MEASUREMENT OF THE POTENTIAL DIFFERENCE AND THE DISTRIBUTION OF ELECTROLYTES ACROSS THE MEMBRANE OF THE SHAY CHLOROLEUKEMIC TUMOR CELL

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TABLE OF CONTENTS

INTRODUCTION page 1

MATERIALS and METHODS

I. Maintenance of the Tumor 8

II. Examination of the Tumor by Electron Microscopy 8

III. Measurement of the Potential Difference (PD) of Tumor Cells 9

IV. Determination of the Ion(s) Responsible for the Maintenance of the PD 11

V. Measurement of the Fluxes of Labeled-Inulin, -Sulfate, -Chloride and -Sodium in the Tumor 13

VI. Quantitative Methods 16

RESULTS

I. Examination of the Tumor by Electron Microscopy 22

II. Measurement of the Potential Difference (PD) of Tumor Cells 22

III. Determination of the Ions Responsible for the PD 22

IV. Uptakes of Labeled-Inulin, -Sulfate, -Chloride and -Sodium 25

DISCUSSION

I. Compartmentation of the Shay Chloroleukemic Tumor 28

II. The Concentrations of Ions in the Shay Chloroleukemic Cell 33

III. The Size and Nature of the Potential Difference Across the Membrane of the Shay Chloroleukemic Tumor Cell 35

IV. Surface Properties of Malignant Cells 46

V. Concluding Remarks 52

SUMMARY 54

TABLE OF CONTENTS

BIBLIOGRAPHY 55

TABLES 62

FIGURES

APPENDIX

MEASUREMENT OF THE POTENTIAL DIFFERENCE AND THE DISTRIBUTION OF ELECTROLYTES ACROSS THE MEMBRANE OF THE SHAY CHLOROLEUKEMIC TUMOR CELL

ABSTRACT

Since cellular activities are influenced by the ion concentrations which in turn are important in the maintenance of the potential difference (PD) across the membrane, the behavior of malignant cells may result from abnormal membrane function. In the present investigation, the size and nature of the PD of the Shay chloroleukemic cell was examined. These leukemic cells grow into a solid tumor when injected intraperitoneally into rats. The tumor was used in all of the experiments. Measurements of the PD were made using intracellular microelectrodes and standard electrical recording apparatus. In an initial investigation, the mean PD of Shay chloroleukemic tumor cells in normal Ringer medium was -9.4 mV. In order to determine which ion is responsible for this PD, experiments were designed to yield the information necessary for use in the Nernst equation. The Nernst equilibrium potential for an ion distributed passively across the membrane is given by this equation. If the equilibrium potential for an ion is equivalent to the measured PD, then the PD can be said to depend on the distribution of that ion. In general, the procedure used to determine the extent to which potassium or chloride ions are responsible for the PD was to measure the PD of tumor cells bathed in media containing either an increased concentration of potassium or a decreased concentration of chloride. Intra- and extracellular concentrations of ions were measured and the extent of equilibration of tissue sodium or chloride with labeled-sodium or labeled-chloride in the medium was measured. The extracellular space of the tissue used was determined with C^{14}-inulin.

A small change from the normal distribution of the PD was observed when PD measurements were made in 20-, 80-, or 120 mM K Ringer medium. The Nernst equilibrium potential for potassium was -73 mV. Therefore, the potassium ion is probably not a major contributor to the maintenance of

this PD. However, when pieces of tumor bathed in media in which chloride was replaced by sulfate were examined, a marked depolarization of the membrane to zero or a reversal of the sign occurred. Therefore, the chloride ion distribution is of major importance in the maintenance of the PD. Since the Nernst equilibrium potential for chloride was about three times greater than the observed PD, -21 mV, it was proposed that another ion, namely sodium, also played a role in this respect. To test this possibility, the fluxes of sodium and chloride ions across the cell membrane were determined. These fluxes were based on the kinetic analysis of the uptake of Na^{22} and Cl^{36} by pieces of tumor. The kinetics of isotope distribution among the compartments of the system (medium, extracellular space, and intracellular space) were solved by analog computation. The flux between each compartment was calculated from the equation: flux = (rate constant) (compartment size). It was found that the sodium flux across the cell membrane (0.4 to 0.8 micromoles/minute) was almost twice as fast as that for chloride (0.3 micromoles/minute). Calculation of the Goldman equation using this data predicted a PD of +8.7 mV in one case and +9.3 mV in another. The calculation of a predicted PD which is positive may be due to the use of a value for the relative permeability of sodium to chloride which in all likelihood is too great. However, it is postulated that there is enough of a sodium leak flux to shunt the chloride equilibrium potential and together with the potassium ion distribution result in the observed PD.

Similarly low membrane potentials have been reported for other malignant cells. Aull (1967) found the PD of Ehrlich mouse ascites tumor cells was -11 mV and determined primarily by the chloride ion distribution shunted by sodium ions. The PD of mouse L cells, -15 mV, is also predicted by the chloride ion distribution (Lamb et al., 1970). It may well be that a change in membrane structure and physiology which leads to increased sodium permeability occurs in malignant cells.

INTRODUCTION

A fundamental characteristic of all cells is the maintenance of ionic concentrations inside the cell which differ from those in the extracellular fluid. An unequal distribution of ions across a membrane will give rise to an electrical potential difference which is maintained ultimately by active transport processes and the differential permeability of the cell membrane. The ability of malignant cells to proliferate, metastasize and invade normal tissues may be related to the molecular character of the cell surface and membrane.

In the present study, the potential difference of a malignant cell membrane was examined. The Shay chloroleukemic animal provides a convenient source of malignant cells which can be obtained from tumors or from the circulation of the animal. Shay et al. (1951; 1952) succeeded in producing this myelogenous leukemia in Wistar rats by gastric instillation of 20-methylcholanthrene. The transfer of this leukemia is accomplished by the administration of intact leukemic cells into normal young rats. Intraperitoneal or subcutaneous injection of tumor cells results in a solid tumor at the site of injection. Intravenous injection of tumor cells causes a predictable sequence of events in the animal, including invasion of the bone marrow by leukemic cells, where they proliferate. The Shay chloroleukemia represents a transplantable myelogenous leukemia, of which there are very few, whose progression is comparable to the myelogenous leukemia in man. This experimental leukemia has served as a useful model for the study of normal as well as pathologic hematopoiesis (Handler and Handler, 1970).

Usually the leukemias are limited to a single cell line, indicating that the leukemic process acts beyond the progenitor cell either at or beyond the differentiated stem cell level. This indicates impairment of the normal process of maturation. In many instances, the leukemias are characterized by an essentially normal or even slower generation time, impaired maturation, altered cell release into the circulation, and prolonged cell survival,

resulting in a progressive accretion of the potentially divisible cell population arrested in an intermediary phase of development (Bierman, 1967).

The Shay chloroleukemic cell proliferates without maturation and invades normal hematopoietic tissues. These characteristics make it a good model for a malignant cell. In this cell, differentiation appears to be arrested in either the myeloblast or promyelocyte stage, and proliferation occurs leading to a solid tumor, when the cells are placed intraperitoneally or subcutaneously. In the case of intravenously injected cells, invasion of the bone marrow occurs to such an extent that in the late stage of the leukemia almost no other cell types can be found in the femoral bone marrow (Handler et al., 1968).

Cone (1970a; 1971) has proposed a theory which relates the two fundamental characteristics of malignant cells, i.e. uncontrolled proliferation and the ability to metastasize and invade normal tissues. This theory predicts that the malignant cell membrane has a much lower potential difference than the corresponding normal cell. This is observed in the case of rhabdosarcoma cells which have a potential difference of about -15 millivolts while normal muscle has a resting potential of about -90 millivolts (Balitsky and Shuba, 1964). Cone suggests that the source of this lower potential difference may be related to the molecular character of the cell surface and its degree of interaction and bonding with other cells. Thus, in normal tissue, the surfaces of similar cells are highly compatible and tightly bonded by surface adhesion. The potential difference is high and the rate of division of the cells is very low. Dissociation of normal tissue cells, e.g. by enzyme treatment, results in a depolarization of the membrane followed by increased proliferation in culture. Therefore, the fundamental implication is that an alteration in the molecular architecture and specificity of the cell surface is an essential change occurring in malignant transformation.

