparing results of experiments performed even on the same cell line if these experiments are long separated in time. Such changes in cells used may explain some of the contradictory findings periodically reported.

A further advantage of tissue culture is that the cells may be grown in a controlled environment. Many established lines will grow in chemically defined media. Also, if a solution of the test drug in nutrient medium is continuously perfused around the cells, the exact concentration of drug is known to which each cell is exposed for the duration of the experiment.

Surprisingly little use has been made too of cultures of diseased cells, other than cancer cells, most of the work so far having concerned either the killing of these, or freedom from toxicity on the part of the test drug toward healthy cells. There is also little work on mixtures of drugs to study synergism or antagonism, which could also be observed *in vitro.*

The tissue culture method has, however, the limitation that a drug metabolized to an active form *in vivo* could not be tested *in vitro* unless the active form were first prepared. A second disadvantage is that, although the cultured material may be structurally unchanged, it may have changed functionally (4, 8), *e.g.*, loss of neoplastic properties, loss of transplantability, respectively. Third, in systems which involve several cells, such as the central nervous system, the gross effect of a drug may not be demonstrable. Rather in this instance the often overlooked effects of the drug on the component cells of the system may be more easily elucidated by biochemical and cytophysiological means.

Finally, like the results of whole animal tests, those from tissue culture tests must of course be correlated with clinical findings. Once the need for such correlation and the limitations of the method are recognized, tissue culture may be seen to provide a useful means of assessing activity and toxicity of many drugs (9–12).

## TISSUE CULTURE STUDIES ON DRUG ACTION

**General**—Perhaps the most complete list of publications on all aspects of tissue culture is that of Murray and Kopech (13) covering the years 1885–1950 and including some 15,000 references. There has since been a supplement to this. At present a bibliography by the same authors of more recent work is being published in parts, the first part having contained 1,213 references and the second part 1,244 references, this part coming out only 4 months later. This, taken in conjunction with the 1,461 Medlars references on drug action in tissue culture alone, is an indication of the vast increase in tissue work as a whole which has recently taken place. Obviously, a complete list of references even on this one aspect of tissue culture is beyond the scope of a single review article. Only the most important references since 1953 will be mentioned with in addition a few earlier works necessary for the explanation of the more recent work.

## METHODS USED IN ASSESSING THE BENEFICIAL AND ADVERSE EFFECTS OF DRUGS ON CELLS

Many microscopic methods have been used for this purpose, varying from observation of cell death to observation of minor, reversible changes. When the end point of the experiment is death, vital staining has been used (14) to indicate the percentage killed. On the other hand, attention has been drawn (15) to the fact that increased cell permeability by a dye is not necessarily synonymous with loss of viability. Thus, some other index of cell death is preferable. Direct observations by microscope of hanging drop preparations of cells have been used (16, 17) either to follow slowing or stopping of growth, or to see changes within the cell. Chromosome examination is another measure sometimes used.

Usually phase-contrast microscopy is used to allow observation of live, unstained material, and this may be usefully allied to time-lapse filming to obtain a permanent continuous record of the experiment. The authors' design of such apparatus allows for controls being recorded simultaneously (18–22). Electron microscopy examinations are being undertaken with increasing frequency as will be seen from later sections of this paper.

In many experiments, use is made not of cell lines which have grown for many generations *in vitro*, but of primary explants of tissue fragments (23). These are sometimes preferred because of the changes, morphological, chromosomal, or metabolic, which may occur, apparently spontaneously, in cells after some time in culture.

A great amount of work on such explants of various tissues has been carried out by Pomerat and Leake (1), who measured the concentrations of many pharmaceutically important substances which would inhibit the outgrowth of cells from such explants. Others (24, 25) have also measured the area of explant growth, or the time of closing of a small hole punched in the explant (26). Such measures of area, however, take no account of the depth of the growth or of whether the cells in the halo of outgrowth are loosely or tightly packed.

One disadvantage of using fragments of tissue or small organs, as distinct from separated cells, is that the drug may not have access to the cells at the center of the piece or organ. This may lead to false results if the experiment is of the type where the material is exposed to the drug for a fixed time and then is returned to growth medium without drug. On the other hand, such conditions may be desirable for some experiments because they more nearly approximate to *in vivo* conditions where each cell, for example in a tumor, is not necessarily bathed in drug solution. Another point that must be kept in mind in examining outgrowths from explants is that the rate of growth of the different cell types in the explants will probably vary—for example, a small number of epithelial cells may first be seen and later be completely overgrown with a halo of fibroblasts. Adequate control experiments are necessary before any conclusions can be drawn about a drug having a differential toxicity for epithelial and fibroblast cells under such conditions.

Inhibition of macrophage migration from spleen explants has also been used as an index of toxicity (27), as has the rate of migration of white blood cells in cultures of these alone (28). An interesting technique (29) overlays monolayers of cells with semisolid agar, through which the drug diffuses from paper disks as in an antibiotic assay. The agar is then poured off, the cells incubated, and examined for death or other changes. A comparison can be made with control cells on the same monolayers since the agar localized the drug. The only criticism of the method would be that it is necessary to verify chemically, where the result is unchanged cells, that the drug did elute from the paper.

Other methods of measuring growth in tissue cultures have been fully described (30, 31) or reviewed (32). These methods include total cell counts, viable counts, nuclear counts (in hemocytometer chambers or electronic counters), cell size measurements, packed cell volume or dry weight measurements, estimation of cell constituents such as protein, DNA, RNA, measurements of glucose utilization, mitotic index or duration of mitosis, or of respiration (33–37). Among respiration techniques, the authors' experience shows that micro-techniques using Cartesian diver types of respirometer are particularly useful and give a relatively rapid result.

Thus there are many indices from which to choose. Those chosen should always in the authors' experience include looking for simple morphological change because this is usually more sensitive than other indices. However, a change capable of being expressed objectively is better than one that can be described only subjectively, *e.g.*, number of polynucleated cells produced would be better than simply change of shape of cell. In using the other indices, *i.e.*, cell number, weight, volume, or biochemical measurements, which can all be expressed mathematically, it is necessary to do sufficient experiments and simultaneous controls for results to be statistically sound. Regrettably, the extent of variation among normal, untreated, replicate tissue cultures is all too often not recognized. This is surprising, because in recent years the need for good design in conventional, whole animal, pharmacological experiments has become generally appreciated, and one would have thought the same principles would have been applied to tissue culture methods. It is impossible to evaluate work where insufficient replicates and no controls are done, nor under such circumstances can one know what relative "weight" to assign to the all too many papers showing contradictory results.

Finally, whatever method is adopted for assessing the effect of a drug on cells, fully detailed reporting of that method is absolutely essential. Even very minor experimental variations may affect the results. Items which should be included in reports are details of the cell type, strain, origin, duration and treatment between obtaining and growing, duration since explanting, temperature and atmosphere of growing, medium in which growing, and frequency and methods of feeding and subculture. Whether material is fresh or frozen/thawed may affect its appearance (5), and also the effect of a drug may vary with the growth phase of the cells when treated. If synchronized cultures are used, this should be stated. Growth can be affected even by whether the cultures are exposed to light and by whether centrifuging is a stage in the subculturing procedure (38). The converse effect, weightlessness, is still of course under active investigation!

Only if all these qualitative and quantitative aspects are planned for and reported is a paper of present or future value and able to be put in true perspective in relation to other papers.

## GROUPS OF DRUGS WHICH HAVE BEEN EXAMINED BY TISSUE CULTURE METHODS

The various drugs which have been so examined fall into four main groups—anticancer agents, antimicrobial agents, hormones, and alkaloids. Although the latter is generally regarded as a chemical group, as opposed to the first three being pharmacological groups, from the point of view of change in cells, this too is a pharmacological group. There are fewer references to other drugs, and here these will be grouped under *Other Drugs*, including both chemical and biological substances.

There are few references to the substances, other than the active ingredient, in pharmaceutical formulations. Such items as vehicles, ointment bases, adjuvants, diluents, stabilizers, emulsifying agents, substances which increase viscosity (39), surface-active agents, tablet lubricants, disintegrants, coatings and polishes, flavorings and dyes, and materials used in surgical dressings and sutures—especially where the materials are new—are all items which could properly be examined by tissue culture. It is just as important that they be free from toxicity as it is for the active ingredient to be.

**Anticancer Agents**—It is extremely reasonable to use a cellular method for testing such drugs, since primarily a differential action on normal and malignant cells is being sought. Some of the material taken as "normal" in some experiments, however, scarcely merits this description, *e.g.*, benign growths which have been removed and varicose vein material (40).

The field of anticancer agent screening is in fact the place where the most active work is taking place using the tissue culture method. Such works may be said to have increased explosively since it was said (40) in 1961 that "the application of human cell cultures for the evaluation of cytotoxic activity of new carcinostatic agents has been reported by a few investigators."

The earlier papers in this field tended to emphasize the ease with which malignant cells could be grown *in vitro*, resulting in the availability of large amounts of uniform test material. This idea should be treated with reservation, however, from the viewpoints of both ease and uniformity. In considering ease of growth, although undoubtedly cells planted into a bottle or flask and grown to form a monolayer usually, in the case of normal cells, cease growing once intercellular contact is established—and in the case of malignant cells may not so cease—yet the proportion of explants of malignant tissue actually producing growth *in vitro* is far from 100% (41–44). Figures like 20–60% are more common. There has even been a report on a means of growing sarcoma cells free from healthy cells (45). Also, concerning uniformity of material produced, this has been taken for granted too much. Recent work has shown that variations do in fact occur over a period in cultured material. For example, cells have been shown to fail to maintain their blood groups in tissue culture (46), although this is not a unanimous finding, and chromosome variations have been observed (4). The heterogeneity of response of individual cells in neoplastic populations has also been discussed (47).

Clinical observations on the variation in response to the same drug in different patients with histologically indistinguishable tumors has resulted in the suggestion that it is useful to test drugs on primary explants of an individual patient's tumor at its particular stage of development (40, 42, 48–50). Also, in view of the current idea that an immune component may be concerned in some tumors, the concept of individual host specificity must be borne in mind (51). The idea of using the patient's own material may be very pertinent if the patient has been receiving chemotherapy because of the reported (52) difference between tumor materials from treated and untreated sources in terms of biochemistry, growth kinetics, and karyotype. On the other hand, it has been suggested (40) that prior *in vivo* chemotherapy made no difference to the tissue culture findings.

