tive in decreasing nuclear basophilia [see, *e.g.*, (4)].

The finding that unpurified pyronin revealed the actions of enzymes much less clearly than purified pyronin suggests that at least one of the contaminating reddish pigments stains cellular materials different from those stained by pyronin itself. On the other hand, the mauve contaminant in unpurified thionin appears to stain the same cellular materials as does the bluer thionin since the only noticeable effect of its removal was a shift in hues of stained cellular materials toward the blue.

Use of digestions with these and other enzymes may prove useful in cytochemical studies of virus-infected cells in tissue culture.

Summary. A large decrease in cytoplasmic pyroninophilia and thioninophilia resulted from digestion of formalin-fixed monkey kidney tissue culture with either chymotrypsin or ribonuclease. This pair of enzymes decreased cytoplasmic pyroninophilia and thioninophilia more than either enzyme alone. Digestion of similar cultures with desoxyribonuclease gave a large decrease in nuclear pyroninophilia and thioninophilia. The effects of these 3 enzymes on pyroninophilia of these cells were more clearly shown when a purified pyronin was used.

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Long-Term Culture of Tissue Cells in Magnet Stirred Fluid Suspensions. (25013)

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One of the most important contributions to development of tissue culture technics has been the observation that tumor cells can multiply as freely suspended cell populations in fluid media(1,2). This discovery served to dispel the concept that tissue cells require anchorage to a solid substrate for multiplication, and methods were subsequently developed by Earle and co-workers(3) for first practical cultivation of animal cells on a large scale. Using 1-liter boiling flasks on Brunswick rotary shakers, these investigators successfully propagated massive fluid-suspensions of a number of cell lines and studied growth characteristics and metabolism of these free cell populations(4,5,6). There have been numerous subsequent reports on cultivation of animal cells in suspension(7-13), and other systems have been used. Preliminary experiments* indicated that tissue cells may be

* P. A. Eichorn and A. Der Aris (unpublished data), Amer. Cyanamid Co., Pearl River, N. Y.

propagated in fluid suspensions agitated by means of a magnetic stirring bar resting on floor of culture bottles. This system has been used by us for continuous propagation of 3 cell lines required in large quantity for preparation of nucleoproteins in immunological studies with tissue culture cells(14). This method of cultivation has proven practical for the long-term culture of these cell lines and an economical means of providing large numbers of cells for various needs. Methods used and some results are reported here.

Materials and methods. Cell-strains and culture medium. The cell strains chosen for cultivation by these methods were the MCN line, derived from human leukemic bone marrow(15), and 2 human epidermoid carcinoma strains, H. Ep. #1(16), and HeLa(17). The culture medium was composed of either 30% calf or horse serum and 0.5% lactalbumin hydrolysate, incorporated into Earle's balanced salt solution(18) and supplemented with vitamins in concentrations reported essential for HeLa cells(19). To control bacterial and fungal infection, penicillin at 250 μ/ml, neomycin at 20 μ/ml and nystatin at 50 μ/ml were incorporated into all culture media. Culture vessels were silicone-coated,† 1-, 2-, or 9-liter Pyrex bottles, carefully selected with slightly convex bases. Each bottle contained a magnet encased in Pyrex glass (Fig. 1, O) designed to provide smallest possible grinding surface against the convex curvature at bottom. Since an early experiment showed desirability of continuous gas exchange (Table I), all vessels were thereafter fitted with rubber stopper‡ which provided an inlet and outlet for continuous flow of 5% CO_2 in air, approximately 140 ml/hr during incubation. Culture bottles were incubated at 37°C in portable incubator units designed for magnet stirring (Fig. 1). Briefly, the unit consists of portable wooden box insulated with 1-inch Celotex wallboard which serves as incubator. Heat is provided by a DeKhotinsky thermoregulator and an Allen-Bradley automatic starter. The magnet stirring assembly is mounted in a compartment beneath the incubator and consists of 3 metal rotating holders for three 2½ x 2½ inch magnets, each posi-