The results obtained in potential difference simulation experiments with Chinese hamster cells in culture demonstrate a direct relationship between the potential difference and mitotic activity (Cone and Tongier, 1970). It

was found that culture medium compositions designed to impose intracellular ionic conditions simulating high potential difference levels (about -70 millivolts) reversibly blocked DNA synthesis and mitosis, while lower levels (in the range of those observed to exist normally for these cells in culture, about -10 millivolts) produced maximum proliferation rates. According to Cone (1970b), the two primary factors which determine the potential difference, sodium conductance and sodium efflux, are intimately associated with the metabolic state of the cells, which in turn is influenced by the potential difference, thus leading to a dynamic feedback circuit. There is some data which imply that sodium concentrations in excess of those used normally in culture media have a stimulatory effect on mitotic activity in vitro. Hypotonic pulsation of synchronized, monolayered mouse cells with sodium chloride produced a striking increase in mitotic activity and a shortening of interphase (Cone, 1969).

Thus, the maintenance of the malignant state could be the result of a dynamic self-sustaining, metabolic feedback system. For example, the autonomous division characteristic of malignant cells could be envisioned as the result of a stable feedback circuit in which surface polymers (protein or mucopolysaccharide) produced by the cell as a result of induced malignant transformation cause a low negative potential difference. This low potential difference brought about by high sodium conductance or decreased sodium efflux, in turn, insures continued activation of metabolic pathways involved in the production of the particular polymer forms necessary to maintain the low potential difference and associated mitosis (Cone, 1970b; 1971). This theory is compatible with some experimental observations and it provides a basis for the design of further experiments to test its validity.

As a result of physicochemical investigations of membrane structure, Wallach (1969) proposed that there is a common feature underlying the diversity of neoplasia; alterations in one or more of their membrane systems may be a regular feature of tumor cells. This hypothesis results from his theory of membrane structure in which large regions of the cell membrane

are believed to be lattices of interacting lipoproteins. In such a system any one subunit would of necessity modulate the behavior of other members of the lattice. Wallach postulates that an oncogenic agent acts to introduce an inappropriate protein into cell membranes, either in replacement of or in addition to normal components. Numerous membrane functions, including transport and immunologic recognition, would be modified as a consequence, accounting for the membrane changes seen in various cancers (Wallach, 1968; 1969).

Relatively little study has been directed to membrane transport as to whether or not it influences or reflects abnormal maturation in leukemic cells. Rigas (1961) studied intracellular cation concentrations in human leukemic leukocytes and found a relatively low sodium to potassium ratio. Block and Bonting (1964) examined the Na-K-activated ATPase system present in normal and leukemic leukocytes and found that its activity was highest in acute leukemic leukocytes, chronic lymphatic leukemic cells, and lowest in normal leukocytes. This activity was correlated with the morphologic immaturity of cells in the granulocytic line. In other studies of this kind, Lichtman and Weed (1968) found that small lymphocytes and myeloblasts have a higher activity than polymorphonuclear granulocytes. Chronic granulocytic leukemic cells and chronic lymphocytic leukemic leukocytes resembled their normal counterparts in cation content and ATPase activity. In general, higher cell sodium, higher ATPase activity and higher cell surface charge density (as measured by electrophoresis) were found in young granulocytes (myeloblasts) as compared with mature granulocytes. In these studies, the small lymphocyte resembled the myeloblast more closely than the polymorphonuclear granulocyte. Therefore, the observed differences between normal and leukemic cells may reflect differences related to the degree of maturation of the cell and not to their leukemic condition.

Other studies have been made relating the influence of ion transport, ion concentrations and membrane physiology on the metabolism of normal and tumor cells. Tosteson (1966) studied ion transport in sheep red blood cells

and found that active transport of sodium and potassium proceeds about four times more rapidly in HK (high potassium) than in LK (low potassium) cells. In addition, ATPase activity was greater in HK red cells. When LK sheep were subjected to massive hemorrhage, the cells formed during the subsequent rapid hematopoiesis contained much more potassium than cells released normally. Cells were separated by density gradient centrifugation and it was found that the lightest cells were the youngest and had the higher pump activity. Therefore, it was proposed that the LK membrane develops relatively late in cytodifferentiation.

It has been shown that ouabain inhibits the blastogenic action of phytohemmaglutinin as well as the incorporation of tritiated nucleosides into nucleic acids in human lymphocytes (Quastel and Kaplan, 1968). Excess potassium in the culture medium diminished or prevented this effect of ouabain. Lubin (1967) examined the effect of potassium on the inhibition of protein synthesis in mouse sarcoma and L cells caused by amphotericin B. It was inferred from his results that the depression of macromolecular synthesis in the presence of antibiotic can be reversed by increasing the level of intracellular potassium. Also, only a small decrease in intracellular potassium was needed to produce a proportionate decrease in macromolecular synthesis. However, as pointed out by Lubin, the results do not distinguish between a direct effect of potassium levels and an indirect effect, such as inhibition of synthesis or utilization of ATP. Moreover, the loss of potassium from mammalian cells is known to be compensated for by a gain in sodium and therefore, the results may be attributed to a change in the potassium to sodium ratio.

Levinson and Hempling (1967) presented data which suggest that ion transport plays a role in the regulation of respiration in the Ehrlich mouse ascites tumor cell. They found stimulation of respiration by potassium and inhibition of this effect by oligomycin. It DNP was added after the oligomycin, this inhibition was reversed and the resulting respiratory rate was higher than that prior to the addition of any potassium. Since DNP relieved the

inhibition of respiration produced by oligomycin but external potassium did not, it was concluded that DNP and potassium have to be acting on two different forms of respiratory intermediates. Thus, DNP acts above the oligomycin sensitive site and external potassium acts below it.

Clearly, the intracellular concentrations of ions are important in the regulation of the metabolic activities of the cell, which in turn affect the degree of proliferation and maturation of the cell. The electrical potential difference is a reflection of the distribution of ions across the membrane. Since the activities of the cell are influenced by these ions, aberrant behavior such as seen in malignancy, may have its basis in the permeability characteristics of the cell membrane and its resultant potential difference.

The classical view of the distribution of ions across a cell membrane is based on the study of frog muscle and holds that the ions are distributed according to the Donnan equilibrium. Boyle and Conway (1941) proposed that the distribution of potassium and chloride ions was caused by a Donnan equilibrium which existed across the fiber membrane as a result of the high concentration of impermeant sodium ions outside the fiber and the large number of impermeant intracellular organic anions. If potassium and chloride are distributed passively, the concentration ratios and the potential difference at rest would be predicted by the Nernst equation. Later it was found by Adrian (1956) and Hodgkin and Horowicz (1959) that at low concentrations of external potassium, the Nernst equation and Donnan relationship could not explain the observed resting potential of frog muscle. They applied the Goldman equation and showed that at low or physiologic external concentrations of potassium as well as at higher concentrations, the experimentally measured potential differences fit those predicted by the Goldman equation.

The basic assumptions of the Goldman or "constant field" equation are the following (Hodgkin and Katz, 1949): (i) that ions in the membrane move under the influence of diffusion and the electric field in a manner which is essentially similar to that in free solution; (ii) that the electric field may be

regarded as constant throughout the membrane; (iii) that the concentrations of ions at the edges of the membrane are directly proportional to those in the aqueous solutions bounding the membrane; (iv) and, that the membrane is homogeneous. From the first assumption, which leads to the equations for the current carried by sodium, potassium or chloride, the Goldman equation is derived.

In the present investigation the potential difference of the Shay chloroleukemic cell was measured and the ions responsible for maintaining the potential difference, as assessed by the Goldman equation, were determined. The results are discussed in the light of the relationship of the tumor cell potential difference to that of normal cells.