A further advantage of the tissue culture method for finding the best available drug for any one patient is that it saves involving the patient in the drugs, whose effects may be cumulative, synergistic, or antagonistic (51). In the authors' opinion it is erroneous to decry the tissue culture screening method (40) because of inconsistencies between clinical findings and tissue culture results with apparently identical samples. It would be more correct to realize that samples histologically the same might differ physiologically and functionally. A further advantage of using primary explant material from the patient is that it has not yet become overgrown with one dominant cell type at the expense of others. This is important in view of the numerous reports of many substances having different toxicities toward different cell types.

Whatever type of material, experimental method, and index of toxicity is used, it is necessary that all reports be presented in complete detail so that valid comparisons may be made. For example, results have varied (53) with the percentage of serum in the medium, and even undue chopping of the original material may

produce regenerative hyperplasia (54). Wilson and Barker (49) support the authors' opinion that one index of toxicity used should be morphology. They find it preferable to enzyme inhibition or $^3H$ uptake, and Morasca (55) finds it more sensitive than mitotic index change and $^3H$ uptake. Also George *et al.* (56) caution that antimitotic activity is not necessarily equivalent to antitumor activity, basing this conclusion on the fact that various antimitotic substances had very different antitumor activities.

The use of a tissue culture screening method has of late produced a flood of publications with the negative conclusion that "no differential effect was observed between this substance's action on normal and malignant cells." The greatest bulk of screening experiments is that carried out by and for the Cancer Chemotherapy National Service Center Screening Laboratories, the results of which are published in *Cancer Research* supplements. Both synthetic and natural substances are reported. For example, several of the monthly 1965 and 1966 issues contain reports on between 1,000 and 2,000 substances of either type. These reports cover only substances with which it was found not worth proceeding. There are now several years of experience behind the experiments of these workers, who have streamlined their test to use only one cell line (KB cells derived from a carcinoma of the nasopharynx). They did this after tests on more than 1,000 substances indicated less variation among different cell lines used simultaneously than with successive tests using one cell line. A note of caution must here be introduced, however, to mention the variations more recently found to occur in various KB lines with time (4). Only one index of toxicity is routinely used in these experiments: finding the dose inhibiting protein synthesis to 50% of the level found in controls. While this measure by no means exhausts the effects that these substances may produce in cells, it has been considered an adequate indication of whether further *in vivo* testing is likely to lead to any valuable findings. By mid-1966 these reports spoke of decreasing the amount of tissue culture work performed because it had not yet yielded any useful drug. In the authors' opinion decreasing is the wrong solution to this—a diversification of indices of toxicity used would be likely to lead to other results. Also, regrettably, tissue culture is not the only research method which has failed to yield useful anticancer drugs.

Other recent reports of anticancer screening are now numerous (44, 51, 54, 56–67). The gradual accumulation of contradictory findings among these reports further indicates the importance of detailed reporting of cells, media, cultural conditions, and index of toxicity used—for example, not simply referring to techniques as "standard" (44). The authors think, however, that this need for detail is very slow in being realized. Very few papers (68) draw attention to the advantages of using human rather than experimental animal material or to the good mathematical planning of experiments. For example, at least three dose levels separated in geometric progression (61, 64, 69–71), a principle long recognized (72) in whole animal techniques, are used.

There are many other publications dealing with the effects of drugs on malignant cells, but these were conducted to advance knowledge on the nature of the differences between these cells and normal cells, and thus the drugs were incidental to these experiments and not the main object. Such papers are therefore not within the scope of the present article. Often it is interesting to observe the comparison of malignant to embryonic cells in seeking factors affecting cell differentation—a large part currently played by tissue culture in cancer research.

However, clearly anticancer screening is a field in which much work remains to be done, both in seeking new substances and in choosing the best existing one for a particular patient. In the first case morphological change seems to be the most sensitive index to use, and in the second case, where time presses, the differential respirometer apparatus seems, in the authors' experience with melanomas, to form a suitable measure. Results may be obtained from this index more quickly than from simple growth or from such measures as dehydrogenase activity (51). The large number of replicate experiments possible *in vitro* facilitates the simultaneous examination of various modifications of a molecule found somewhat active in this field.

**Antimicrobial Agents**—Both antibacterial and antiviral drugs have been tested *in vitro* for freedom from toxicity toward cells. The method is particularly applicable to antiviral drugs where the viruses are growing intracellularly both *in vivo* and *in vitro*.

It is rather surprising to find some very early work (73) on antibacterials, including reports on *in vitro* tests for freedom from toxicity, and then to observe that there followed a long period when no such tests were performed. Only now is the method again being appreciated.

A notable early example of such work was its use by Sir Alexander Fleming in 1929 (28) in his original investigations of penicillin. He used the activity of leucocytes (species of origin not stated)

in broth as his criterion of toxicity, and reported that he could find no impairment of function in the presence of penicillin. Similar work with purer penicillin was later reported (74, 75). The latter work established the routine inclusion of penicillin in tissue culture media to prevent the growth of microorganisms, which multiply all too easily in any media supporting the growth of other cells. One group of organisms particularly troublesome in tissue culture is the *Mycoplasmataceae* (pleuro-pneumonia-like organisms). A by-product of work on their elimination is further information on the concentration of many antibiotics which are harmless to various cells, one report alone (76) mentioning 50 antibiotics. Other reports on relative toxicities of various antibiotics indicate (25) that chloramphenicol and chlortetracycline HCl[1] are more toxic in culture than penicillin, and actinomycin and xanthomycin (77, 78) more toxic than other antibiotics.

In the field of antimicrobial testing, nonhuman material is too often used, especially chick embryo material. This seems a needlessly indirect procedure. Early work by Sallé and co-workers (79–82) unfortunately used such material, but they made an advance in the field by reporting results in terms of "toxicity index," relating cell toxicity to antibacterial effect. This was based on the idea that a substance, although very active against bacteria, would have no clinical application if it were at the same time toxic to tissue. The work was extended (83, 84) to measure the maximum time of contact with a given concentration of drug compatible with cell growth, rather than the less realistic measure of maximum concentration allowing cell growth, and was later made quantitative (85) by measuring the number of tissue fragments out of 10 surviving in each part of the experiment and control.

Much of the work on antibacterials has involved ranking currently available substances in order of freedom from toxicity to cells, for example, the comparison of sulfonamides and proflavine (86, 87) on several cell types—the sulfonamides being found to be much less toxic than proflavine—or the comparison of several sulfonamides (88). More recent papers investigate in more detail how these substances affect cells, *e.g.*, proflavine has been reported to produce chromatid breaks (89) or the synthesis of an abnormal RNA (90), and recent work on sulfonamides (91) finds a contradiction in a non-increase in protein synthesis associated with an increase in ribosomes. Rightly, the conclusion is that much further work is required on the action of sulfonamides on cells. Several dyes have also been compared. For example, brilliant green, gentian violet, and acriflavine have been reported to be more toxic than proflavine (92).

Some experiments have been rather more complicated (93–97), being designed to study the effects of antibiotics on live bacteria once these were inside phagocytes. It was found that the phagocytes did, to a certain extent, prevent access of some antibiotics to the bacteria, *e.g.*, penicillin and streptomycin (93), penicillin, streptomycin, and erythromycin (94), streptomycin (95), ampicillin, erythromycin, and oleandomycin (96), but not benzylpenicillin (96), or chloramphenicol, or tetracycline (94). The other report (97) indicated that although the monocytes protected *B. abortus* against streptomycin alone, they did not protect if chlorpromazine were also present. The conclusion is that it helped the streptomycin to penetrate the cells. Similar work not confined to antibiotics, on ingested *M. tuberculosis* (98, 99), reported protection against streptomycin, viomycin, and neomycin B, but not oxytetracycline HCl[2] and isoniazid. The later work on antibiotics also deals with the more detailed effects of these on cells; *e.g.*, streptomycin has been reported to produce nonrandom chromosome breaks in leucocytes (100) and puromycin to inhibit RNA synthesis (101). Such work indicates that new antibiotics might well require careful screening *in vitro* before being marketed.

With all the work on antibacterial substances, only one paper (102) seems to have considered the toxic effects on skin cells of by-products of bacteria multiplying in wounds. This paper reported on pyocyanin from *Ps. pyocyanea* and concluded, from its effects on human skin cells and leucocytes *in vitro*, that it probably would not affect phagocytosis but that it might affect wound closure.

There is less available work on antiviral drugs, from which any useful conclusion can be drawn, than on antibacterial drugs, despite the large amount of work that has been carried out on antiviral chemotherapy. Most materials so far have unfortunately turned out to be too cytotoxic to use. It had been thought that the discovery of the existence of cell receptor sites for adsorbing viruses would have been helpful, but so far metabolic inhibitors once the virus is in the cell seem most useful (103). Many of the

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[1] Marketed as Aureomycin by Lederle Laboratories, Pearl River, N. Y.

[2] Marketed as Terramycin by Chas. Pfizer and Co., New York, N. Y.

substances investigated are merely protein synthesis inhibitors, inhibiting virus only by inhibiting host cells. It seems unlikely (103) that there is any chance of inhibiting a pathway that occurs uniquely in infected cells, a problem analogous with that previously mentioned: finding a substance with a differential action on malignant and normal cells. In antiviral work, however, there is perhaps less scope because of greater species specificity (23), for using some of the exotic species of cells that have been chosen for antibacterial work simply because these cells happen to grow well in culture. Nevertheless, there are reports of the use of goldfish cells (104) and plant and insect cells (105) in the study of human pathogens.

Aside from the innumerable papers on various synthetic chemicals, there are also a few on natural products. Algal extracts (106) and bacterial extracts (107) have been screened, a few of each being reported as worth pursuing. Two other groups of workers (108, 109) have reported the interesting discovery of antiviral substances produced by apparently uninfected cells in culture, as distinct from the well-known production of interferon by virus-infected cells. The substances were effective against some enteroviruses (108) and influenza (109). The authors are cautious about interpreting their unexpected results. Tsilinsky's work (108), however, is extensively mathematical, and his conclusions seem entirely reasonable.