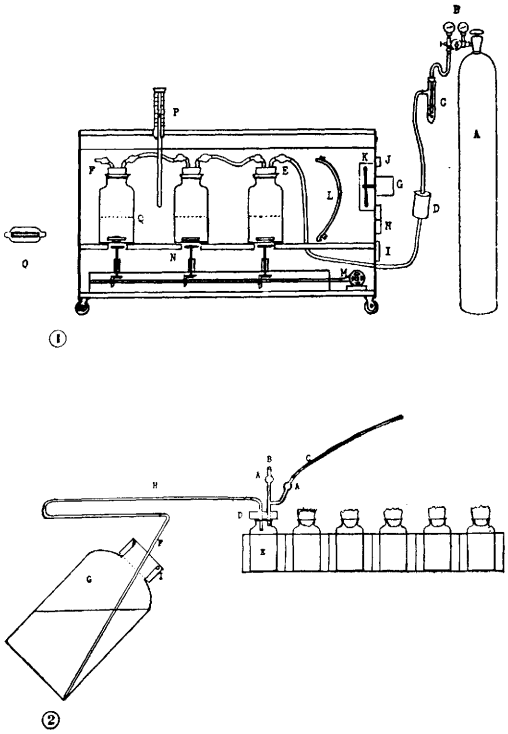

FIG. 1. Diagram of incubator and magnet stirring unit. A, Tank of 5% CO_2 in air; B, high and low pressure gauge (Matherson Co., East Rutherford, N. J.); C, bubbler tube containing Carbowax 400 (polyethylene glycol) (Carbide & Carbon Chemical Co., N. Y.); D, air purifier and flow equalizer (Koby Corp., Boston); E, cotton-packed gas inlet of culture bottle; F, cotton-packed gas outlet of culture bottle; G, electric motor (1750 r.p.m.) for fan; H, DeKhotinsky thermo-regulator (Central Scientific Co., Chicago, Ill.); I, Allen-Bradley automatic starter (Allen-Bradley Co., Milwaukee, Wis.); J, pilot light; K, 10-in. fan and nichrome heating coil; L, insulated panel to circulate heat over culture bottles; M, Bodine electric motor with changeable gears for 200, 250 and 300 r.p.m.; N, adjustable magnet holders; O, magnet encased in Pyrex glass; P, thermometer; Q, the level of medium.

FIG. 2. Apparatus for aseptic transfer of cell suspensions to centrifuge tubes. A, Cotton-packed filter bulbs; B, finger tip control; C, rubber tubing to vacuum line; D, rubber stopper fitted with glass tubing connections from culture bottle to vacuum line. (The cell suspension is transferred to the centrifuge bottle by placing the stopper securely onto the neck of the centrifuge bottle and filling the bottle to the desired level by use of vacuum and fingertip control "B."); E, centrifuge bottles; F, glass tubing to culture bottle; G, culture bottle; H, rubber tubing; I, protective cover with small hole for entry of glass tubing.

† General Electric, SC-87, Silicone Products Dept., Waterford, N. Y.

‡ Cafe Au Lait rubber, Baxter Rubber Co., Newark, N. J.

TABLE I. Effect of Gas Flow on Multiplication of H.Ep. #1 Cells in Magnet Stirred Fluid Suspension Cultures.

Culture #	Gas flow during incubation	No. of nuclei/ml × 1000 — 0 days	3 days	7 days	10 days	14 days	17 days	21 days
1	None, cultures sealed	300	326	333	520	346* 173	275	331
2		"	410	533	933	489* 245	300	410
3		"	250	510	553	379* 189	210	280
4	5% CO_2 in air, 140 ml/hr	300	400	1,973	3,950	3,306* 1,653	4,150* 2,075	4,210
5		"	280	1,773	3,224	2,457* 1,228	3,600* 1,800	3,822
6		"	405	1,670	3,191	2,580* 1,290	3,200* 1,600	3,502

