sensitivity of the receptor membrane (slow muscle fiber) to released transmitter substance from the nerve endings.

Summary. Effects of thyroxine and thiourea upon optically evoked potentials in the midbrain of goldfish were tested. Thyroid hormone treatment reduced latency, time to reach maximum response, and time to complete the response, and it increased response amplitude. Thiourea had the opposite effects. Thyroxine shortened the "recovery" time between successive stimuli, and thiourea prolonged it. Supramaximal second responses were found when light flash stimuli were repeated at 1/5 to 1/10 second intervals in some of the thyroxine-treated fishes. The data suggest that thyroxine has a facilitating action upon tectal synaptic events.

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Serial Cultivation of Human Leukemic Cells.* (31166)

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The use of leukemic cells in studies of avian and murine leukemia has illuminated virus-cell relationships and antigenic properties of the cells(1,2). Long term serial cultures of leukemic cells have afforded convenient tools for study of the chicken and mouse diseases(1,3,4). The first human neoplasm of lymphocytic origin grown as a continuous suspended cell culture was the lymphoma of Burkitt(5,6). More recently, serial cultivation of human leukemic leukocytes has been achieved(7,8,9). This report concerns 3 serial cultures of human leukemic cells which have been initiated from pediatric patients, 2 with acute lymphoblastic leukemia and one with acute myeloblastic leukemia. The cultivation of the cells in suspension and observations on the cultures are described.

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Materials and methods. Medium. Medium RPMI1629(7), a variation of McCoy's medium(10) was used as the growth medium in the development of all 3 serial cultures. The medium was supplemented with 25% inactivated (56°C, 30 minutes) fetal calf serum, 100 units of penicillin and 100 μg of streptomycin sulphate per ml. After cells were multiplying, they were grown also on Eagle's basal medium (BME) and Eagle's minimum essential medium (with glutamine 2 mM per ml and non-essential amino acids 0.2 mM per ml) supplemented with inactivated fetal calf serum, 10 to 25%, and the antibiotics in the concentrations mentioned.

Preparation of cells for culture. Blood (10 to 30 ml) was drawn from peripheral veins of leukemic patients into plastic 30 ml syringes containing 0.5 ml of heparin. After

gentle agitation, the syringe was set upright (the covered needle facing upward) and the red cells allowed to settle for 1 to 2 hours. Plasma and buffy coat were then expelled into a 40 ml centrifuge tube through a bent needle with the syringe still upright. The cells were sedimented at 800 rpm for 20 minutes at 4°C in an International centrifuge, resuspended in growth medium and recentrifuged under the same conditions. This procedure was repeated twice more unless otherwise indicated. After the third washing in growth medium the viable cells were counted, using 0.1% trypan blue, and diluted in growth medium to the desired concentration. Cultures were incubated at 36°C in stationary Erlenmeyer flasks using fluid volumes amounting to 20% of the total volume. All cultures were refed twice weekly by carefully removing the top one-half of the growth medium and replacing an equal amount of fresh growth medium.

Morphological studies. Cells were centrifuged, growth medium poured off and the packed cells gently smeared on glass slides. The slides were air-dried and stained with May-Gruenwald Giemsa or tetrachrome stain.

Microbiological cultures. Cultures for mycoplasmas were done using broth and agar described by Chanock *et al*(11). Cultures for bacteria and mycobacteria were done in the Bacteriology Laboratories of The Children's Hospital of Philadelphia. Attempts at virus isolation were made by serial passage on human dipoid (WI-38), human embryonic kidney, and HeLa cells. Tests for resistance to vesicular stomatitis virus (VSV) were done as described by Henle and Henle(12).

Animal inoculation. Newborn hamsters (Lakeview Farms, N. J.) were inoculated subcutaneously and intracerebrally and observed for development of illness or tumors.

Sources of leukemic cells. Through the courtesy of Dr. Esshagh Abir and Dr. Irving Wolman of the Department of Hematology, The Children's Hospital of Philadelphia, peripheral blood was obtained from the 3 pediatric patients with high-count acute leukemia, as well as many marrow specimens from leukemic patients.