Thus, there is scope for much more work on antimicrobial substances for both systemic and topical use. One side effect of the large amount of tissue culture work done in connection with wound healing and skin grafting is that there are many papers on the growing of skin *in vitro*, either in fragments or reduced to cells (18, 26, 102, 103, 106, 107, 110–128). Also remnants of skin from grafts are readily available from surgical units. Thus, it is a practical proposition to grow skin *in vitro* in quantities suitable for testing ranges of potential topical antimicrobial substances for their effects on skin (129). In all reports on antimicrobial substances, as on anticancer substances, full details of materials and methods and statistics are required so that various papers may be compared. For example, the use of calcium-free medium to allow the cells to stay in suspension (130) could well create differences invalidating comparisons with other papers unless such points are kept in mind. The same authors remark on another important point: the differences of inhibition of respiration found, as between the use of tissue slices and individual cells. This again raises the problem of the drug's access to the cells, a point already noted in work on anticancer drugs. A further precaution to be observed in testing antiviral drugs for their effects on the morphology of cells is to distinguish these effects from the effects due to the viruses themselves. That such distinction is possible is seen from the work of Schauer and Likar (131). The principal advantages of the tissue culture method for antiviral screening of antimicrobial substances then appear to be: first, the number of replicates that can be set up; second, the overcoming of species differences, by the use of human material, in respect to both different species susceptibility to various viruses and different effectiveness of different drugs in different species. A further advantage is that quoted by Nemes (132) that one can find by histochemical techniques if the drug is being incorporated into the host cells.

**Hormones**—The use of tissue culture techniques in the study of the physiological and pharmacological properties of hormones is well established. As has been pointed out (133), the freedom from interference by nervous and other humoral influences prevents much confusion and conflicting conclusions. When one restricts the limit of perspective to those experiments which use pharmacological rather than physiological dose levels, one finds that the scene is dominated by the mass of work carried out on the steroids, natural or synthetic, although insulin and—to a lesser extent—thyroid hormone have received some detailed attention. These substances are used to replace natural deficiencies, and others are used in connection with hormone dependent or influenced tumors. They may therefore be classified as "drugs" in these circumstances. A recent review by LeDouarin (134) covers much recent work on the influence of most of the hormones and vitamins on the growth and differentiation of embryonic bone in culture.

*Steroids*—Considerable confusion existed in the literature concerning the early work on the adrenal steroids and their toxicity in tissue culture systems. Reports ranged from complete inhibition of growth at 100 mcg./ml. with slight inhibition at 50 mcg./ml. down to a marked restriction in growth at 0.3 mcg./ml. (135–140). The cells used in these experiments were largely fibroblasts derived from embryonic chick or mouse sources, although Geiger *et al.* (135) could only demonstrate inhibition using fibroblasts derived from adult sources. The variation in response obtained by the earlier workers could in part be attributed to the variation in cell type and culture systems used. It was subsequently shown that cells of epithelial origin were less af-

fected by steroids than were fibroblasts subjected to similar concentrations (138, 141, 142), although the closely related synthetic steroid, prednisolone, has been shown to increase the proportion of fibroblasts in cultures of bone marrow (143). This apparent anomaly is not substantiated by evidence from other sources, since prednisolone is regarded as more cytotoxic than cortisone (144, 145) and there must be some reserve until the complexities of a marrow culture system are better understood. There was also evidence that the observed effect was dependent upon growth vigor of the culture (146). Poor cultures were stimulated, while rapidly multiplying ones were inhibited. Whether the addition of steroid to the cultures returned suboptimal conditions to optimal and raised optimal to lethal, respectively, was not clarified, and the solution to the problem awaits further work. Pihl and Eker (142) obtained evidence for a tolerance period, after which a strain of liver cells was capable of multiplying even in the presence of a tenfold increase in prednisolone concentration.

Such dependence of results on the details of methods used in toxicity studies can only serve to emphasize the importance of standardizing the conditions of experiments before valid comparisons can be made.

The consensus of the reports would appear to point to a toxic or lethal concentration of 100 mcg./ml. below which the results are variable and also reversible (139). Below 10 mcg./ml. little or no effect was discernible by optical methods or by measuring cellular "growth" in one of its forms.

A similar series of studies was carried out on the related mineralocorticoid, deoxycorticosterone (DOCA), where doses of 35 and 70 mcg./ml. resulted in cellular hyperplasia and an accumulation of intracellular lipid. The changes were not reversible, but as with the glucocorticoids, fibroblasts showed much greater sensitivity than epithelium or endothelium (147, 148). When DOCA and cortisone acetate were added simultaneously to cultures, the effect of the former predominated (149). Whole thickness skin was used in these experiments, and its ability to serve as an autograft after a period in culture was used as a parameter in assessing the effect of these substances. It was found that the skin could be grafted back on the donor after 3 to 4 weeks of culture in a cortisone enriched medium as compared to the 10–14-day maximum time after which controls could not be regrafted. DOCA, on the other hand, while not prolonging the regrafting time, did maintain the structural integrity of the skin better.

This preservation effect of cortisone and related compounds in lower concentrations has been documented from other sources. Embryonic bones in organ culture (150) were maintained better in the presence of hydrocortisone than in its absence, while other reports, dealing with isolated cells in culture, have shown that at lower concentrations (0.1–10 mcg./ml.) the corticosteroids preserved the cells from the degenerative effects of culture exhibited by controls (151, 152). Others claim a positive stimulatory effect rather than simple preservation, using fibroblasts and low concentrations of drug (138, 153).

Not only fibroblasts but also glial cells in cultures of embryo rat cord ganglia (154) showed increased migration and better survival than controls, while in large pieces of tissue central necrosis, which is normally expected, was diminished. The production of myelin in this experiment was unaffected. It is of interest to note that the cells which show greatest sensitivity to the presence of steroid are those responsible for the structural framework and support of the tissues, a possible pointer to the mechanism of the anti-inflammatory properties of the steroids. It has been suggested that these cells may in normal culture be existing at a subphysiological level of steroid, and by adding low concentrations to the medium, the optimal level is attained. This suggestion has much to commend it in this instance where we are dealing with a drug which is also a normal constituent of the *in vivo* environment.

The fundamental reason for low concentrations being beneficial, however, is as obscure as ever. To answer the obvious question, attempts have been made to assess the influence of the corticosteroids on biochemical systems in cultivated cells.

The results of the simple toxicity tests are in part reflected in the data obtained at this level. High concentrations of cortisone were shown to depress the respiration of cells and increase glycolysis, while at lower concentrations aerobic respiration was stimulated (140). The level of alkaline phosphatase in epithelioid cells was raised by low concentration of prednisolone (155), while there was seen to be an increase in RNA synthesis which was not reflected in the RNA/DNA or protein/DNA ratios of the cultures (156). It must be assumed therefore that RNA turnover was increased. Such a fragmentary picture of the influence of the steroid on the molecular biology of the cell must be completed before any fundamental understanding of the influence of these drugs is possible.

The biochemical approach has been used by those interested in the use of steroids to alleviate inflammatory conditions. Mucopolysaccharide production by cells derived from articular or dermal tissue was found to be suppressed, despite a slightly increased rate of growth (153), and studies on the synthesis and subsequent sulfation of chondroitin in tissue culture (157) showed an inhibitory effect of steroids to be exerted at the latter stage.

Thus it becomes evident that while cells can be stimulated to reproduce more vigorously and maintain their morphology under the influence of the corticosteroids, the laying down by cells of a matrix for connective tissue is inhibited.

Kuchler and his co-workers have put forward an interesting theory on the mechanism whereby the corticosteroids achieve their effects (158, 159). The writers postulate, on the evidence obtained from experiments with synthetic steroids, such as estradiol and stilbestrol, that the drug molecules form a monolayer on the surface of the cell, thus preventing by occlusion of active sites, the transfer of ionized or highly polar substances across the cell membrane, as for example, amino acids. The work cited is rather small on which to base a theory so profound, but it is certainly worth pursuing. If this explanation is shown to be correct, then the lethal effects could be explained by a starvation of the cell, denied its nutrients by a blockade of steroid. However, the stimulating effect of lower concentrations awaits a rational explanation.

The chemical modifications which can be made to the steroid nucleus have led several workers to investigate the relationship between structure and activity. Substitution of a hydroxyl in the 11 position increased the toxicity markedly (160), while a keto group in the same position had little effect. Biswas and his colleagues (161) tested 99 different steroids in a simple system consisting of observing outgrowth of fibroblasts from explanted fragments of embryonic chick hearts, and of these substances 36 showed some degree of toxicity which could not be related to the chemical structure of the molecules. The stereochemical configurations were, however, not considered, and it is feasible that some evidence may be obtained to indicate a common mode of action. The addition of a fluorine atom to the molecule increased its toxicity despite the presence of other chemical groupings at other sites (162).

Berliner (163, 164) has observed that different types of cells vary in their metabolic substitution of the basic steroid molecule. On this basis, he postulates that the varying susceptibilities of cells may stem from the different cellular metabolites, which will vary in their toxicity. This work also deserves closer attention, and it is to be hoped that attempts will be made to place the suggestion on a firmer foundation than that upon which it presently rests.

The role of hydrocortisone in suppressing the immune mechanism of the body has been a source of considerable speculation and investigation resulting *inter alia*, in the discovery that cells pretreated for 1 hr. with hydrocortisone are more susceptible to infection by polio virus (165), although simultaneous addition of virus and steroid depressed the infectivity of the virus.

This latter observation received support in a series of experiments (166, 167) which showed that cortisone protected cells from the cytopathic effect of the virus normally caused possibly by lysis of lysosomal membranes, while Holowczak (168) showed that hydrocortisone and actinomycin D together substantially reduced the number of virus particles entering the cells.

The production of interferon by cells in culture is under intensive study, and the production of an antiviral substance by cells growing in the presence of hydrocortisone and virus (169) has been noted, although others claim that the presence of subtoxic concentrations of steroid depress the production of interferon by cells when in contact with pox or arbor viruses (170). Such an observation is in line with clinical observations, but the work is in its early stages and the whole question of interferon production and viral infectivity must be better understood before a rational explanation of the influence of steroids or any other drug is forthcoming.