* First figure represents No. of nuclei/ml of culture medium before half the cells were harvested.

tioned directly beneath glass-covered opening in floor of incubator. Holders for magnets are mounted on Monel metal gear system and rotated at 200 rpm by an electric gear-reduction motor. *Planting of cultures, culture medium changes and cell harvests.* One- and 2-liter bottles were generally seeded with 100-300 ml of cell suspension prepared with aliquot of cells scraped from glass floor of stock strain culture flasks (modified T-60 flasks of Earle and Highhouse) (20). These cells were resuspended in culture medium at 300,000 cells/ml with approximate error of ± 30,000 cells(21). At consecutive culture medium changes, the volume of medium was increased slowly to provide a maximum of 300-400 ml in each one-liter bottle and 600-800 in each 2-liter bottle. When no further increase in cell concentrations occurred in the maximum volume of medium, either one-half of cell population was removed, or the entire cell population was used as seed for a 9-liter culture bottle. The 9-liter bottles were seeded with not less than 300,000 cells/ml in one liter of medium. This volume was increased at subsequent culture medium changes to a maximum volume of 3 liters/bottle. The culture medium was changed twice weekly, *i.e.*, once each 3-4 days. At this time, cell suspensions were removed from bottles, collected in 250 ml centrifuge tubes and sedimented in an International #2 centrifuge at 600 rpm for 5 minutes. A diagrammatic sketch of the apparatus, operated by vacuum, which permits aseptic transfer of cell suspensions to centrifuge tubes by one operator, is shown in Fig. 2. Following centrifugation the supernatant was removed and discarded. The packed cells were taken up in a volume of fresh medium, pipetted back and forth through tip of a 10 ml serological pipette to disperse clumping resulting from centrifugation and returned finally to their respective culture bottles. The bottles were filled with desired volumes of fresh medium, stoppered, and placed again over a rotating magnet at 37°C. When cells were to be collected from cultures, the gently packed cells resulting from centrifugation were resuspended in a given volume of medium and a measured portion of this suspension returned to original culture bottle. At time of medium changes and collection of cells, cell nuclei counts were carried out as a measure of cell numbers in each culture. This was accomplished by aseptic removal of a 5-ml sample from each culture which was agitated over a small "Mag-Mix"§ to insure removal of a representative sample. Cell nuclei were prepared and enumerated by the method of Sanford *et al.*(21).

Results. When culture bottles were initially seeded and no cells collected at subsequent culture medium changes, the following phases in the life of cell population were ob-

§ Magnetic motor stirrer, Catalogue #18852, Central Scientific Co., Chicago, Ill.

served for all 3 cell strains: a) an initial lag phase of 2-3 days. b) a phase of active cell multiplication reaching maximum concentrations around 9-10 days and c) a phase beginning around 12 days consisting of no further increase in cell numbers and characterized by presence of many dead cells. These results appear generally to coincide with those obtained by Kuchler and Merchant with L strain cell suspensions in shaker cultures(8). It was soon noted that highest yields of cells could be obtained by collecting one-half of the cells from populations in phase (b) and repeating this each 3-4 days or semi-weekly. Comparative cell yields of cultures from which cells were collected weekly and semi-weekly revealed that although one-half of the cells are removed from these cultures each 3-4 days, maximum cell populations are almost regularly attained at end of this period. One 9-liter bottle culture of MCN cells, when one-half of its cells were harvested each 3-4 days, yielded an average of 21.5 ml of centrifuge-packed cells each week for 7 months of continuous cultivation. Maximum cell concentrations in these cultures ranged from 3-5 million cells/ml of culture medium.

In one experiment, an attempt was made to study variation of multiplication of cells suspended freely in fluid medium with and without continuous gas exchange. For this study H. Ep. #1 cells harvested from one 9-liter culture bottle were used as an inocula for 6 one-liter bottles, each containing 300 ml of medium. Subsequently 3 of these bottles were sealed with rubber stoppers and 3 were closed with stoppers which provided an inlet and outlet for a continuous flow of gas during incubation. Medium changes or cell harvests were made semi-weekly; each culture was sampled at this time for cell nuclei counts (Table I).