Cultivation of the cells. Lymphoblast culture LK1D. Twenty ml of blood was obtained from a 13-year-old girl with acute lymphoblastic leukemia of 4 years duration, previously treated with a variety of antileukemic drugs and on prednisone (30 mg daily) at the time the blood was drawn. Her peripheral leukocyte count was 40,000 per mm^3 with 90% lymphoblasts. After preparation as outlined above, washed cells were distributed in a 500 and 250 ml Erlenmeyer flask and in addition in six 2-ounce prescription flasks at a concentration of 2 to 5 $\times$ 10^6 cells per ml. Cultures were refed twice weekly. Both Erlenmeyer flask cultures and at least one prescription bottle were counted weekly.

Myeloblast culture LK57. Twenty ml of blood was obtained from a 5½-year-old girl with newly diagnosed, untreated acute myeloblastic leukemia. Her peripheral leukocyte count was 288,000 per mm^3 with 85% myeloblasts. Washed cells were distributed in various size Erlenmeyer flasks at counts varying from 1 to 10 $\times$ 10^6 per ml.

Lymphoblast culture LK60. Eight ml of blood were drawn from a 3½-year-old girl with newly diagnosed, untreated acute lymphoblastic leukemia. Her peripheral leukocyte count was 100,000 per mm^3 with 92% lymphoblasts. Cells were washed only once and placed in one 500 ml Erlenmeyer flask at a count of 6 $\times$ 10^6 per ml. While preparing this culture, the red cells had settled further in the syringe and additional plasma and buffy coat was expelled, uncounted, into 25 ml of growth medium in a 125 ml Erlenmeyer flask.

Results. All 3 leukocyte cultures from the 3 leukemic patients began to multiply after a latent period of 4 to 10 weeks. The cell cultures then took on the characteristics of continuous growth, all 3 doubling their cell number in approximately 48 hours. The cultures have now been maintained, at 1 to 3 to 1 to 5 divisions once or twice weekly for 10 months (LK1D), 9 months (LK57) and 6 months (LK60).

Lymphoblast culture LK1D. Cell counts in all cultures fell rapidly from initial viable counts of 2 to 5 $\times$ 10^6 per ml, to about 3 $\times$ 10^5 per ml and then slowly to the

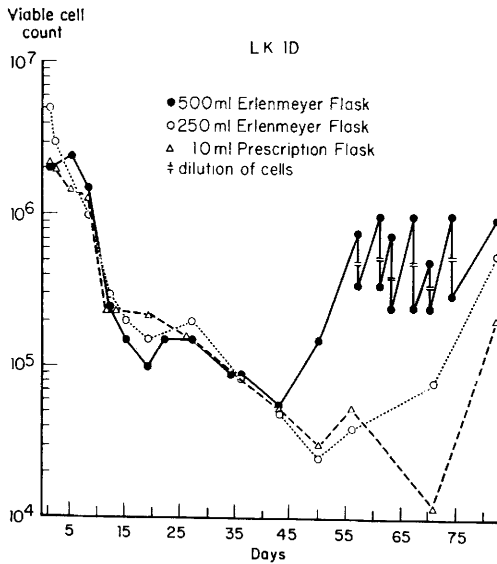


FIG. 1. Schematic representation of cultivation of lymphoblast cell culture LK1D.

3×10^4 per ml range (Fig. 1). Between days 43 and 49, the cells in the 500 ml Erlenmeyer flask doubled in number and at the same time it was noted that the medium became more acid and clumps of cells about 1 to 2 mm in diameter were forming. Between days 49 and 55, the 250 Erlenmeyer flask culture began to increase in cell number (Fig. 1) and again the medium became more acid and the cells formed clumps. Five of the 6 prescription bottle cultures were manipulated in various ways, either adding medium to decrease, or deleting medium to increase, cell concentration without effect on the decline in cell number. One bottle was merely refed biweekly and between days 70 and 80 the cells began to multiply and form clumps (Fig. 1). The cells from the original 500 ml Erlenmeyer flask are in their 37th passage and if seeded at 5×10^5 cells per ml in medium 1629 with 25% inactivated fetal calf serum, the doubling time is a little more than 48 hours. Using other media or less fetal calf serum, doubling times are somewhat longer. Cell counts seldom rise above 1×10^6 per ml in the same growth medium despite length of time in culture. It was found most convenient with all cultures to seed at 3×10^5 cells per ml and split 1:3 every 4 to 5 days at which time cell counts were usually 1×10^6 ml.