The corticosteroids have not been the sole group of steroids to be examined for toxicity or activity in tissue culture systems. Of the sex steroids, estrone is unusual in that no toxic properties could be shown under the conditions of a simple outgrowth experiment (161). On the contrary, it was responsible for stimulating normal cell division in hamster ascites cells which would otherwise form multinucleate giant cells (171). This activity was suppressed by the incorporation, along with estrone, of progesterone or a chemical derivative. The degree of suppression could not be related to either chemical structure or progestational activity. Progesterone itself, and also the synthetic estrogen, estradiol, show varying toxicity to different cell types ranging from toxic to chick fibroblasts to growth stimulatory to other lines at similar concentrations (172, 173). Of the steroids tested on mouse mammary tumors, progesterone was the most toxic (174), although such a tissue, which is

normally influenced by the sex hormones, cannot be directly compared with the tissues used in other work. Testosterone and androsterone have also been shown to be toxic to most cells at low concentrations compared to the corticosteroids (151 ,173), although Lasnitzki (175), using organ culture, has maintained the differentiation of young rat prostate glands. Older glands display edepithelial hypertrophy and abnormal mitoses which raised the question of testosterone acting as a carcinogen to senile glands *in vivo*, a point which might be remembered when elderly patients are under treatment with the drug.

Such work, however, borders on the mass of study which has been undertaken, using cell and organ culture, on the physiological actions and relationships of the steroids and associated hormones. The line of demarcation between pharmacology and physiology is at best tenuous and ill-defined. Nevertheless, an account of this work must fall outside the scope of this review.

*Thyroid*—As in the case of steroids, there are conflicting reports here too, some finding stimulation and some finding inhibition. This difficulty has been mentioned in a general review on the mechanism of action of thyroid hormone by Barker (176). Much of the work involving thyroid has dealt with the development of embryonic bone in culture. Although originally undertaken as work on embryology, this type of work could serve as a basis for assay or as a means of comparing related substances. One early established fact (177) in work resulting from the observation of bone changes in hyper- and hypothyroidism was that different embryonic chick bones, in the same experimental conditions, showed different susceptibilities. For example, radii and humeri responded to the growth promoting activity of thyroxine, while to femora and tibiae a harmful effect was observed. This could well be one factor accounting for conflicting reports. An interesting later work by the same authors (178) compared the effects of L-triiodothyronine and of thyroxine on the same tissue, reporting that both substances inhibited growth, but that the former was about four times as potent as the latter. Both, however, showed more effect on the more rapidly growing bones. A further factor reported to affect the result is the age of the chick embryo, stimulation changing to retardation with increasing age, considering radii and ulnae. Different effects on different bones or on the same bones at different stages in development are noted also by Dieterlin-Lievre (133), who mentions that increase in dose can even change stimulation to inhibition. Thus, in any quantitative experiments investigating the effects of thyroid on bone, the bone used, its age, and its growth rate would have to be considered.

The effect of thyroid on tissues other than bone has been investigated less, although stimulation of nerve tissue has been reported (179) and also retardation of embryonic lung and kidney tissue (180). This report, however, mentions that no retardation was observed in lines of cells that had been long in culture, indicating again that these may change in metabolism and therefore be of less value in observing and measuring drug effect than are primary explants.

*Parathyroid*—There are reports too of bone resorption experiments in connection with parathyroid. One comprehensive and mathematically sound paper (181) lists the various factors affecting the result, which would have to be kept constant in a quantitative assay. The tissue culture method is compared with the rat assay method and found to be more sensitive and to require less material, but to be less precise and to take longer. However, the author cautions that even fuller investigations into the factors affecting the result by tissue culture are required since changes in sera in the media affected it, with no reason for this being evident. Another report (182) suggests that constituents in the medium which bind protein may affect the results, and a further report (183) indicates that sulfated polysaccharides may stimulate bone resorption by parathyroid—though it could not be determined whether it was by virtue of the sulfate *per se*.

*Insulin*—A hormone which has received much attention from a tissue culture point of view is insulin. It is in fact fairly often included in tissue culture media for a growth-promoting effect. As will be seen, however, many cell types have been used in investigations of insulin, and again contradictory results of stimulation are found in these reports, some of which are difficult to evaluate for want of adequate information.

Organ culture of chick bones has been used (184) to demonstrate the ability of insulin to inhibit longitudinal growth of the bones while the epiphyses became abnormally large. A curious phenomenon was described whereby a second shaft formed alongside the original bone shaft. The histological differentiation and maturation of the bones was retarded, with poorer staining of cartilage. The level of 0.16 I.U. insulin per ml. of medium is however far in excess of any therapeutic dose which tissues *in vivo*, other than the subcutaneous tissues at the site of injection, are likely to encounter. Further work on bone (133) indicated that previously

reported micromelia was in fact directly due to insulin and not an indirect effect of hypoglycemia.

The same organ culture technique has been used (185) to study the effects of insulin on brown fat. Both glycogen and lipid contents of the cells showed an increase, although the glycogen fell after 3 days while the level of intracellular lipid remained high. The response was most marked in a medium of serum, but such results could be explained by the presence of insulin in the serum itself in addition to the added hormone, or to selective adsorption as suggested by Waymouth (186). She concluded from her own work and the examination of that of numerous others that, since insulin is adsorbed onto glass especially in the absence of other protein, in media containing protein the effect of insulin would probably be seen at lower levels.

An attempt to observe cellular uptake of fluorescein-labeled insulin (187) was unsuccessful. Failure was attributed to differing sensitivities of cell types (those used here being hamster kidney, both normal and tumor and Landschütz ascites cells) since Paul (188) had noted a greatly increased rate of pinocytosis (*i.e.*, uptake) in HeLa cells. This he conjectured to be due to either more energy made available to the cell by insulin for this kind of activity or a direct action of insulin on the cell membrane. On the other hand, Danes and Freshney (189) did not find insulin increasing pinocytosis, their measure of pinocytosis being not vacuole counts but uptake of iron–dextran.

This sensitivity difference of different cell types toward insulin and variation with media used was again recorded (190), insulin being found to promote growth of human fetal skin fibroblasts in maintenance medium but having no effect on their growth rate in growth-promoting medium. HeLa cells, on the other hand, increased their cell material but not cell number under the influence of insulin in maintenance medium, and their growth rate in growth-promoting medium was also significantly increased in the presence of the hormone. Insulin has also been found to have a significant effect on cell numbers in chick heart explants. The increase in the rate of cell division was coupled with a rise in RNA phosphate per cell (191). Waymouth (186) reported also an elongating effect of insulin on L cells. Since this took place after a few hours, it is considered a direct effect on existing cells and not selective survival of long cells.

The metabolic effects of insulin have been studied, using fresh chick embryo and L cells (188). Carbohydrate metabolism was stimulated, with a rise in glucose utilization and lactate production, while pyruvate levels in the medium fell. Stimulation of synthetic processes was found to be erratic, especially with fresh explants. The explanation of this unpredictable reaction was not forthcoming, but the suggestion was made that it might be associated with the ability of the cells to produce high energy compounds dependent on the previous treatment of the cells. Glucose utilization increase by HeLa cells in growth-promoting medium, was reported by Leslie *et al.* (190), and the rate of incorporation of various amino acids into protein has also been reported (192).

Briefly, the effect of insulin is most marked at a metabolic level, stimulating carbohydrate, lipid, and protein metabolism. The effects of insulin are due solely to its hormonal action since the concentrations of the hormone were normally too low for it to be utilized by the cells as a normal protein.

*Gonadotropic Hormones*—Much work has been done on these also in tissue culture (193). Human chorionic gonadotropin or prolactin separately were almost inactive in incorporating acetate-$^{14}C$ into cholesterol in normal rat corpus luteum *in vitro*, but together they increased the cholesterol labeling by 90%. Such synergism would need to be taken into account if either was being assayed *in vitro*. Follicles and stromal cells of rat ovary are also reported (194) to be stimulated by gonadotropic hormone in culture, but this work comments that species of origin of tissue may be important in assessing these substances, different results having been obtained with mouse tissue.

**Alkaloids**—These substances and other weak bases, although differing widely in their pharmacological action *in vivo*, mostly seem to produce similar morphological change, vacuolization, in cells in culture. Tissue culture could not so far be used to distinguish many alkaloids from one another, although it could distinguish them from toxins or metabolic inhibitors whose effect is irreversible. There is one extremely comprehensive article on substances producing vacuolization (195) showing that this is an easily observed cell change and the extent of it varied with concentration. The article goes on to show that changes with concentration are in fact caused by this and not by osmotic pressure changes. From the point of view of making a quantitative assay the occurrence or nonoccurrence of vacuolization would be preferable as an end point to a rating system for amount of vacuolization.

Alkaloids and other weak bases producing vacuolization include procaine hydrochloride and amide, cocaine, atropine, ephedrine, pilo-

carpine, and ammonium chloride. However, dibucaine and quinidine, in similar concentrations, did not produce vacuoles in similar cells, although the cells did die (195). A further report of Buchsbaum and Kuntz (196) also refers to vacuolization, and makes the very useful contribution of an excellent design of equipment for such study. Another useful article on drug-induced vacuolization (197) describes similar reversible effects produced by various autonomic drugs in malignant but not in normal cells, and only if the cells were initially undamaged. Structure of the drug molecule could not be correlated with production of vacuoles, and some drugs antagonistic *in vivo* had additive effects in respect of vacuolization.

The present authors have never observed, or seen illustrations of, drug-induced vacuolization as extensive as the state produced by vacuolating virus. The actual cell size does not seem to enlarge in the former. It may be as Yang, Strasser, and Pomerat (195) reported that the vacuole is an attempt to dilute out the drug, and if this is so, it may be that the cell is overcome ultimately by lethal effects of the drug setting in before vacuolization becomes as pronounced as with virus. None of the above experiments, however, was continued to cell death, since the reversibility of this effect was one of the objectives of this investigation. Vacuole production is not specific to alkaloids and chemically similar substances, having been observed also from plant polysaccharides (197). Nevertheless, its nonspecificity does not prevent its being a useful index of toxicity. A more fundamental investigation of precise causes of vacuolization and nature of vacuole contents is difficult owing to the intangible nature of the vacuoles. Morphine sulfate also produced vacuoles (198) and in fact the cells developed a tolerance to it, becoming able to withstand doses which would have been lethal to fresh cells. In addition, the cells exhibited withdrawal symptoms, adopting morphological characteristics of unhealthy cells. Another alkaloid inducing vacuolization is nicotine (199, 200), but unlike the experiments with morphine, those with nicotine showed neither dependence nor withdrawal symptoms. Another effect of nicotine on cells has also been reported (201). It depressed ciliary activity in ciliated cells, doing this to a greater extent in the presence of influenza infection than when alone. Nevertheless, these works show that ciliary activity is a useful change to observe in cells, and that it can be observed with normal cinemicrographic apparatus, high speed photography of the cilia not being necessary. Another work (202) on inhibition of cilia by nicotine unfortunately used amphibian cells growing at room temperature. The effect of berberine has also been observed (17). The cells rippled, frothed, and bubbled violently, similar to the effects produced by the dibucaine and quinidine mentioned above.