MATERIALS and METHODS

I. Maintenance of the Tumor

Intraperitoneal tumors of rats bearing the Shay chloroleukemia were selected for use in all of the following experiments. Ten days after the injection of tumor cells into the peritoneal cavity of a rat, a tumor develops which is approximately 5 cm long x 2.5 cm wide x 1.5 cm in depth. This tumor consists of nodules or strands of cells closely layered together and surrounded by a thin sheet of transparent connective tissue. There are no major blood vessels in the tissue and no visible necrotic areas.

The Shay chloroleukemia has been maintained in our laboratory by means of subcutaneous or intraperitoneal transplants of leukemic cells into 40 to 60 gram male rats of the Long-Evans strain. The method of transplantation is described below. All glassware and surgical equipment are sterilized by dry heat (220^{o}C for three hours), and sterile, pyrogen-free saline is used as the suspending medium for the cells. Approximately 14-day old tumor tissue is collected from a rat bearing a subcutaneous tumor or 10-day old tumor tissue is collected from a rat bearing an intraperitoneal tumor, and placed in a loose-fitting, 30 ml, hand homogenizer containing a ground-down teflon pestle. Moving the pestle up and down slowly about five times, without turning it, frees the tumor cells from the surrounding stroma and places them in suspension. The cell suspension, thus prepared, is filtered through glass wool and the number of cells per unit volume is assessed using a Spencer Brite-Line hemocytometer and 1% acetic acid as diluent. Each rat then receives 20 x 10^{6} tumor cells either intraperitoneally or subcutaneously.

II. Examination of the Tumor by Electron Microscopy

Since measurements of the potential difference (PD) across tumor cell membranes were to be made, it was important to examine the tumor tissue to see if the cells had intact membranes. Some information about the

nature of the spaces between the cells could also be obtained in these studies. The procedure for the examination of the tumor tissue by electron microscopy is outlined below. Table I in the Appendix lists the components of the solutions and mixtures used. Small pieces of tumor, measuring about 1 mm square, were placed in the fixation mixture and kept in an ice bath for about one hour. The tissues were then rinsed in saline and post-fixed in uranyl acetate solution for 30 minutes in an ice bath. After rinsing in saline, the tissues were dehydrated in a graded series of ethanol-water solutions (30, 50, 70, 95 and 100% v/v) for about 5 minutes each. The tissues were then placed in two changes of propylene oxide for 10 minutes each and infiltration of the tissue was started in a 1:1 mixture of propylene oxide and complete resin mixture for 1 hour. To complete the embedding process, the tissues were placed in dry gelatin capsules to which complete resin mixture was previously added and allowed to harden in an oven at 60^{o} for about two days. Epon-embedded sections were cut in a Sorvall Porter-Blum ultramicrotome using glass knives and placed on uncoated copper grids. The sections were stained with a saturated uranyl acetate solution and examined with an RCA EMU 3-H electron microscope operated at 50 kV with a 45 micron diameter objective aperture.

III. Measurement of the Potential Difference (PD) of Tumor Cells

A unique characteristic of different types of cells is the size and nature of the electrical PD which exists across the cell membrane. The electrical PD of Shay chloroleukemic tumor cells was measured in pieces of tissue from nine different tumors bathed in a normal Ringer solution (prepared as described in table II of the Appendix). The intracellular microelectrodes and techniques of electrical recording used for these measurements are described below.

Micropipettes are prepared from pyrex tubing using a vertical pipette puller (David Kopf Instruments, model 700B). These are filled with 3M KCl by initially allowing the electrolyte to enter the tip by capillary action

and then back-filling with distilled water. The micropipettes are then placed in an oven at 55^{o} overnight during which time the space between the KCl and the water becomes a bubble which is easily removed with a thin wire. The micropipettes are then back-filled with 3M KCl just prior to their use as microelectrodes.

In order to measure the PD across a tumor cell membrane, a piece of tumor measuring about 2 cm long x 1 cm wide x 0.5 cm in depth (or about 0.5 grams) is placed in a chamber constructed by pouring paraffin into the bottom of a Petri dish and then carving out an area to accomodate the tissue at one end and the reference electrode at the other end. The depression can hold about ten ml of the solution in which the PD measurements are made.

The complete set-up is depicted in figure I of the Appendix. The microelectrode used to impale a cell is held by a 3M KCl-agar bridge which in turn is held by micromanipulators (Narishige Co.) and connected to the input of a Bioelectric NF1 preamplifier via a chloridized silver wire. This preamplifier, is then connected to the input of a Tektronix 503 oscilloscope and a Keithley electrometer (model 610B). A permanent record of the PD is obtained by feeding the output of the electrometer to a Keithley recorder (model 670). While a PD is being measured the recorder is run a a speed of 12 inches per minute and the electrometer is used on the 30 or 10 millivolt scale. The reference electrode, consisting of a bridge containing Ringer-agar in the end in contact with the bathing medium and 3M KCl-agar in the other end, is connected to the ground terminal of the Bioelectric preamplifier via a chloridized silver wire. The tissue-micromanipulator set-up is supported on lead bricks to minimize vibration and kept within a copper mesh Faraday cage to minimize pick-up. In some experiments this set-up was modified by having the output of the preamplifier connected to the negative input of a Tektronix storage oscilloscope (type 3A3 Differential Amplifier and type 2B67 Time Base) and a record of the PD measured was made using a Polaroid camera attached to the screen of the oscilloscope. This was found to be superior to using the Keithley electrometer and recorder because the recorder has a time lag of

0.3 seconds.

The selection of proper microelectrodes for use in measuring a PD is very important. Typical microelectrodes used for this purpose had tip resistances of about 10 to 30 megohms and tip potentials of about 1 to 3 millivolts. Conveniently, the Bioelectric preamplifier has a circuit which passes 10^{-9} amps of current through the microelectrode. The resulting deflection on the oscilloscope represents the resistance of the microelectrode, and is calibrated such that millivolts equals megohms. In order to examine the tip potential of a microelectrode, the whole set-up minus the microelectrode is balanced at zero with the preamplifier while both agar bridges are immersed in the Ringer solution. Then the microelectrode is placed in the agar bridge which is connected to the input of the preamplifier and is held in place by the firm agar. The potential that exists when the microelectrode is immersed in the Ringer solution is called the tip potential.

The criteria used for the successful measurement of a PD are the following: (i) As the microelectrode is moved into the tissue, the potential changes abruptly from that of the tip potential to a new level. (ii) The new potential difference remains fairly steady for at least one second, usually it remains so for 5 to 10 seconds and has been observed to last as long as 1 minute. (iii) As the microelectrode is slowly withdrawn, the potential rapidly returns to its original value or very close to it.

IV. Determination of the Ion(s) Responsible for the Maintenance of the PD

The equilibrium potential for an ion distributed passively across a membrane is given by the Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{C_o}{C_i}$$

where E = the equilibrium potential or the potential inside - the potential outside (ground), R = 8.31 volt coulombs/mole^{o}K, T = 296^{o}K (room temperature), F = 96,500 coulombs/mole, z = valence, C_o = concentration of the ion in the external medium, and C_i = concentration of the ion in the

cells. If the equilibrium potential of an ion is equivalent to the measured PD, then the PD can be said to depend on the distribution of that ion.

In general, the procedure used to determine the extent to which potassium or chloride ions are responsible for the PD was to measure the PD of tumor cells bathed in media containing either an increased concentration of potassium or a decreased concentration of chloride. These media were prepared by modifying a basic normal Ringer solution (Aull, 1967) as described in table II of the Appendix. The data needed are the intra- and extracellular concentrations of ions and the PD under circumstances in which the tissue had equilibrated with the outside medium. The PD measurements were made as described above. To calculate the intracellular concentrations of ions, the extracellular space of the tissue must be known and the concentration of ions in the extracellular space was deducted from the concentration measured in the whole tissue in order to obtain the intracellular concentration. The extracellular space of the tissue was measured as described below in section V (A) using S^{35}-sulfate or C^{14}-inulin. A measure of the extent of equilibration of the tissue with the external medium was obtained by adding Na^{22} or Cl^{36} to the medium and comparing the specific activity (in cpm/uM) of the tissue to that of the medium at the time when the PD measurements were made. The methods used to extract the ions and labels from the tissues, to measure the concentrations of ions and count the labels are described in section VI. All calculations and equations used are given in section II of the Appendix.