Morphologically the cells resemble immature blast-like cells at various stages of early development (Fig. 2a) with large nuclei and prominent nucleoli. Clear areas resembling vacuoles in the cytoplasm did not stain with May Gruenwald Giemsa. Red granules were seen in the cytoplasm of some of the large cells. Smaller, lymphocytic appearing cells with more dense cytoplasm and a few large cells with 2 to 4 nuclei were also present.

Myeloblast culture LK57. Cell cultures seeded at 1 to 10×10^6 cells/ml became very acid within 24 hours. Two of 3 cultures were diluted to 3 and 4×10^5 cells/ml, and one was maintained at 1 million cells per ml. Manipulation of cell concentrations is shown diagrammatically in Fig. 3. Cell counts dropped slowly over a 4-week period, after which multiplication began in 3 of four 500 ml Erlenmeyer flasks. Generation times were found to be shorter than those of lymphoblast cultures LK1D. The myeloblast cell counts doubled in somewhat less than 48 hours and after reaching counts of 1×10^6 cells per ml leveled off with increases in non-viable counts.

On microscopical examination, after staining with May-Gruenwald Giemsa or tetrachome stain, the myeloblast culture resembled the lymphoblast culture (Fig. 2b).

Lymphoblast culture LK60. The 500 ml Erlenmeyer flask seeded at 6×10^6 cells/ml showed an initial rapid drop in cell count to 5×10^5, followed by a slow decline to 1×10^5. Between 6 and 7 weeks after initiation this culture began to multiply (Fig. 4). In the smaller (125 ml) Erlenmeyer flask initiating cells were not counted. By the fourth day there were only 5×10^4 viable cells per ml. Subsequently, the cultures stabilized or grew very slowly (differences in counts were within the limits of error) until definite multiplication began between the 38th and 41st day (Fig. 4). With this culture, as with LK1D, the doubling time has stabilized at slightly more than 48 hours. The morphology of the cells is similar to the other 2 cultures and a representative smear is illustrated in Fig. 2c.

Additional observations. Cultures LK1D, LK57, and LK60 were inoculated as live cells and as extracts at counts of 1×10^6 viable cells per tube, onto monolayers of human dipoid (WI-38), HEK, and HeLa cells. The monolayers used as the "indicator" cells, showed no CPE after 3 weeks of observation and subinoculation of the supernatant fluids onto new "indicator" monolayers also gave negative results at 3 weeks.

Inoculation of newborn hamsters intracerebrally and subcutaneously with 1×10^6 viable cells of LK1D and LK57 per animal has not resulted in specific illness nor tumors over a 6-month observation period. Cultures for mycoplasma, bacteria and mycobacteria were uniformly negative on all 3 serial cultures.

One $\times 10^6$ viable or disrupted LK1D cells were transferred to tubes of HeLa, WI-38 or HEK, and the cultures were tested for development of resistance to vesicular stomatitis virus (VSV) using 10^3 to 10^4 $TCID_{50}$. Resistance to the challenge virus was evident only in tubes of HEK inoculated with viable LK1D cells before VSV challenge (Table I). This type of resistance occurred in repeated tests using HEK and viable LK1D cells. In a limited number of tests, due to a limited total cell number, the LK57 myeloblastic cells, freshly prepared from the patient, failed to induce resistance in HEK cells to VSV.