One alkaloid with a very characteristic effect on cells is, of course, colchicine, which arrests mitosis in the metaphase. This effect and its inhibition have been studied (17) using phase contrast cinemicrography, and a method has been developed (203) for measuring movements of isolated cells under the influence of colchicine or other substances. Experiments on combinations of colchicine and other substances showed that narcotine, bulbocarpine, and some apomorphine derivatives exerted a synergistic effect on the action of colchicine and related compounds (204), while tropolone and myo-inositol specifically antagonized its action (205). Such results with mixtures would have to be borne in mind in assays for any of these substances, just as in the case of chorionic gonadotropic hormone and prolactin mentioned above.

**Other Drugs**—No other drug or group of drugs has been studied nearly so extensively as the above in respect to its effects on cells.

Some antihistamine drugs have been studied (206) since they may be applied directly to nasal mucosa cells. These cells unfortunately were not used in the experiments, but in all the types of cells that were used (heart, intestine, and lung fibroblasts) clinical concentrations of the drugs produced no inhibition of growth, although the cells were vacuolated, however. On the other hand, the same workers (1) comment on the high toxicity of various antihistamines toward outgrowth from primary explants of normal adult human skin. Inhibition of tumor cells *in vitro* has also been reported (207), the authors speculating on the relation of this to reduced histamine found in cancer tissue.

One drug, sodium fluoride, has been used in experiments which have received more publicity than was perhaps expected. While the effects of sodium fluoride on cells are of interest to cytologists, they are of interest also to those concerned with adding the substance to water supplies deficient in it, to prevent dental decay in young children. There has been controversial correspondence on whether growth was slowed by the concentrations advocated for drinking water or only by higher concentrations (208–213). A further report (214) deals with inhibition of oxygen uptake and glycolysis but not inhibition of phagocytosis by sodium fluoride. So far it

can be said that inhibition does occur at the various concentrations reported on the cell types used, but it is too early to correlate this with any effect *in vivo.*

Salicylate analogs, cinchophen and chloroquine derivatives, and phenylbutazone and related compounds were examined by Whitehouse (215) using his technique of culturing and assaying cartilage. Anomalies between his results and *in vivo* findings were encountered and probably point to intermediate metabolism of the drugs into active forms by other organs. Sodium aurothiomalate and saligenin, for example, were inactive, but thiomalic acid and salicylate were effective.

Chloroquine has been further investigated for cell-inhibiting properties (62, 216) using chick embryo cells. Vacuolization and decreased growth were observed at 1/40,000, although lower concentrations stimulated growth. The cells also disaggregated to individual cells, like trypsinized cells. A detailed work on chloroquine (217) was undertaken, in view of its use in some conditions with abnormalities of the immune response, to find exactly what its mode of action in such cases might be. It was concluded that it "stabilized" lysosome membrane preventing enzyme release which would start lymphocyte derepression. It appeared to act only on lymphocytes responding to a stimulus. This paper throughout seems logically reasoned and mathematically sound, and for these reasons, as well as its topic, it is of interest.

Organophosphorus substances have been studied (218), their amount being estimated by the effect on ciliary activity of cells *in vitro.* Decrease of ciliary activity is not of course a specific effect of these substances, having been observed also in connection with local anesthetics (219) and nicotine (201).

An interesting use has been made of isolated, beating, chick embryo heart cells to compare digoxin, quinidine, and procaine amide (220). The action of digoxin was to produce arrhythmias, and this is in keeping with the view that digitalis preparations have an intracellular action. Quinidine and procaine amide had a depressant action on beating, and the potentiating effect of quinidine on digitalis recorded *in vivo* was reflected in tissue culture, indicating the potential usefulness of tissue culture model in studying cardiac drugs. Very little use has been made of this so far. In fact, the comment made in 1954 (1) is still applicable: "Similar (i.e. tissue culture) screening may be made of the action of cardiac glucosides on beating heart fragments. This possibility has not yet been adequately explored, but it offers many promising opportunities for fruitful observations."

Acetylcholine has also been used (221) in a study of the sensitivity to the drug of rat diaphragm muscle fibers when grown by organ culture methods. It was concluded that muscle fibers themselves produced changes in the fiber membrane, once nervous connection had been lost, to enable it to react to cholinergic drugs, thus confirming previous *in vivo* findings.

The irritant action of a number of substances, including podophyllin, croton oil, turpentine, and peptone was studied (222), and their effect on the motility of leucocytes was observed.

Anticoagulants have not been widely studied (223, 224). The former studies compared the effect of clinical concentrations of heparin, polyanetholsulfonic acid, and sodium citrate on leucocyte migration and phagocytosis. Only the heparin did not affect these. However, the author found no correlation between migration and phagocytosis, though later work usually describes a cessation of movement during pinocytosis. The other work involving heparin was with hamster sarcoma cells where effects were seen on the morphology, contents, mitotic index, and chromosomes, leading the authors to wonder if these were caused by a combination of sodium citrate with the proteins of the mitotic spindle. Further evidence on a relation between heparin and cell division was described by Lippman (225), who considered that, if mucopolysaccharides were removed from the cell surface, $Ca^{2+}$ could then penetrate, increase cytoplasmic viscosity, and initiate chromosome condensation, and thence cell division. This paper includes a list of many sulfated polysaccharides reported to block cell division in many types of cells.

The tissue culture method offers scope for the development of assay methods for some immunological preparations. As is well known (23), there are still several such products for which there is no assay which correlates well with clinical results. The group of immunological products most obviously and directly lending itself to this assay method is the antisera, whose protective capacity against the relevant toxin may be assessed on cells. There are of course already some officially recognized tests of this nature, *e.g.*, the Biological Assay of Poliomyelitis Neutralising Antibody of the "British Pharmacopoeia," where the content of this antibody in a sample of serum is determined by comparing the dose of serum necessary to protect tissue cultures against infection from a test dose of poliomyelitis virus with the dose of a standard serum necessary to give the same protection (226). The cells

used are usually monkey kidney and the index of toxicity is cell damage as seen by light microscope. Tissue culture work has included also work on antibody production *in vitro* (227) performed with a view to gathering further knowledge on the mechanisms of antibody production and therefore irrelevant to this paper.

The application of tissue culture in active immunity preparations would be in testing toxoids and vaccines for freedom from toxicity and, where appropriate, freedom from live viruses. There are five recent works on a tissue culture assay for diphtheria preparations. The indices of toxicity investigated have been demyelination of fetal rat cord ganglion cells (154) or inhibition of protein synthesis in KB cells (228), a result being obtainable here in 90 min., which is usefully quick. Also the cellular method has been reported (229) to be quicker and cheaper than the use of guinea pigs. However, one important point still to be cleared up is whether different cell types affect the result. There are reports both for and against this (*References 230* and *231*, respectively).

Staphylococcal toxins have also been investigated in tissue culture (232, 233) on various cell lines, using both morphological and biochemical indices of toxicity. Although the work was done to gain knowledge on the relation between pathogenicity and the production of various toxins, the method is equally applicable to assay, the basis being there already in the mathematical detail quoted in these papers.

In connection with diagnostic immunological preparations, a proliferative effect of tuberculin purified protein derivative on human peripheral macrophages has been reported (234), but no correlation was established between this effect and the donor's skin sensitivity state toward ordinary tuberculin testing.

In the field of nonmicrobiological immunologicals, spider venom has been reported (235) to produce degenerative changes in HeLa and L cells within a few hours, the changes not occurring if the venom was neutralized with antiserum. This again could serve as a basis for assay of the antivenom.

It seems then that the most promising "growth points" of tissue culture as applied to immunological preparations are the quantitative assays of antisera and the testing for freedom from toxicity of viral vaccines and bacterial toxoids.

Drugs acting on nerve cells are probably the most difficult to investigate by tissue culture (236), since these cells in culture, if normal, probably move and divide less than most other cell types. A biochemical rather than a microscopical index of effect is therefore indicated. However, other nervous system cells have been used—for example, oligodendrocytes (237)—to study the effect of chlorpromazine, an increase in contraction rate being the effect observed. The toxicity of barbiturates toward outgrowths from embryonic chick spinal cord and heart tissues has also been measured (238). It was noted that heart tissue was more resistant to the range of barbiturates tested. The toxicity of convulsant barbiturates was in keeping with their *in vivo* toxicity, but anomalies were blatant in the case of hypnotic barbiturates. Such anomalies would probably indicate an *in vivo* metabolic pathway synthesizing a more or a less toxic molecule.

When the discovery was being investigated that the antiepileptic drug phenytoin (B.P.) aided wound healing, it was found (239) that it stimulated the growth of a line of human gingival fibroblasts, although experiments with analogs failed to clarify which part of the molecule was responsible. The mode of action was not cleared up by a later comprehensive work (240), using primary explants from dental surgery from 15 patients to set up 596 test experiments and 533 controls.

Finally, the tragedy of thalidomide stimulated work into its effects on various tissues grown *in vitro*, resulting in the report (241) that the soluble hydroxy derivative of the drug was capable of inhibiting the synthesis of protein by embryonic cells. Other work (242) has endeavored to find the mechanism of action whereby thalidomide inhibited small lymphocytes in culture using the two racemates and various degradation products, all in concentrations similar to those teratogenic in animals. It was hoped that this might give a clue to the teratogenic mechanism. However, more work is required for any proof, and only a theory could be put forward, that thalidomide might be antagonistic to glutamic acid or glutamine.

## CONCLUSION

The use of tissue culture techniques in the study of drug action and toxicity is as yet in its infancy. The bulk of the work presented in this publication has dealt with the toxicity of materials toward explants of tissues, often embryonic avian tissues whose metabolism cannot be comparable in every respect to the metabolism of human tissues.