To determine the role of potassium in maintaining the PD of the tumor cell, the concentration of potassium in the external medium was increased, and the procedure described below was used. A tumor was removed from a rat, placed in normal Ringer medium and four pieces were cut from it. One piece was placed in each of four paraffin chambers containing 10 ml of either the normal (7 mM K), 20, 80, or 120 mM K Ringer medium each with added Na^{22}. PD measurements were made using the electrical recording apparatus described above, in a random sequence. After one hour,

each piece of tissue was removed and the remaining supernatant medium was saved for analysis. The tissues were extracted by the ashing procedure. The concentrations of sodium and potassium were measured, and the Na^{22} counted in the extracts and supernatant media. This experiment was performed four times.

In order to determine whether or not the chloride ion distribution across the tumor cell membrane is important in the maintenance of the PD, four experiments were performed in which PD measurements were made in pieces of tumor bathed in media containing sulfate in place of chloride ions. The experiments was performed as follows: Ten ml of each medium, i.e. the normal (160 mM Cl), sulfate (7 mM Cl), and high K-sulfate (Cl-free) Ringer, containing Cl^{36} was placed in paraffin chambers. A tumor was removed from a rat and cut into pieces weighing about 0.4 grams wet. One piece was placed in each chamber and the time was noted. One piece was also placed in a vial containing C^{14}-inulin-normal Ringer medium to determine the extracellular space of a representative piece of tissue. PD measurements were made as described above. After two hours, the tissues were removed from their media and extracted by the acetic acid procedure. The supernatant media were saved for analysis. The chloride concentrations and the Cl^{36} present in the extracts and supernatants were then measured.

V. Measurement of the Fluxes of Labeled-Inulin, -Sulfate, -Chloride and -Sodium in the Tumor

In the experiments described here, the terms influx, uptake and distribution will be used according to the following definitions. Influx refers to the unidirectional transfer of a substance from the medium into the cells for a steady-state system. Uptake is defined as the net transfer of a substance from the medium into the tissue. Distribution refers to the final concentrations of a substance in all the compartments of a system after equilibration has occurred.

A. C^{14}-Inulin and S^{35}-Sulfate Uptake

The uptakes of C^{14}-inulin and S^{35}-sulfate by pieces of tumor were measured for the following two purposes: (i) in order to determine the extracellular space (ECS) of the tissue; and (ii) to compare the distribution of inulin and sulfate and calculate their respective rate constants.

Quantitation of the ECS of a tissue is necessary for the determination of the intracellular ion concentrations. In order to measure the ECS of a tissue, a marker is used, such as a radioactively-labeled substance. Ideally the cells in the tissue should be completely impermeable to this substance which is then confined to the ECS. The C^{14}-inulin molecule and the S^{35}-sulfate anion were added to normal Ringer solution in trace amounts and the uptake of these labels was followed using the procedure described below. A tumor was removed and cut into the desired number of pieces, each weighing about 0.5 grams. Each piece was then placed in a vial containing 5 ml of either the C^{14}-inulin-normal Ringer medium or the S^{35}-sulfate-normal Ringer medium. The tissue was thus incubated either at room temperature (23^{o}) or in an ice bath (0^{o}) for time intervals ranging from 0 to 120 minutes. After this time, the tissue was removed and the labels were extracted by the homogenization procedure and counted. This procedure was used for one kinetic study of the uptake of S^{35}-sulfate at 23^{o} and two at 0^{o}. One kinetic study of the uptake of C^{14}-inulin was done at each temperature. At a later time, another C^{14}-inulin uptake was performed at room temperature and the tissues were extracted by the acetic acid procedure instead of homogenization. The data from these uptake experiments were used to calculate the ECS of the tissue using the equation shown in section II of the Appendix. A kinetic analysis of these data was made and compared to a similar analysis of Cl^{36} and Na^{22} uptake data.

B. Cl^{36} Uptake

The fluxes as well as the distribution of chloride were determined in order to assess the contribution of chloride to the PD. To calculate the fluxes of chloride ions between the medium and the ECS and across the cell

membrane, a kinetic analysis of the data from three Cl^{36} uptake experiments was made as described below.

To examine the uptake of Cl^{36} by tumor cells, the following procedure was used. A tumor was removed from a rat and cut into pieces weighing about 0.4 grams, and each piece was placed in a vial containing 5 ml of normal Ringer medium containing Cl^{36}. The tissue was incubated at room temperature for one of the following time intervals: zero time (tissue was quickly dipped, removed and blotted), 5, 15, 30, 60, 90, 120, or 150 minutes. At the same time, a piece of tissue cut from the same tumor was placed in a vial containing 5 ml of C^{14}-inulin-normal Ringer medium for 90 or 120 minutes to measure the ECS of the tissue. Then the tissues were removed from their media and the medium was saved for analysis. The chloride and inulin were extracted from the tissue by the acetic acid method and the concentrations of chloride in the extracts and supernatant media were measured. The Cl^{36} and C^{14} labels were counted.

C. Na^{22} Uptake

The possibility existed that sodium ions were also involved in the maintenance of the PD. In order to compare the relative fluxes of sodium to chloride, the data from three uptake experiments were used. These data were then analyzed and the fluxes calculated as described below.

The procedure used for the Na^{22} uptake experiments was similar to that used for Cl^{36}. Pieces of tumor weighing 0.4 to 0.5 grams were placed in vials containing 5 ml of normal Ringer medium to which Na^{22} was added. These vials were left at room temperature for one of the following time intervals: zero, 1, 5, 15, 30, 60, 90, 120, or 150 minutes. At the same time, a piece of tissue was placed in a vial containing 5 ml of C^{14}-inulin-normal Ringer medium for 120 minutes to measure the ECS of the tissue. The tissues were then extracted in acetic acid and the supernatant media were saved for analysis. The concentrations of sodium were measured and the labels counted in the extracts and supernatants.

D. Kinetic Analysis of the Uptake Data

Since the data were not compatible with a simple exchange of chloride or sodium ions between free cells and the medium, but included an extracellular space between the cells and the external medium, a model of three compartments was proposed. The kinetics of isotope distribution among the components of a three compartment system can be solved by analog computation. The model and derivation of the analog computer program are given in section III of the Appendix. Inulin and sulfate uptake data were analyzed using the same program, in which the ECS consisted of two compartments, and the medium constituted the third compartment.

VI. Quantitative Methods

A. Method of Measuring Tissue Water

For all analyses, wet and dry weights of tissues were measured to determine the amount of tissue water. At the completion of an experiment, the tissue used was blotted well on filter paper, placed in a pre-weighed glass vial or platinum boat, and weighed. The tissue was dried by placing the vial or boat in an oven maintained at 110^{o} overnight. Then the vial or boat containing the dried tissue was weighed and used in all subsequent procedures.

B. Methods of Extracting Ions from Tissues

1. Dry Ashing Technique

For all dry ashing, the platinum boats containing the dried tissues were placed in a muffle furnace at 550^{o} overnight. The resulting ash was then dissolved by adding 0.2 ml of 0.1N nitric acid to the boat, followed by 5 ml of distilled water. In order to assess the effect of the dry ashing on the extraction of ions, recovery experiments were run with known quantities of sodium or potassium. A sample of sodium standard containing 25 uM (micromoles) was ashed, diluted to a final volume of 65 ml and then analyzed in the flame photometer as described below. In one case, 24.4 uM of sodium were recovered (2.6% error) and in a duplicate case, 24.5 uM of sodium were recovered (2% error). Therefore, essentially no loss of sodium ions

was incurred by the ashing process. In another recovery experiment for sodium, 0.2 ml aliquots of each of the normal, 20, 80, and 120 mM K Ringer media containing the same amount of Na^{22}, as used in the experiment described above (IV), were ashed and diluted with 0.2 ml of 0.1N nitric acid and 5 ml of distilled water. A set of comparable samples which were not ashed were prepared by adding 0.2 ml of each of the media to 5 ml of distilled water. In order to count the Na^{22}, four ml of each of the diluted samples were counted as described below. The mean percent error between the ashed and unashed samples was 2.5%. Therefore, no Na^{22} was lost in the ashing process.