Discussion. The technique described was successful for establishment of continuous cultures from the peripheral blood of 3 out of 3 patients investigated with high-count acute lymphoblastic and myeloblastic leukemia. Similar techniques using similar cell concentrations, but by necessity in smaller flasks, were used in attempts to grow several leukemic bone marrows as suspended stationary cultures, but resulted only in the growth of fibroblast-like cells on the bottom and along the sides of the containers. Marrow cultures were maintained and observed for as long as the peripheral leukocyte cultures had been (up to 3 months). Supernatant fluids from marrow cell cultures were sampled in search of cells growing in suspension, but none were seen. Most of the marrows cultured were from patients under treatment, while 2

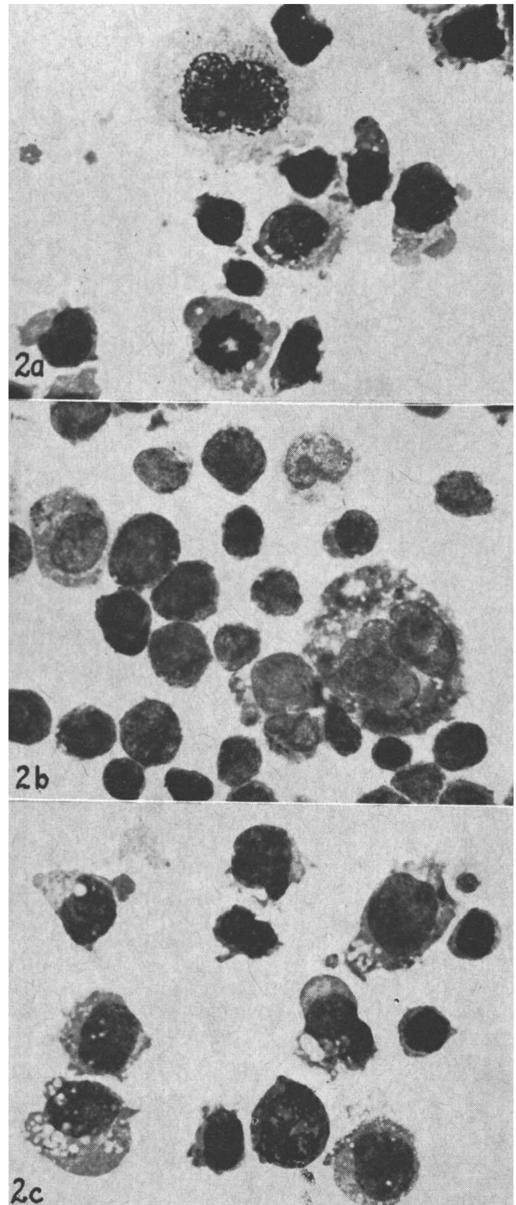
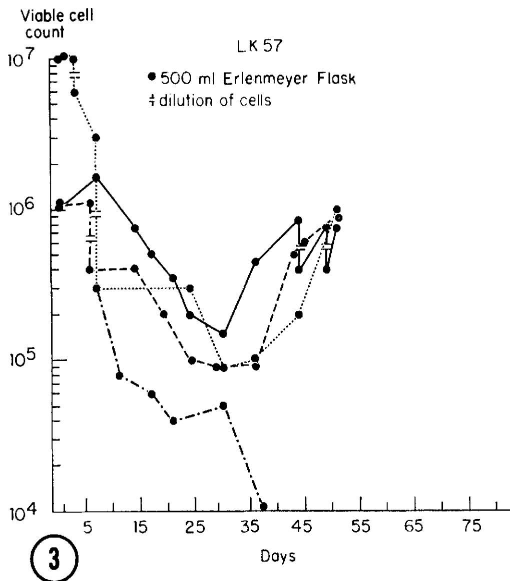


FIG. 2a. Lymphoblast cell culture LK1D. Cells of varying morphology including different sized cells undergoing division. Tetrachrome stain. Magnified $\times$ 520.

FIG. 2b. Myeloblast cell culture LK57. The smear also shows varied cells, some with dark staining cytoplasmic granules and a large multinucleated cell. Tetrachrome stain. Magnified $\times$ 520.

FIG. 2c. Lymphoblast cell culture LK60. Similar variable appearing cells as seen in the other cultures, including some with both intracytoplasmic and intranuclear vacuoles. Tetrachrome stain. Magnified $\times$ 520.