Generally, toxicity is recognized by failure of a tissue or cell culture to grow. However, growth in tissue culture systems is difficult to define. It may be taken to mean an increase in cell number,

increase in cell size, or increase in metabolic rate. These criteria, however, may be completely independent and affected by unrelated factors. It has been repeatedly emphasized that the different indices of growth are not necessarily synonymous. Cunningham and Kirk (32) found area of explant, cell size, and cell number may vary independently, and Ritter (243) found that protein and cell counts could also vary independently. The authors feel confident that, as the effect of more drugs in tissue culture are investigated, more, and more specific, effects will be observed. For example, in their own work, a curious arcing of H.Ep. 2 cells has been observed with spermine (22). Effects on stages of cell division are the group of effects investigated in greatest detail as yet, leaving much to be done on all the other aspects of cell biology.

The advent of time-lapse cinemicrography and the sophistication of many techniques in cytology and biochemistry have led to the possibilities of investigation into the detailed action of medicinal substances on tissues and on cells, and of the metabolism of the drugs by the cells. For too long, this has been a subject where isolated workers scratch at the outside of a particular problem when the possibilities of reaching a fundamental solution have been numerous. Fortunately, in the laboratories of the world, attention is now being paid to those basic phenomena essential to the life of the cell. Ultimately there will be increased knowledge of the effect of drugs in their widest sense on those phenomena, a logical sequence to the more elementary gross toxicity tests which have been hitherto carried out. The rewards of such a study are potentially great, and tissue culture techniques have clearly an increasing part to play in the study of the interaction between the organism and pharmacologically active substances.

## SUMMARY

This review investigates the effects of drugs on animal cells grown *in vitro*. The cells include separated individual cells, tissues, and small organs, both primary explant material and serially propagated lines, and also material from various species. The techniques used to examine the effects of the drugs on the cells include simple microscopy, time-lapse cinemicrography, electron microscopy, and many biochemical estimations.

It is shown that only a small fraction of available drugs have as yet been examined for direct cellular effect. Also it is shown that the method can, with value, be included in routine examination of new drugs. The advantages and limitations of this technique are listed and compared with other pharmacological techniques.

## REFERENCES

(1) Pomerat, C. M., and Leake, C. D., *Ann. N. Y. Acad. Sci.*, **58**, 1110(1954).
(2) Diamond, L., and Koprowski, H., *Am. Assoc. Cancer Res. Proc.*, **6**, 15(1965).
(3) Coriell, L. L., Greene, A. E., and Silver, R. K., *Cryobiology*, **1**, 72(1964).
(4) Foley, G. E., Handler, A. H., Lynch, P. M., Wolman, S. R., Stulberg, C. S., and Eagle, H., *Cancer Res.*, **25**, 1254 (1965).
(5) Malinin, T. I., and Perry, V. P., *Cryobiology*, **2**, 18 (1965).
(6) Malinin, T. I., Perry, V. P., and Dolan, N. F., *ibid.*, **2**, 19(1965).
(7) Narula, P. N., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19**, No. 105 (1965).
(8) Gillette, R. W., Goulian, D., Jr., and Conway, H., *J. Natl. Cancer Inst.*, **34**, 643(1965).
(9) Pomerat, C. M., *Methods Med. Res.*, **4**, 198(1952).
(10) Pomerat, C. M., *J. Am. Vet. Med. Assoc.*, **129**, 458(1956).
(11) Fell, H. B., *J. Clin. Pathol.*, **11**, 489(1958).
(12) Osgood, E. E., *J. Lab. Clin. Med.*, **24**, 954(1939).
(13) Murray, M. R., and Kopech, G., "A Bibliography of the Research in Tissue Culture," Academic Press Inc., New York, N. Y., 1953.
(14) Phillips, H. J., and Andrews, R. V., *Exptl. Cell Res.*, **16**, 678(1959).
(15) Kay, E. R. M., *Cancer Res.*, **25**, 764(1965).
(16) Verne, J., *Ann. N. Y. Acad. Sci.*, **58**, 1195(1954).
(17) Lettré, R., *ibid.*, **58**, 1085(1954).
(18) Dawson, M., *J. Roy. Microscop. Soc.*, **82**, 1(1963).
(19) Dawson, M., Dryden, W. F., and Matthews, J. E., *ibid.*, **83**, 391(1964).
(20) Dawson, M., Dryden, W. F., and Matthews, J. E., *Proc. Roy. Microscop. Soc.*, **1**, 156(1966).
(21) Dawson, M., and Dryden, W. F., "Toxicity Study of Phagocytin on Human Skin Cells," film shown at annual meeting of British Association for the Advancement of Science, Cambridge, England, September 1965.
(22) Dawson, M., and Dryden, W. F. "Toxicity Testing in Tissue Culture," film shown at meeting of Research Film Section of International Scientific Film Association, Roscoff, France, September 1966.
(23) Dawson, M., "Immunological and Blood Products," Heinemann, London, England, to be published.
(24) Hsu, T. C., *Texas Rept. Biol. Med.*, **10**, 336(1952).
(25) Lépine, P., Barski, G., and Maurin, J., *Proc. Soc. Exptl. Biol. Med.*, **73**, 252(1950).
(26) Ulloa-Gregori, O., Blocker, T. G., Nowinski, W. W. and Pomerat, C. M., *Texas Rept. Biol. Med.*, **8**, 400(1950).
(27) Heilman, D. H., *Bacteriol. Proc.*, Abstr. No. M.8, 1965, p. 41.
(28) Fleming, A., *Brit. J. Exptl. Pathol.*, **10**, 226(1929).
(29) Chabbert, Y. A., and Vial, H., *Exptl. Cell Res.*, **22**, 264(1961).
(30) Paul, J., "Cell and Tissue Culture," 3rd ed., Livingstone, Edinburgh, Scotland, 1965, pp. 304–343.
(31) Merchant, D. J., Kahn, R. H., and Murphy, W. H., Jr., "Handbook of Cell and Organ Culture," 2nd ed., Burgess Publishing Co., Minneapolis, Minn., 1964.
(32) Cunningham, B., and Kirk, P. L., *J. Cellular Comp. Physiol.*, **20**, 343(1942).
(33) Cruickshank, C. N. D., "Report of the Medical Research Council for the Year 1959–1960," Her Majesty's Stationery Office, London, England, 1961, p. 40.
(34) Cruickshank, C. N. D., *Exptl. Cell Res.*, **7**, 374 (1954).
(35) Glick, D., "Quantitative Chemical Techniques of Histo- and Cytochemistry," Interscience, vol. 1, London, England, 1964.
(36) Danes, B. S., Christiansen, G. S., and Leinfelder, P. J., *Exptl. Cell Res.*, **5**, 234(1953).
(37) Danes, B. S., Broadfoot, M. M., and Paul J., *ibid.*, **30**, 369(1963).
(38) Edwards, B. F., and Lee, J. B., "Tissue Culture Association Annual Meeting Abstract Papers, in *Excerpta Med.*, **19**, No. 63 (1965).
(39) Holmes, R., and Eliassen, B. P., *ibid.*, **19**, No. 61 (1965).
(40) Cobb, J. P., Walker, D. G., and Wright, J. C., *Cancer Res.*, **21**, 583(1961).
(41) Paul, J., *ibid.*, **22**, 431(1962).
(42) Hurley, J. D., and Yount, L. J., *Am. J. Surg.*, **109**, 39(1965).
(43) Wojewski, A., and Przeworska-Kaniewicz, D., *J. Urol.*, **93**, 721(1965).
(44) Wright, J. C., Cobb, J. P., Gumport, S. L., Golomb, F. M., and Safadi, D., *New Engl. J. Med.*, **257**, 1207(1957).
(45) Chýle, P., *Folia Biol. (Prague)*, **9**, 312(1963).
(46) Kuhns, W. J., Bramson, S., Chessin, L., and Hirschhorn, K., *Federation Proc.*, **24**, 368(1965).
(47) Caspersson, T., Farber, S., Foley, G. E., Killander D., and Zetterberg, A., *Exptl. Cell Res.*, **39**, 365(1965).