The ashing technique was also proved satisfactory for recovering potassium. Ringer solutions were prepared to contain normal (7 mM K), 20, 80, or 120 mM K as described in table II of the Appendix. Samples of these solutions were ashed and the mean actual concentrations of potassium measured four different times were 6.9, 20.8, 80.7, and 119.2 mM. These results show that the procedure for extracting and measuring potassium was satisfactory.

2. Homogenization

One ml of distilled water was added to the vial containing the dried tissue and this was allowed to stand overnight. Then the tissue, which was now rubber-like and easily held with forceps, was placed in a ground-glass, motor-driven, 3 ml capacity homogenizer with the 1 ml water extract and an additional 1 ml of distilled water. The tissue was then homogenized and the homogenate was poured into a test tube. The glass vial and the homogenizer were rinsed with distilled water and the rinse water was added to the test tube. The final volume of the tissue extract (homogenate) was 5 ml. To test the efficiency of the homogenization procedure for the extraction of S^{35}-sulfate or C^{14}inulin, an aliquot of either was added to a piece of tissue which was then homogenized and the label was counted. The label was recovered in both cases (98 to 99%) when compared to an equal aliquot of label which was not extracted from a piece of tissue.

3. Acetic Acid Extraction

Five ml of dilute acetic acid reagent (2 drops of glacial acetic acid added to 10 ml of distilled water) were added to the vial containing the dried tissue. The vial was then covered, placed in a boiling water bath for about two hours and then allowed to stand overnight (adapted from Dunham and Gainer, 1967). The resulting extract was clear and the extracted tissue remained behind as an intact rubber-like mass. The acetic acid procedure was shown to be satisfactory for sodium by a recovery experiment in which 0.2 ml of normal Ringer medium containing Na^{22}, alone or with a piece of tissue was extracted. It was found that 99.8% of the Na^{22} was recovered from the tissue when compared to the extracted control which contained acid but no tissue. About 4 % error was found when this control was compared to a 0.2 ml aliquot of the medium comparably diluted but with only distilled water. Since there were slightly more cpm/ml in the extracted control than in the distilled water control, there was no quenching by the acid.

Tissues containing labeled-inulin were extracted by the acetic acid procedure in some experiments. In a recovery experiment for C^{14} extracted by this procedure, a known amount of label was added to a piece of tissue which was then extracted and 95% of the label was recovered.

The recovery of chloride by the acetic acid extraction procedure was tested by the following experiment. Two pieces of tissue were soaked in sulfate Ringer medium for 90 to 120 minutes during which time the cells lost chloride to the bathing medium and attained a base concentration of chloride which was low. The tissues were removed and dried and 1 ml of 20 uM/ml KCl standard was added to one tissue while 1 ml of distilled water was added to the other tissue (control). The chloride in each tissue was extracted with acetic acid. In one such experiment 95% of the 20 uM of chloride was recovered while 90% were recovered in a duplicate experiment. Part of the 5 to 10% error was due to a method which assumes that the tissue to which the 20 uM of chloride were added had the same concentration of chloride to begin with as the control tissue. Therefore, the acetic

acid extraction was considered satisfactory for chloride. In another experiment, a known amount of Cl^{36} was added to a piece of tissue which was then extracted with acetic acid. It was found that 98% of the Cl^{36} was recovered.

Much difficulty was encountered when attempts were made to extract chloride by ashing or homogenization techniques. The ashing technique was unsatisfactory because considerable quantities of chloride (10 to 70%) were lost in this process. The homogenization technique could not be used because the protein left in the homogenate interfered with the analysis of chloride with the chloride titrator. When attempts were made to remove the protein with perchloric acid, there was poor recovery of chloride ions.

C. Chemical Analyses

1. Sodium

The sodium concentration in the extract resulting from dry ashing or acetic acid extraction was measured using a Baird-Atomic flame photometer with an internal lithium standard. One ml of the extract was removed and brought to 10 ml with 2 ml of 1250 ppm lithium nitrate and water. This dilution gave a final concentration of 250 ppm lithium nitrate and a concentration of sodium sufficient to be measured within the linear part of the standard curve for sodium (0 to 1 mEq/liter).

2. Potassium

Potassium was analyzed in extracts prepared by the ashing technique. One ml of the extract was brought to 25 ml with 5 ml of 250 ppm lithium nitrate and distilled water. This dilution yielded a final concentration of 50 ppm lithium nitrate and a concentration of potassium sufficient to be measured in the linear range of the standard curve for potassium (0 to 1 mEq/liter. In using the flame photometer for both sodium and potassium measurements, the concentration of ion in the unknown sample was determined by measuring it between the next higher and next lower concentration of standard and calculating the concentration of ion in the unknown by interpolation. The dilutions used for measurements of sodium and potassium concentrations in samples of the Ringer solutions used are given in table III

of the Appendix.

3. Chloride

The concentration of chloride in the tissue extracts was measured with a Buchler-Cotlove chloridometer. Two ml of extract was removed and brought to 10 ml. Then, 0.5 ml of this dilution was added to 4 ml of chloride reagent (prepared by mixing 900 ml of water, 100 ml of glacial acetic acid and 6.4 ml of concentrated nitric acid) along with two drops of gelatin reagent (prepared by dissolving 6.2 grams of a dry mixture in one liter of hot water. This dry mixture, which was supplied with the instrument, consists of gelatin, thymol blue and thymol in a weight ratio of 60:1:1. The time it takes for the chloride in the sample to be titrated with silver ions is measured by the chloridometer, and this time is proportional to the concentration. The concentration in the sample is then determined from a previously prepared standard curve. In order to measure the concentration of chloride in the Ringer solutions it was necessary to dilute them 250 times.

D. Methods of Extracting Radioisotopes from Tissues

Radioactive sodium was extracted from tissues in the same manner as non-labeled sodium, either by ashing or by treatment with acetic acid. The acetic acid procedure was also used to remove Cl^{36} from the tissues. S^{35}-sulfate was extracted from tissues by the homogenization procedure while C^{14}-inulin was extracted either by homogenization or acetic acid .

E. Methods of Counting Radioisotopes

1. Radioactive Sodium

To count the Na^{22} in tissue extracts, four ml of the extract was placed in a lusteroid test tube and counted in a well-type scintillation counter. To count the Na^{22} in supernatant Ringer solutions, a sample was diluted 25 times and four ml of this dilution was counted.

2. Radioactive Chloride, Sulfate and Inulin

Extracts and Ringer solutions containing Cl^{36}, S^{35}-sulfate or C^{14}- inulin were counted by taking 0.2 ml and adding it to 10 ml of scintillation fluor and counting in a Packard liquid scintillation counter. The scintillation

fluor was prepared by adding the following compounds to 3 liters of p-dioxane (spectroquality) and stirring for at least three hours: 180 g naphthalene, 36 g 2,5-diphenyloxazole, 1.8 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and 600 ml 2-ethoxyethanol.

RESULTS

I. Examination of the Tumor by Electron Microscopy

Figure 1 is an electron micrograph of a section of tumor. The Shay chloroleukemic cells have some features typical of immature blast cells. These features include a large nucleus comprising about 75% of the cell. Usually a single nucleolus is seen and accumulations of chromatin material surround the inner periphery of the nuclear membrane. This membrane is interrupted by many nuclear pores. The cytoplasm holds many free ribosomes and some particles having a dense core and a limiting membrane. These particles resemble C-type viruses. The cells are tightly packed with very narrow channels between closely apposed membranes and small lacunae comprising other areas between the cells.

II. Measurement of the Potential Difference (PD) of Tumor Cells

Measurements of the PD were made in nine separate experiments on nine different tumors bathed in normal Ringer solution. The distribution of the PD measurements is shown in the histogram in figure 2. Values for the PD ranged from -4 to -22 mV and the mean PD for 143 measurements was -9.4 ± 0.3 (SE) mV.