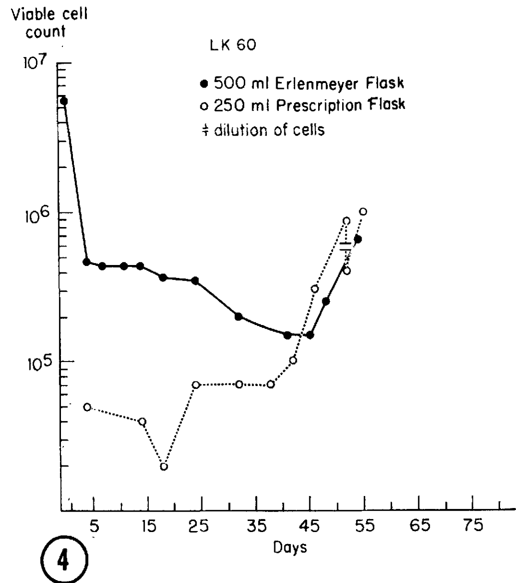


FIG. 3. Schematic representation of cultivation of myeloblast cell culture LK57.
FIG. 4. Schematic representation of cultivation of lymphoblast cell culture LK60.

of the 3 peripheral blood cultures were from untreated patients and one was on prednisone only. Treatment with antimetabolites might well influence the chances of initiating serial cultures. Persistence in maintaining and refeeding the peripheral blood cultures appears to be one of the more important factors in achieving a consistently growing culture. A long latent period before the onset of continuous multiplication was observed by Ikwata and Grace(7) and by Clarkson(9) in developing their cultures. An irradiated cell feeder layer used by Ikwata and Grace, was not necessary to induce growth of the leukemic cells reported here. This is similar to the experience of Foley *et al*(8) and Clarkson (9). In most of the culture flasks, a large percentage (80-90%) of the cells initially placed in culture died during the first week. This was followed by a slower decline in viable cells down to 1 to 5% of the starting cell number, before multiplication began.

The majority of cells in the initial suspension cultured were blastic and similar in appearance, but with obvious differences in *in vitro* growth potentials. After multiplication

TABLE I. Tests for Induction of Resistance in Various Indicator Cells.

Indicator cell	No. of LK1D cells inoculated	Day of VSV challenge (10,000 $TCID_{50}$)	CPE, days after challenge† 1	2	4	6
HEK	1×10^6	5	0	0	0	0
	1×10^6	3	0	0	0	0
	1×10^6	2	0	0	0	0
	1×10^6*	2	2	4		
WI-38	1×10^6	2	0	4		
	1×10^6*	2	4	4		
HeLa	1×10^6	2	3	4		
	1×10^6*	2	3	4		
Mouse fibroblasts (strain L)	1×10^6	2	–	4		

* Cells disrupted by sonication.
† 0 (no lesions) to 4 (complete destruction of culture).
– Not read.

had been established, the morphology of the cells within a culture has not been entirely uniform at a given time. This could be the result of different stages in the maturation of a single cell type or of different clones of cells growing simultaneously. This can only be decided by cloning or possibly histochemical studies.

Examination of the 3 leukemic cultures for microbial agents has been unrewarding. There is indirect evidence suggesting latent viral infection of LK1D. HEK cells which readily produce interferon on appropriate stimulation and are highly sensitive to interferon, developed definite resistance to VSV challenge after incubation with viable LK1D cells. Less sensitive indicator cells for interferon action (WI-38) showed only slight differences from control cells and a HeLa line (JJH) which does not produce interferon(13, 14) showed no resistance to VSV when tested in parallel with the HEK cultures described above (Table I). The necessity for using viable LK1D cells to obtain resistance of the HEK cultures appears comparable to previous work by Henle and Henle(12) with Burkitt lymphoma cells on various indicator monolayers which demonstrated protection to VSV challenge. In these studies a substance meeting current criteria for an interferon was detectable in supernatant fluids. It is possible that a similar mechanism accounts for the transfer of resistance from LK1D to HEK cells as observed in the present studies. Two of the 3 serial cultures (LK1D and LK57) have been reported(15) to react in complement fixation tests with various human sera. This phenomenon is presently of unknown significance. In previously reported studies(16), LK1D failed to show indirect immunofluorescence with various human sera similar to that observed with a proportion of Burkitt lymphoma cells of various lines.