(48) Di Paolo, J. A., *Cancer Chemotherapy Rept.*, **44,** 19(1965).
(49) Wilson, C. B., and Barker, M., *ibid.*, **44,** 9(1965).
(50) McDonald, G. O., and Cole, W. H., *Surgical Forum*, **X,** 67(1959).
(51) Di Paolo, J. A., and Dowd, J. E., *J. Natl. Cancer Inst.*, **27,** 807(1961).
(52) Johnson, R. E., Zelen, M., and Kemp, N. H., *ibid.*, **34,** 265(1965).
(53) Kupiecki, F. P., *Cancer Res.*, **25,** 417(1965).
(54) Lasnitzki, I., *J. Natl. Cancer Inst.*, **35,** 339(1965).
(55) Morasca, L., *European J. Cancer*, **1,** 141(1965).
(56) George, P., Journey, L. J., and Goldstein, M. N., *J. Natl. Cancer Inst.*, **35,** 355(1965).
(57) Eagle, H., and Foley, G. E., *Am. J. Med.*, **21,** 739 (1956).
(58) Limbosch-Rolin, S., *Ann. Biol.*, **74,** 63(1963).
(59) Cobb, J. P., *Ann. N. Y. Acad. Sci.*, **84,** 513(1960).
(60) Galbraith, W., Mayhew, E., Roe, E., Handel, J., and Sugár, J., British Empire Cancer Campaign for Research, 41st Annual Report, 1963, p. 126.
(61) Yarnell, M., Ambrose, E. J., Shepley, K., and Tchao, R., *Brit. Med. J.*, ii, 490(1964).
(62) Hiraki, K., and Kimura, I., *Acta Med. Okayama*, **17,** 231(1963).
(63) Toplin, I., *Cancer Res.*, **19,** 959(1959).
(64) McBride, T. J., Schmitter, R. W., Oleson, J. J., and Toplin, I., *ibid.*, *Suppl.*, 23, 1(1963).
(65) Kim, J. H., and Eidenoff, M. L., *Federation Proc.*, **24,** 331(1965).
(66) Rich, M. A., Meyers, P., Weinbaum, G., Cory, G. C., and Suhadolnik, R. J., *Biochim. Biophys. Acta*, **95,** 194(1965).
(67) Antikajian, G., Wright, L. T., Plummer, J. I., and Weintraub, S., *J. Natl. Cancer Inst.*, **12,** 269(1952).
(68) Talyzina, V. A., Timofeevskaya, E. A., Urazova, A. P., and Firsova, G. A., *Antibiotiki*, **X,** 722(1965).
(69) Mungyerova, G., Babusikova, O., Kalafut, F., and Jacz, K., *Neoplasma*, **12,** 289(1965).
(70) Umeda, M., *Acta Pathol. Japon.*, **14,** 373(1964).
(71) Toplin, I., *Cancer Res.*, **21,** 1042(1961).
(72) Perry, W. L. M., Design of Toxicity Tests, Medical Research Council Special Report Series, No. 270, His Majesty's Stationery Office, London, England, 1950.
(73) Fleming, A., *Proc. Roy. Soc. Sci., Ser. B*, **96,** 171 (1924).
(74) Abraham, E. P., Chain, E., Fletcher, C. M., Gardiner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, ii, 177(1941).
(75) Gey, G. O., Gey, M. K., Inui, F., and Vedder, H., *Bull. Johns Hopkins Hosp.*, **77,** 116(1945).
(76) Perlman, D., and Brindle, S. A., *Bacteriol. Proc.*, Abstr. No. V136, 1965.
(77) Brotzu, G., *Minerva Chir.*, **17,** 1254(1962).
(78) Perlman, D., Guiffre, N. A., Jackson, P. W., and Giardinello, F. E., *Proc. Soc. Exptl. Biol. Med.*, **102,** 290(1959).
(79) Sallé, A. J., and Lazarus, A. S., *ibid.*, **34,** 371(1936).
(80) Sallé, A. J., McOmie, W. A., and Schechmeister, I. L., *J. Bacteriol.*, **34,** 267(1937).
(81) Sallé, A. J., McOmie, W. A., Schechmeister, I. L., and Foord, D. C., *ibid.*, **37,** 639(1939).
(82) *Ibid.*, **37,** 694(1937–1938).
(83) Witlin, B., *Am. J. Pharm.*, **113,** 203(1941).
(84) *Ibid.*, **113,** 353(1941).
(85) Paff, G. H., Lehman, R. A., and Halperin, W. H., *Proc. Soc. Exptl. Biol. Med.*, **58,** 323(1945).
(86) Jacoby, F., *Brit. J. Exptl. Pathol.*, **26,** 137(1945).
(87) Jacoby, F., Medawar, P. B., and Willmer, E. N., *Brit. Med. J.*, ii, 149(1941).
(88) Pomerat, C. M., Capouya, M., and Greenleaf, F. I., *Texas Rept., Biol. Med.*, **2,** 97(1944).
(89) Ostertag, W., and Kersten, W., *Exptl. Cell Res.*, **39,** 296(1965).
(90) Scholtissek, C., *Biochim. Biophys. Acta*, **103,** 146 (1965).
(91) Kutzsche, A., Merker, H.-J., and Schwarz, W., *Naturwissenschaften*, **52,** 118(1965).
(92) Robson, J. M., *J. Physiol.*, **102,** 7P(1943–1944).
(93) Baker, H. J., *Ann. N. Y. Acad. Sci.*, **58,** 1232(1954).
(94) Shaffer, J. M., Kucera, C. J., and Spink, W. W., *J. Exptl. Med.*, **97,** 77(1953).
(95) Zhukov, V. G., and Navashin, S. M., *Antibiotiki*, **X,** 318(1965).
(96) Zak, A. F., *ibid.*, **X,** 733(1965).
(97) Goldenbaum, E. G., and Fukui, G. M., *Bacteriol. Proc.*, Abstr. No. M. 176, 1965, p. 69.
(98) Mackaness, G. B., *J. Pathol. Bacteriol.*, **64,** 429 (1952).
(99) Mackaness, G. B., and Smith, N., *Ann. Rev. Tuberculosis*, **66,** 125(1952).
(100) Cohen, M. M., Shaw, M. W., and Craig, A. P., *Proc. Natl. Acad. Sci. U. S.*, **50,** 330(1963).
(101) Holland, J. J., *ibid.*, **50,** 436(1963).
(102) Cruickshank, C. N. D., and Lowbury, E. J. L., *Brit. J. Exptl. Pathol.*, **34,** 583(1953).
(103) Kaufman, H. E., *Progr. Med. Virol.*, **7,** 116(1965).
(104) Kroeker, H. M., and Bird, T. J., *Bacteriol. Proc.*, Abstr. No. V137, 1965.
(105) Maramorosch, K., Mitsuhashi, J., Streissle, G., and Hirumi, H., *ibid.*, Abstr. No. V138, 1965.
(106) Starr, T. J., Deig, E. F., Church, K. K., and Allen, M. B., *Texas Rept. Biol. Med.*, **20,** 271(1962).
(107) Carver, D. H., and Naficy, K., *Proc. Soc. Exptl. Biol. Med.*, **116,** 548(1964).
(108) Tsilinsky, Ya. Ya., *Acta Virol. (Prague) (Engl. Ed.)*, **7,** 350(1963).
(109) Pumper, R. W., Yamashiroya, H. M., and Alfred, L. J., *Nature*, **192,** 1101(1961).
(110) Billingham, R. E., and Reynolds, J., *Brit. J. Plastic Surg.*, **5,** 25(1952–1953).
(111) Medawar, P. B., *Nature*, **148,** 783(1941).
(112) Bassett, C. A. L., Evans, V. J., and Earle, W. R., *Plastic Reconstr. Surg.*, **17,** 421(1956).
(113) Fell, H. B., *Ann. N. Y. Acad. Sci.*, **58,** 1183(1954).
(114) Fell, H. B., *Brit. Med. Bull.*, **12,** 35(1956).
(115) McGowan, T. R., and Bang, F. B., *Bull. Johns Hopkins Hosp.*, **107,** 63(1960).
(116) Trowell, O. A., *Exptl. Cell Res.*, **6,** 246(1954).
(117) *Ibid.*, **16,** 118(1959).
(118) McGowan, T. R., *Natl. Cancer Inst. Monograph*, **11,** 95(1963).
(119) Boök, J. A., Fraccaro, M., and Lindsten, J., *Acta Paediat.*, **48,** 453(1959).
(120) Harnden, W. G., *Brit. J. Exptl. Pathol.*, **XLI,** 31 (1960).
(121) Gilbert, D., Mier, P. D., and Jones, T. E., *J. Invest. Dermatol.*, **40,** 165(1963).
(122) Medawar, P. B., *Quart. J. Microscop. Sci.*, **89,** 187 (1948).
(123) Parshley, M. S., and Simms, H. R., *Am. J. Anat.*, **86,** 163(1950).
(124) Söltz-Szöts, J., *Arch. Klin. Exptl. Dermatol.*, **214,** 594(1962).
(125) Lewis, S. R., Pomerat, C. M., and Ezell, D., *Anat. Rec.*, **104,** 487(1949).
(126) Penttinen, K., and Saxén, E., *Nature*, **184,** 1570 (1959).
(127) Terasaki, P. I., and Chamberlain, C. C., *J. Exptl. Med.*, **115,** 439(1962).
(128) Bakken, P. C., Evans, V. J., Earle, W. R., and Stevenson, R. E., *Am. J. Hyg.*, **73,** 96(1961).
(129) Dawson, M., Dryden, W. F., and Matthews, J. E., paper presented to the British Tissue Culture Association, Glasgow, Scotland, 1964.
(130) Currie, W. D., and Gregg, C. T., *Biochim. Biophys. Res. Commun.*, **21,** 9(1965).
(131) Schauer, P., and Likar, M., *Pathol. Microbiol.*, **28,** 371(1965).
(132) Nemes, M. M., and Hilleman, M. R., *Proc. Soc. Exptl. Biol. Med.*, **119,** 515(1965).
(133) Dieterlin-Lièvre, F., "Les Cultures Organotypiques," Masson & Cie, Paris, France, 1965, p. 135.
(134) LeDouarin, G., *Année Biol.*, **3,** 233(1964).
(135) Geiger, R. S., Dingwall, J. A., and Andrus, W. de W., *Am. J. Med. Sci.*, **231,** 427(1956).
(136) Kaufman, N., Masson, E. J., and Kinney, T. D., *Am. J. Pathol.*, **29,** 761(1953).
(137) Rozen, V. B., and Chernin, L. S., *Federation Proc. Transl. Suppl.*, **24,** T861(1965).
(138) Grossfield, H., and Ragan, C., *Proc. Soc. Exptl. Biol. Med.*, **86,** 63(1954).
(139) Holden, M., and Adams, L. B., *ibid.*, **95,** 364(1957).
(140) Grossfield, H., *Endocrinology*, **65,** 777(1958).
(141) Kline, I., Leighton, J., Belkin, M., and Orr, H. C., *Cancer Res.*, **17,** 780(1957).
(142) Pihl, A., and Eker, P., *Biochem. Pharmacol.*, **14,** 1065(1965).
(143) Farnes, P., and Barker, B. E., *Metabolism*, **14,** 75 (1965).
(144) Nowell, P. C., *Cancer Res.*, **21,** 1518(1961).
(145) Elrod, L. M., and Schrek, R., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19,** No. T.53 (1965).
(146) Sujard, C., and Chany, E., *Compt. Rend.*, **257,** 973 (1963).
(147) Cornman, I., *Science*, **113,** 37(1951).
(148) Gillette, R. W., and Buchsbaum, R., *Proc. Soc. Exptl. Biol. Med.*, **89,** 146(1955).
(149) Gillette, R. W., Goulian, D., and Conway, H., *J. Natl. Cancer Inst.*, **34,** 643(1965).
(150) Schryver, H. F., *Exptl. Cell Res.*, **37,** 327(1965).
(151) Arpels, C., Babcock, V. I., and Southam, C. M., *Proc. Soc. Exptl. Biol. Med.*, **115,** 102(1964).
(152) Connor, J., and Marti, A., *Federation Proc.*, **24,** 199(1965).
(153) Castor, C. W., *J. Lab. Clin. Med.*, **60,** 788(1962).
(154) Murray, M. R., and Peterson, E. R., *Excerpta Med., Intern. Congr. Ser.*, **94,** E221(1965).
(155) Melnykovych, G., "Tissue Culture Association Annual Meeting Abstract Papers," in *Excerpta Med.*, **19,** No. T14 (1965).
(156) Hijmans, J. C., and McCarty, K. S., *ibid.*, No. T48 (1965).
(157) Whitehouse, M. W., and Lash, J. W., *Nature*, **189,** 37(1961).
(158) Kuchler, R. J., Arnold, N. J., and Graver, R. C., *Proc. Soc. Exptl. Biol. Med.*, **111,** 798(1962).
(159) Campbell, G. C., and Kuchler, R. J., *Bacteriol. Proc.*, **1965,** 120.
(160) Ruhman, A. G., and Berliner, D. L., *Federation Proc.*, **24,** 575(1965).
(161) Biswas, S., MacDougall, J. D. B., and Cook, R. P. *Brit. J. Exptl. Pathol.*, **45,** 13(1964).
(162) Perlman, D., Guiffre, N. A., Brindle, S. A., and Pan, S. C., *Proc. Soc. Exptl. Biol. Med.*, **111,** 623(1962).
(163) Berliner, D. L., *Cancer Res.*, **25,** 1085(1965).
(164) Berliner, D. L., *Ann. N. Y. Acad. Sci.*, **116,** 1078 (1964).