III. Determination of the Ions Responsible for the PD

A. The Effects of Increased External Potassium on the PD

The effects of increased external potassium on the PD were examined in four experiments in which the PD of cells in tissues bathed in normal (7 mM K), 20, 80, or 120 mM K Ringer medium was measured using the procedure previously described. Concentrations of sodium and potassium inside and outside the cells were determined and the value of 0.10 ml "inulin or sulfate water" per g wet wt of tissue was used to calculate the ECS. The extent of equilibration of the tissue with the medium during the experiment was determined from the distribution of Na^{22} in the tissue and in the

medium.

The histograms in figure 3 show how the PD recordings were distributed, and typical recordings of PD measurements are shown in figures 4, 5, 6 and 7. In the normal Ringer medium there was a peak number of PD recordings between -6 and -8 mV; the mean PD was -9.2 mV (see table 1). In the 20 mM K Ringer medium the greatest number of PD recordings were between -6 and -8 mV and the mean PD was -8.2 mV. Measurements between -4 and -6 mV in the presence of 80 mM K were most often obtained and the mean PD in this case was -6.7 mV. However, with even higher concentration of potassium in the medium (120 mM), the mean PD was -7.3 mV and the greatest number of recordings were obtained in the range of -6 to -8 mV. This represents no change in the distribution of the PD in cells bathed in media containing high concentrations of potassium.

The data in table 1 show that in such media, the cells lose sodium as the external sodium concentration is lowered. The concentration of sodium in the cells bathed in the 120 mM K Ringer medium was only 26.2 mEq/liter cell water as opposed to the value of 48.8 for cells in the normal Ringer medium. The difference between these values is significant with $P < 0.01$. The statistics used are described in section I of the Appendix. Therefore, the cells are losing sodium to the medium when the concentration of external sodium is decreased from 165.8 to 75.3 mEq/liter. In the case of potassium, the cells bathed in the 120 mM K Ringer medium had 137.7 mEq/liter cell water while the value for those cells in normal Ringer medium was 121.6. Although the difference between these two values may not be significant ($P < 0.20$), some potassium may be entering the cells along its electrochemical gradient when the potassium ion concentration in the external medium is raised. The percent cell water given for the tissues in each medium is about 78%. For each Ringer medium, at least 80% of the cells whose sodium and potassium ion concentrations and PD were measured had equilibrated with the outside medium.

If potassium were the ion responsible for maintaining the PD across the

tumor cell membrane then the measured PD would be equivalent to the Nernst equilibrium potential for potassium calculated as follows:

$$E_K = \frac{RT}{zF} \ln \frac{K_o}{K_i}$$

where R = 8.31 volt coulombs/mole^{o}K, T = 296^{o}K, F = 96,500 coulombs/mole, z = +1, K_o = 7.0 mEq/liter (from table 1), and K_i = 121.6 mEq/liter (from table 1). The equilibrium potential (E_K) is -72.8 mV. Since the measured PD is only -9.2 mV in normal Ringer medium, the potassium ion could not be principally responsible for the PD.

B. Effects of Decreased External Chloride on the PD

The PD of tumor cells bathed in media containing sulfate in place of chloride ions was measured in order to examine the role of chloride, if any, in the maintenance of the PD. Four experiments were performed in which pieces of tumor were allowed to equilibrate in normal, sulfate, or high K-sulfate Ringer medium containing Cl^{36} and the PD of the cells was measured. Concentrations of chloride were determined and the ECS was measured with inulin in each tumor used.

As seen in table 2, after two hours in the sulfate or high K-sulfate Ringer medium, the tumor cells lost chloride to the external medium and reached a concentration of 20.5 and 19.3 mEq/liter cell water, respectively. A student "t" test showed that the intracellular chloride concentration (71.1 mEq/liter cell water) of cells in normal Ringer medium differed significantly from those concentrations in either of the sulfate media ($P < 0.0005$).

The histograms in figure 8 show that in some cells in the sulfate media, small positive PD recordings were made but most cells showed no measurable PD. Typical PD recordings are seen in figures 9 and 10. The difference between the mean PD for cells in the normal Ringer medium (-7.4 mV, from table 2) and that of cells in sulfate Ringer medium (+0.7 mV) or in the high K-sulfate Ringer medium (+0.2 mV) was significant ($P < 0.0005$).

Since the cells were depolarized or even showed a reversal of PD, the

distibution of chloride ions across the tumor cell membrane was considered to be of significance. However, when the Nernst equilibrium potentials for chloride were calculated for the intra- and extracellular concentrations of chloride in each medium, they were found to differ significantly from the corresponding mean measured PD, with $P<0.0025$ for the cells in normal Ringer medium, $P<0.005$ for the cells in the sulfate Ringer medium, and $P<0.0005$ for the cells in the high K-sulfate Ringer medium. This means that some other ion, perhaps sodium, is also important in the maintenance of the PD across the membrane of the tumor cells.

The data in table 2 also show the extent of equilibration of each tissue with its medium. The mean equilibration of tissues bathed in the normal Ringer medium was 96.9% while only 42.2 and 44.1% equilibration was attained in the sulfate and high K-sulfate media in the 120 minutes allotted for equilibration. Although isotopic equilibration was incomplete at low external chloride concentrations, for reasons to be discussed later, measurements of the PD were made within 10 to 15 minutes after the end of the equilibration period and the chloride measurements made for the cells at this time were used in the Nernst equation. Therefore, the intracellular concentrations measured represent a reasonable estimate of the values associated with the PD.

IV. Uptakes of Labeled-Inulin, -Sulfate, -Chloride and -Sodium

A. C^{14}-Inulin and S^{35}-Sulfate

The extracellular space (ECS) of the tumor was measured from an examination of the distribution of C^{14}-inulin and S^{35}-sulfate. In these experiments, ECS is defined as the volume of fluid, in ml, occupied by inulin or sulfate and has the dimensions of ml "inulin or sulfate water" per g wet wt of tissue. The results of the uptakes of C^{14}-inulin at room temperature and at 0^{o} were very similar (figure 11) indicating that the distribution of inulin in pieces of tissue is independent of temperature. At both temperatures equilibration was reached in 60 minutes and the maximum value for the ECS

ranged from 0.089 to 0.116 ml "inulin water" per g wet wt (table 3).

Figures 13, 14 and 15 show the curves for the uptake of S^{35}-sulfate by the tissues equilibrated either at 0^{o} or 23^{o}. The distribution of sulfate was found to depend on temperature. At 23^{o}, (figure 15), the sulfate space reached a maximum value of 0.39 ml "sulfate water" per g wet wt at 60 minutes. Since this value is almost four times the value of "inulin water" it probably includes a sizeable fraction of the intracellular water and indicates that sulfate can enter the cells. This observation is supported further by the finding that temperature influences the size of the sulfate space. The two other graphs (figures 13 and 14) represent data from experiments performed at 0^{o}. Maximal values comparable to those obtained with inulin were reached. Since sulfate could penetrate the cells at room temperature, it was not used to measure the ECS. The inulin marker was used in all experiments in which the ECS of the tissue was determined.

B. Cl^{36} Uptake Studies

The relative specific activity (s.a.) defined as the ratio of the s.a. of the tissue to that of the medium, vs. time for the mean of three experiments was graphed in figure 16. About 90% equilibration was reached in 90 minutes and a final value of 93% equilibration was reached by 150 minutes. The value for the relative s.a. at time zero was 0.085 instead of zero and probably represents an uptake during a time interval of about 30 seconds. This interval was the time it took to dip the tissue in the medium and blot off the excess medium from the surface.

As seen in table 4, except for the value at time zero, the intracellular concentration of chloride remained fairly constant. The extracellular chloride concentration also remained constant at about 154 mEq/liter. The percent cell water was about 79% (v/w) for all the tissues.