These results show an initial lag in all cultures of about 3 days, after which a phase of active cell multiplication began. Maximum cell concentrations were reached at about 10 days. Rate of gas exchange appears all-important to cultures which under these conditions are restricted to a renewal of culture medium each 3-4 days. These observations appear to conform with those of Earle *et al.*(3), who found that sealed shaker cultures which were flushed out with 5% CO_2 in air only each 2-3 days exhibited evidence of cell death from lack of oxygen and from acidity.

Discussion. Cells derived from human leukemic bone marrow (MCN) remained freely dispersed in these cultures, exhibiting little evidence of clumping. However, HeLa cells and to a lesser extent, H. Ep. #1 cells, tended to form spherical aggregates of less than 1 mm, such as that described by Earle *et al.* in shaker cultures(3). These aggregates appeared healthy, whereas many of the singly occurring cells appeared fragmented and full of cytoplasmic granules. Often, cells could be seen adhering to inside walls of culture bottle, forming a ring where medium swirled over them. The collection of cells at this site was not excessive, however, and could generally be dislodged by gentle shaking of culture at room temperature.

Earle *et al.*(3) reported extensive injury to cells when using a cylindrical magnetic stirrer which rolled over a flat bottom culture container. Little evidence of such destruction was noted with the methods here described, nor of protein thread-precipitate from culture medium which sometimes has been observed in shaker cultures. No foaming of culture medium was observed at rate of agitation used.

Cells collected from these cultures have on occasion been transferred to stationary culture flasks. When this is done, the viable cell clumps attach themselves to the glass, leaving broken cell fragments and debris floating free in supernatant medium. This debris can be removed upon the first change of medium; such cultures require one or 2 passages, with use of trypsin for detaching cells from glass, before the clumps become evenly dispersed and uniform cell sheets are obtained.

Summary. Human leukemic bone-marrow-derived (MCN) cells and human epidermoid carcinoma cells, (HeLa) and (H. Ep #1), have been continuously cultured over 1 year as suspended cell populations in a magnet-stirred fluid medium. This method of cultivation is a practical means of providing large numbers of freely suspended tissue cells for immunochemical and other studies. Cell mul-

tiplication for all 3 strains studied is characterized by: a) an initial lag phase of 2-3 days, b) a phase of active cell multiplication, reaching maximum concentrations around 9-10 days, and c) a phase beginning around 12 days with no further increase in cell numbers. Under conditions described, maximum cell yields were obtained by collecting one-half the cells from populations in phase (b) each 3-4 days.

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Radioautographic Study of Interkinetic Nuclear Migration in the Neural Tube.* (25014)

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Mitotic figures in the early neural tube occur only adjacent to central canal. This led to the view that cells near the lumen constitute a distinct type, which alone divide, and thus serve as "mother cells" for the entire wall. However, histological studies(1,2) indicated a migration of nuclei in the neural tube, *i.e.*, after division at luminal margin, a nucleus moves into the depths of wall during interkinetic period, and returns to the free surface as it passes into the prophase of the next division. Nevertheless, a recent discussion(3) continues to favor the long held germinal cell theory. The response of the early neural tube to colchicine(4,5) gave the first experimental confirmation of the migration concept. Additional evidence came from measurements of amount of deoxyribonucleic acid (DNA) in cells of neural tube. Sauer and Chittenden(6) found that mean amount of DNA in nuclei at some distance from the lumen considerably exceeded the 2n value. Since synthesis of DNA precedes each cell division during duplication of chromosomes, this indicated that deep nuclei undergo changes preliminary to division, as the concept of migration requires. A demonstration of the site of formation of DNA in the early neural tube with tritium-labeled thymidine, known to be incorporated only into new DNA,

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