Contamination of experimental cell cultures with established cell lines is a known danger in laboratories working with various tissue cultures. With this in mind, all 3 experimental cultures were refed on different days than established leukemic cell lines under investigation. As soon as multiplication became evident in an experimental culture, it was refed after the others, at a separate time at the end of the working day. Ordinary routine precautions such as separate media bottles for each culture were maintained. Under these conditions contamination of the experimental culture by cells of an established leukemic cell line is highly unlikely. The only certain method of ruling this out would be by a cytological (*i.e.*, chromosome) or immunological marker. A small percentage of cells of the Burkitt lymphoma cell lines and Clarkson's SKL1 and SKL2 leukemic cell lines stain with fluorescent labeled human gamma globulin(16). The 64-10 myeloblast line of Ikwata and Grace and the 3 serial cultures developed in this laboratory do not, indicating some similarity between them and dissimilarity with the others and tending to exclude the others as possible conaminants. The 64-10 myeloblast line cannot be excluded on these grounds but since it does not protect HEK cells against VSV it differs from LK1D. In addition, all 3 leukemic cultures began multiplication in more than one flask, which along with the above precautions and biological and immunological data almost precludes cell contamination.

Summary. Serial suspension cell cultures have been established from the peripheral blood of 3 out of 3 patients with high-count acute lymphoblastic or myeloblastic leukemia. A period of latency from 4 to 10 weeks, during which the cultures were regularly refed, preceded the start of multiplication. The techniques for initiation and maintenance of the cultures are described as well as observations made during these procedures. Preliminary attempts at virus isolation from all of the cultures were negative. Evidence suggesting a possible latent virus infection in one of the serial cultures is presented.

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Effects of Filling Pressure, Outflow Resistance, Heart Rate and Coronary Perfusion on Ventricular Compliance.* (31167)

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In relating the Starling law of the heart to cardiac performance the end diastolic pressure is often used in place of the diastolic volume of the ventricle. The assumption is made that the pressure-volume relationship of the ventricle remains constant, despite experimental manipulation, so that a given filling pressure will induce the same amount of stretch of the myocardial fibers in each situation examined. This assumption has been questioned in the past and cases in which end diastolic pressure and diastolic volume varied independently from each other have been presented(1,2). Changes in diastolic compliance have been described during post-extrasystolic potentiation in the isolated isovolumic right ventricle(3). Moreover, a change in stroke volume may take place at a constant filling pressure(4). Such a change in cardiac performance could be due to changes in cardiac contractility(5) or to an altered metabolic state(6). Another explanation could be found however in a change of the distensibility or compliance of the ventricle, such that a different diastolic volume would occur at the same end diastolic pressure.

The experiments to be reported were designed to determine whether the pressure-volume relationship of the heart is indeed constant over a physiological range of conditions, and to study in some detail some of the parameters which influence it.

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Methods. Mongrel dogs (20-30 kg) are anesthetized with Nembutal (30 mg/kg i.v.). The procedure for the isolation and metabolical support of the heart has been previously described in detail(6). Briefly, the aorta is cannulated and the coronary arteries are perfused with whole blood, under conditions of either constant pressure or constant flow. The coronary venous blood and the left ventricular thebesian flow drain in a gravimetric flowmeter. After the heart is isolated and put on a tray, complete heart block is induced by ligating the His bundle through a small right atrial incision. The ventricular rate is then controlled by a stimulator.

The left atrium is widely opened and a latex balloon inserted into the left ventricle and secured in place by suturing the mitral valve around an attached plastic collar.

The balloon is filled with saline solution to achieve any desired ventricular volume, and is connected *via* a valve system to a specially designed plethysmograph. The instrument consists of a vertical lucite tube (A) (I.D. 6 cm) which is half filled with saline (Fig. 1). Two stainless steel wires are immersed into the solution and connected to the variable arm of a Wheatstone bridge (B). The pumping action of the heart on the fluid filled balloon will alternately increase and decrease the level of the solution in the tube, thus changing the resistance between the wires and unbalancing the bridge. This change