C. Na^{22} Uptake Studies

In figure 17, the mean relative s.a. of the tissue vs. time for three uptake experiments was plotted. The kinetic analysis was done for each individual uptake; this curve serves only to provide a general view of sodium

uptake. The points level off at 60 minutes with a value for equilibration of about 86% and reach 91% equilibration in 150 minutes. In these experiments, as seen in table 5, the intracellular sodium concentration rose from 43.6 mEq/liter cell water at time zero to 76.3 mEq/liter cell water at 120 minutes ($P < 0.05$). The extracellular sodium concentration remained fairly constant at about 162 mEq/liter. The percent cell water was about 78.5% for all the tissues.

D. Kinetic Analysis of the Cl^{36} and Na^{22} Uptake Data

Tracers permit the measurement of unidirectional fluxes. In this case the data for the uptake of Cl^{36} and Na^{22} were analyzed and all fluxes were calculated from the equation: flux = (rate constant) (compartment size), where the rate constant is in dimensions of $minutes^{-1}$, compartment size is in cpm/uM, and the flux is then cpm/uM(minute). Figures 16, 18, 19 and 20 show the curves drawn through the experimental points using the analog computer program described in section III of the Appendix. The compartment sizes were calculated from the concentrations measured in the tissue extracts and media according to the following equations:

$p = uM_{medium}$ = (uM/ml medium) (5 ml medium)

$q = uM_{ECS}$ = (uM/ml medium) (ml inulin water)

$r = uM_{ICS}$ = (uM/ml extract) (ml dilution) - uM_{ECS}.

The rate constants, which were obtained as described in the program, and the fluxes are listed in tables 6 and 7. The flux for chloride between the medium and the ECS compartments was 8.2 uM Cl/minute. The flux for chloride between the ECS and ICS, i.e. across the cell membrane, was 0.33 uM Cl/minute. In the case of sodium, the flux between the medium and the ECS was 3.3-5.7 uM Na/minute, while the flux between the ECS and ICS was 0.4-0.8 uM Na/minute.

DISCUSSION

I. Compartmentation of the Shay Chloroleukemic Tumor

A kinetic analysis was made of the data from the uptake experiments for C^{14}-inulin and S^{35}-sulfate by pieces of tumor. Uptakes were plotted as ml C^{14}-inulin or S^{35}-sulfate water per g wet wt of tissue vs. time as shown in figures 10 - 20. The experimental points were fitted using the analog computer program described in section III of the Appendix. The model proposed for this system, shown in figure 23, consists of the following three compartments: (1) the medium, p; (2) the fast exchanging component of the extracellular space (ECS), q; and (3) the slow exchanging component of the ECS, r. In the case of S^{35}-sulfate uptake at room temperature (23^{o}), the fast exchanging compartment, q, was considered the ECS and the slow exchanging compartment, r, was considered the intracellular space (ICS).

In order to fit the points for S^{35}-sulfate uptake at 23^{o}, shown in figure 15, some estimate of the value for the final equilibration was needed. It was not known if the curve would level off at the last point (0.39 ml sulfate water/g tissue) or continue to rise. An estimate of the relative intracellular sulfate can be made based on the assumption that the S^{35}-sulfate remains in anionic form when intracellular, in the time of the experiment, and does not become incorporated into something else, e.g. sulfur-containing amino acids. It has been observed that about 20 to 30% of the S^{35} label is lost in a protein-free acetic acid extract when compared to a tissue homogenate containing protein. A value for the sulfate anion distribution can be obtained from the Nernst equation by solving for $(SO_4)_o/(SO_4)_i$. If the PD is -21 mV (Nernst equilibrium potential for chloride, table 2), then the ratio of extra- to intracellular sulfate is 5.1. The ratio of intra- to extracellular sulfate is then 1/5.1 or 0.20. If the medium has 1 unit sulfate/ml water and the ECS consists of 0.14 ml inulin water/g wet wt, then the ECS, when equilibrated with this medium, has 0.14 units sulfate/g wet wt. It has been observed

that the tumor contains about 0.80 ml water/g wet wt. If the ratio of the intra- to extracellular sulfate is 0.20, then the cells would have (0.20)(0.80 - 0.14, or 0.66 ml intracellular water)(1 unit sulfate/ml water), or 0.13 units sulfate. Therefore, after equilibration, the tissue would have 0.14 plus 0.13 or 0.27 units of sulfate. However, the observed value is already greater than this (0.39 ml sulfate water/g tissue). The ratio of intra- to extracellular sulfate calculated in the same manner as above for a PD of -7.4 mV (the observed PD, table 2) is 0.57. In this case, the cells would have 0.14 plus 0.38 or 0.52 units of sulfate. This appeared to be a reasonable estimate of the ml sulfate water/g tissue after equilibration at 23°, and the points were fitted by assuming equilibration at this level.

The value of B, or time, was set equal to 1 second of computer time which was equal to 1 minute of real time. Therefore, as described in section III of the Appendix, the rate constants k_{21}, k_{23}, and k_{32} are equal to the potentiometer settings P2, P3, and P4, respectively, and k_{12} is equal to P1(q + r)/p. Amplifiers 2 and 4 represent the proportions of compartments q and r. The values for each of these rate constants are listed in table 8. In order to calculate the rate constant, k_{12}, the ratio of (q + r)/p was gotten from the following equation as shown for the C^{14} inulin data:

$$\frac{(q+r)}{p} = \frac{(0.145 \text{ ml inulin water/g wet wt})(\text{g wet wt})(\text{uM inulin/ml medium})}{(\text{uM inulin/ml medium})(5 \text{ ml medium})}$$

$$= 0.029.$$

As seen in table 8, if the data for the inulin uptake at 0° and 23° and the sulfate uptake at 0° are examined together, the values for each rate constant are in the same range with $k_{12} = 0.003$ to 0.012, $k_{21} = 0.167$ to 0.620, $k_{23} = 0.020$ to 0.060, and $k_{32} = 0.021$ to 0,060 minutes^{-1}. The rate constant k_{12} for sulfate uptake at 23° (0.008 minutes^{-1}) is in the same range as those calculated for sulfate uptake at 0°(0.003 and 0.012 minutes^{-1}) implying that the transport of sulfate from the medium into the ECS is not temperature dependent. In the model for sulfate uptake at 23°, compartment q or 2 is considered the ECS and compartment r or 3 is considered the ICS. In this case, the rate constants k_{23} and k_{32} (0.025 and 0.021 minutes^{-1})

are somewhat smaller than those for sulfate at 0^{o} (0.060).

Figure 23 is a schematic representation of the model proposed for the compartmentation of the Shay chloroleukemic tumor. The similarities between the rate constants for compartments 1 (medium) and 2, k_{12} and k_{21} for sulfate and inulin implies that the nature of the transport process into the first compartment of the ECS does not distinguish between an anion like sulfate and a much larger polysaccharide molecule like inulin. However, the rate constants k_{23} and k_{32} for sulfate are almost twice as large as those for inulin.

To measure the ECS of a tissue, large molecules which do not penetrate the cell membrane are preferred. Inulin has been found satisfactory for this purpose. The characteristics which justify the preference of saccharides rather than ions as extracellular markers are the following (Law and Phelps, 1960): (i) Saccharides are electrically neutral, and cannot thus yield spurious results due to the possible inequality of distribution across vascular and interstitial spaces. (ii) They have relatively high molecular weights, and thus exert far less osmotic pressure at a given concentration. (iii) It appears that inulin cannot penetrate cell membranes, thus any extracellular values obtained should not be in excess of the true value.

Studies on the distribution of inulin in pieces of Shay chloroleukemic tumors indicate that there is an ECS which constitutes about 14% (ml inulin water/g wet wt) of the tissue. Extracellular spaces of some other tissues, as measured with inulin, are greater than this value. Pittman and Debons (1966) found that slices of rabbit thyroid have an ECS of about 24%. A similar ECS (23%) has been found for intact rabbit bone marrow (Michelsen, 1969). An inulin space of 30 to 35% was found in guinea pig intestinal smooth muscle (Goodford and Leach, 1964). Therefore, the ECS of the Shay chloroleukemic tumor appears to constitute a relatively small proportion of the tissue. Examination of the tumor by electron microscopy (figure 1) shows that the tumor cells are very tightly packed, with narrow channels between closely apposed cell membranes and small "lacunae" constituting

the larger areas between cells. Since the kinetics of C^{14}-inulin uptake indicate that the ECS consists of two compartments, it may well be that the first, fast-exchanging compartment consists of the lacunae and the second, slow-exchanging compartment consists of the narrow channels between the cell membranes.