(165) Hellman, A., Kalter, S. S., and Daye, G. T., *Acta Virol. (Prague)*, **9**, 224(1965).
(166) Macieira-Coelho, A., Fernandes, M. V., and Meilman, W. J., *Proc. Soc. Exptl. Biol. Med.*, **119**, 631(1965).
(167) Wheeler, C. E., Harvie, E. A., and Canby, C. M., *J. Invest. Dermatol.*, **36**, 89(1961).
(168) Holowczak, J. A., *Bacteriol. Proc.*, **1965**, 119.
(169) Hannoun, C., Fernandes, M. V., and Macieira-Coelho, A., *Proc. Soc. Exptl. Biol. Med.*, **119**, 153(1965).
(170) Mendelson, J., and Glasgow, L. A., *Bacteriol. Proc.*, **1965**, 98.
(171) Pincus, G., and Gordon, H. L., *Steroids Suppl.*, **1**, 193(1965).
(172) Kawada, I., *Japan. J. Exptl. Med.*, **29**, 615(1959).
(173) von Haam, E., and Cappel, L., *Am. J. Cancer*, **39**, 350(1940).
(174) Rivers, E. M., Elias, J. J., Bern, H. A., Napalkov, N. P., and Pitelka, D. R., *J. Natl. Cancer Inst.*, **31**, 671(1963).
(175) Lasnitzki, I., *J. Endocrinol.*, **12**, 236(1955).
(176) Barker, S. B., *Physiol. Rev.*, **13**, 205(1951).
(177) Fell, H. B., and Mellanby, E., *J. Physiol.*, **127**, 427(1955).
(178) *Ibid.*, **133**, 89(1956).
(179) Hamburgh, M., *Anat. Rec.*, **151**, 357(1965).
(180) Leslie, I., and Sinclair, R., *Exptl. Cell Res.*, **17**, 272 (1959).
(181) Raisz, L. G., *J. Clin. Invest.*, **44**, 103(1965).
(182) Gaillard, P. J., *Develop. Biol.*, **7**, 103(1963).
(183) Goldhaber, P., "Tissue Culture Association Annual Meeting, Abstract Papers," in *Excerpta Med.*, **19**, No. 47 (1965).
(184) Chen, J. M., *J. Physiol.*, **125**, 148(1954).
(185) Sidman, R. L., *Anat. Rec.*, **124**, 723(1956).
(186) Waymouth, C., and Reed, W. E., *Texas Rept. Biol. Med.*, **23**, (Suppl. 1) 413(1965).
(187) Cormack, W. H., and Ambrose, E. J., *J. Roy. Microscop. Soc.*, **81**, 11(1962).
(188) Paul, J., *J. Exptl. Zool.*, **142**, 475(1959).
(189) Danes, B. S., and Freshney, M. S., *Texas Rept. Biol. Med.*, **23**, (Suppl. 1), 221(1965).
(190) Leslie, I., Fulton, W. C., and Sinclair, R., *Biochem. J.*, **63**, 18p(1956).
(191) Leslie, I., and Davidson, J. N., *Biochem. J.*, **49**, XLI (1951).
(192) Singh, V. N., Nataf, B. M., and Chaikoff, I. L., *Life Sci.*, **4**, 1603(1965).
(193) Gospodarowicz, D., and Legault-Demare, J., *Acta Endocrinol.*, **42**, 509(1963).
(194) Pavic, D., *J. Endocrinol.*, **26**, 531(1963).
(195) Yang, W. C. T., Strasser, F. F., and Pomerat, C. M., *Exptl. Cell Res.*, **38**, 495(1965).
(196) Buchsbaum, R., and Kuntz, J. A., *Ann. N. Y. Acad. Sci.*, **58**, 1303(1954).
(197) Belkin, M., Hardy, W. C., Orr, H. C., and Lachman, A. B., *J. Natl. Cancer Inst.*, **28**, 187(1962).
(198) Corssen, G., and Skora, I. A., *J. Am. Med. Assoc.*, **187**, 328(1964).
(199) Bucher, O., *Bull. Schweiz. Akad. Med. Wiss.*, **1**, 252 (1945).
(200) Kokubu, T., and Pollak, O. J., *Exptl. Mol. Pathol.*, **1**, 293(1962).
(201) Dawson, F. W., Harding, H. B., Ballenger, J. J., De Ruyter, M., and Moore, J., *Federation Proc.*, **24**, 302 (1965).
(202) Amenta, P. S., Imbriglia, F. X., and Ferreira, R. C., *Anat. Rec.*. **151**, 481(1965).
(203) Schaerer, P., *Acta Haematol.*, **1**, 178(1948).
(204) Lettré, H., *Ann. N. Y. Acad. Sci.*, **58**, 1264(1954).
(205) Benitez, H. H., Murray, M. R., and Chargaff, E., *ibid.*, **58**, 1288(1954).
(206) Pomerat, C. M., Lay, M., and Emerson, G. A., *Texas Rept. Biol. Med.*, **2**, 316(1944).
(207) Honti, G., and Putnoky, Gy., *Acta Morphol., Suppl. XIII*, **1965**, 70.
(208) Armstrong, W. D., Pollock, M. E., and Singer, L., *Brit. Med. J.*, **i**, 1435(1965).
(209) Berry, R. J., and Trillwood, W., *ibid.*, **ii**, 1064(1963).
(210) *Ibid.*, **i**, 793(1965).
(211) Nias, A. H. W., *ibid.*, **i**, 1672(1965).
(212) Armstrong, W. D., Blomquist, C. H., Singer, L., Pollock, M. E., and McLaren, L. C., *ibid.*, **i**, 486(1965).
(213) Giancotti, M., and Gruddi, A., *Ann. Stomatol.*, **14**, 139(1965).
(214) Selvaraj, R. J., Ouchi, E., McRipley, R. J., and Sbarra, A. J., *Bacteriol. Proc.*, Abstr. No. M173, 1965, p. 68.
(215) Whitehouse, M. W., *Nature*, **194**, 986(1962).
(216) Lipp, R., *Arch. Klin. Exptl. Dermatol.*, **218**, 228 (1964).
(217) Hurvitz, D., and Hirschhorn, K., *New Engl. J. Med.*, **273**, 23(1965).
(218) di Mattei, P., and Melchiorri, P., *Arch. Intern. Pharmacodyn.*, **153**, 339(1965).
(219) Corssen, G., and Allen, C. R., *Anesthesiology*, **21**, 237 (1960).
(220) Mercer, E. N., and Dower, G. E., *J. Pharmacol. Exptl. Therap.*, **153**, 203(1966).
(221) Miledi, R., and Trowell, O. A., *Nature*, **194**, 981 (1962).
(222) Wallbach, G., *Arch. Anat. Microscop.*, **33**, 251(1937).
(223) Allgöwer, M., *Schweiz. Med. Wochschr.*, **77**, 40(1947).
(224) Coatăchel, Q., Fadei, R., and Nachtigal, M., *Exptl. Cell Res.*, **34**, 542(1964).
(225) Lippman, M., *Trans. N. Y. Acad. Sci., Ser. II*, **27**, 342(1965).
(226) "British Pharmacopoeia," Pharmaceutical Press, London, England, 1963, p. 1123.
(227) Ambrose, C. T., *Federation Proc.*, **24**, 377(1965).
(228) Moehring, T. J., Kuchler, R. J., and Solotorovsky, M., *Bacteriol. Proc.*, Abstr. No. M52, 1965, p. 48.
(229) Kyrchikov, B. A., Isupov, F. G., and Egorov, P. A., *J. Microbiol. Epidemiol. Immunobiol.*, **42**, 85(1965).
(230) Gabliks, J., *Bacteriol. Proc.*, Abstr. No. M53, 1965.
(231) Kamzolkina, N. B., Lukashova, N. I., and Borisova, L. V., *J. Microbiol. Epidemiol. Immunobiol.*, **42**, 80(1965).)
(232) Korbecki, M., and Jeljaszewicz, J., *J. Infect. Diseases*, **115**, 205(1965).
(233) Jeljaszewicz, J., Szmigielski, S., Korbecki, M., and Zak, C., *ibid.*, **115**, 421(1965).
(234) Gump, D. W., *Federation Proc.*, **24**, 368(1965).
(235) Morgan, P., and Felton, W. W., *Bacteriol. Proc.*, Abstr., No. V133, 1965, p. 120.
(236) Sato, S., *Tohoku J. Exptl. Med.*, **85**, 346(1965).
(237) Nakazawa, T., *Texas Rept. Biol. Med.*, **18**, 52(1960).
(238) Pomerat, C. M., Drager, G. A., and Painter, J. T., *Proc. Soc. Exptl. Biol. Med.*, **63**, 322(1946).
(239) Shafer, W. G., *ibid.*, **106**, 205(1961).
(240) Nease, W. J., *J. Periodontol.*, **36**, 22(1965).
(241) Miller, Z. B., *Lancet*, **ii**, 1068(1963).
(242) Roath, S., Elves, M. W., and Israëls, M. C. G., *ibid.*, **i**, 249(1963).
(243) Ritter, D., *Pathol. Microbiol.*, **26**, 303(1963).