The results of the kinetic analysis of the Cl^{36} and Na^{22} uptake data are consistent with the model for a tissue with two major compartments, consisting of the ICS and the ECS. From the kinetic analysis the following information was obtained: (i) the relative size of the ICS and ECS, as percent of the total tissue; (ii) the size of these two compartments, as well as the medium compartment, in terms of micromoles of chloride or sodium; (iii) the rate constants and fluxes for the transport of chloride and sodium across the boundary of each compartment. Based on the data obtained from this analysis, the following models for the compartmentation of the Shay chloroleukemic tumor for chloride and sodium were proposed, where compartments: 1 = medium, 2 = fast exchanging compartment or ECS, and 3 = slow exchanging compartment or ICS. These are represented schematically in figures 21 and 22.

As can be seen from these models, the so-called ECS for chloride (44.2 %) is larger than that for sodium (32.3 to 37.4%) with a ratio of chloride to sodium ECS from 1.4 to 1.2. This indicates that perhaps more of this compartment is accessible to chloride than to sodium which may be related to the difference in charge and size of these ions. The chloride anion has a hydrated radius of 1.93 Å while the hydrated sodium cation radius is slightly larger (2.56 Å) (Solomon, 1960). Since the cells are permeable to chloride and sodium ions, this measure of the ECS is probably in excess of the true value for this tissue.

From the values for the compartment sizes and rate constants, the fluxes of chloride and sodium into and out of the compartments of the tissue and external medium can be calculated. As seen in tables 6 and 7, the fluxes for chloride and sodium between the medium and the ECS differ considerably.

A flux of 8.2 uM Cl/minute was calculated for chloride while 3.3, 3.9 and 5.7 uM Na/minute were calculated for the three sodium uptake experiments. This indicates that chloride anions more readily exchange between the medium and the ECS. The flux of chloride ions across the cell membrane, however, was lower (0.3 uM/minute) than that for sodium (0.4 to 0.8 uM/minute). In the case of each ion, the flux between the ECS and the medium was much greater than the flux between the ECS and the ICS indicating that a more effective barrier (the cell membrane) exists between the ICS and the ECS.

Since the tissue compartment sizes for sulfate and inulin are unknown, the fluxes between the compartments could not be calculated. However, the rate constants for exchange between the tissue compartments and the medium can be compared to those for chloride and sodium. For chloride, k_{12} is 0.011 minutes^{-1} and for sodium, k_{12} is 0.005, 0.007 and 0.004 minutes^{-1} for experiments I, II and III respectively. The corresponding values for inulin are 0.003 and 0.004 minutes^{-1}, and 0.003, 0.012, and 0.008 for sulfate. Therefore, on the basis of the rate constants alone, chloride would appear to enter the tissue extracellular spaces most readily, followed by sulfate, sodium and lastly inulin. In the case of each substance studied, the value of k_{21} is about 100 times greater than that of k_{12}. These values for k_{21} are the following (in minutes^{-1}): 1.000 for chloride; 0.460, 0.420 and 0.372 for sodium; 0.184 and 0.353 for inulin; 0.220 and 0.620 for sulfate at 0^{o}, and 0.167 for sulfate at 23^{o}. Since the rate constant refers to the fraction of material in the compartment from which the substance exchanges per unit time, a small compartment has fewer molecules available for exchange than a larger compartment. In the case of each substance, the medium compartment is much greater than the ECS compartment and the rate constant, k_{12}, for transport from the medium into the ECS is about 100 times smaller than that from the ECS to the medium. In the steady-state, with no net flux, a much smaller rate constant would be expected for movement from the large compartment into the smaller compartment. On

the other hand, a much faster rate constant would be expected for movement from the ECS into the larger external medium compartment. The rate constants for transport across the cell membrane, k_{23} and k_{32}, are 0.040 and 0.031 minutes^{-1} for chloride. These values are very similar to those calculated for sulfate anions at 23°, 0.025 and 0.021 minutes^{-1}. If the ECS and ICS compartment sizes for chloride are similar to those for sulfate then the characteristics of the membrane with respect to chloride and sulfate transport would be very similar. In the case of sodium, slightly higher values were obtained for k_{23} in two of the three experiments (0.090, 0.030 and 0.091 minutes^{-1} but the corresponding values for k_{32} (0.036, 0.027 and 0.050 minutes^{-1})are similar to those for chloride and sulfate anions.

As determined for the red blood cell, small anions such as chloride penetrate about 10^6 times faster than cations of comparable size. It is assumed that the capacity of the red blood cell to discriminate sharply between anions and cations is mainly due to the presence of fixed positive charges inside the membrane (Passow and Schnell, 1969). These charges prevent the passage of cations without blocking the movement of anions. As discussed above, the rate constants for sodium are not very different from those of chloride or sulfate, and the flux of sodium ions across the cell membrane is greater than that of chloride. Therefore, the presence of fixed positive charges as proposed for the red blood cell membrane does not seem to be likely for the membrane of the Shay chloroleukemic cell.

II. The Concentrations of Ions in the Shay Chloroleukemic Cell

The Shay chloroleukemic cell has a high intracellular potassium concentration relative to that of sodium. In normal Ringer medium this cell contains about 120 mEq K/liter cell water, and 48 mEq Na/liter cell water (table 1). The intracellular chloride concentration of cells bathed in normal Ringer medium is about 72 mEq Cl/liter cell water (table 2). The results of all experiments indicate that the percent cell water (v/w) is about 78%.

The concentrations of sodium and potassium in the Shay chloroleukemic cell conform to those reported for other leukocytes. Studies on rabbit polymorphonuclear leukocytes collected by peritoneal lavage showed that these cells have a potassium concentration of about 105 mEq/liter water, 68 to 80 mEq Na/liter water, and 79% cell water (Wilson and Manery, 1949; Hempling, 1954). Baron and Roberts (1963) found concentrations of about 60 mEq Na/liter cell water and 100 mEq K/liter cell water with 80.2% cell weight as water in normal human leukocytes. These values for sodium and potassium concentrations in leukocytes are similar to those reported for other types of cells. The following values (in mEq/liter cell water) were found by Morrill et al. (1964): 71 Na and 108 K for mouse pancreas cells; 69 Na and 108 K for frog oocytes; 31 Na and 138 K for HeLa cells; and 25 Na and 137 K for mouse ascites tumor cells. Therefore, in many mammalian cell types, including the Shay chloroleukemic cell high potassium and low sodium concentrations are found.

An investigation of ion concentrations in human normal and leukemic leukocytes was made by Lichtman and Weed (1969). These workers found that potassium and sodium concentrations were the same for human normal and chronic lymphocytic leukemic cells (or small lymphocytes); 120 - 124 mM potassium and 33 - 34 mM sodium/kg water. The percent of weight as water was 79 in both cell types. Leukemic myeloblasts, chronic granulocytic leukemic cells, normal bone marrow cells and normal polymorphonuclear leukocytes had similar potassium concentrations of 112 - 118 mM/kg water. In this same group, leukemic myeloblasts had the highest sodium concentration (40 mM/kg water) and normal polymorphonuclear leukocytes had the lowest sodium concentration (30 mM/kg water). Therefore, there were essentially no differences in sodium and potassium concentrations between normal and leukemic leukocytes. However, the leukemic myeloblast had a slightly higher proportion of cell water (81%) than did the polymorphonuclear leukocytes (76%).

III. The Size and Nature of the Potential Difference Across the Membrane of the Shay Chloroleukemic Tumor

With respect to this investigation, certain problems in the measurement of the membrane potential should be pointed out. One characteristic of the cell which poses a problem is the large nucleus which occupies about 75% of the cell. When a microelectrode penetrates a cell it is likely to be in the nucleus perhaps more often than in the cytoplasm. However, if there were a PD between the nucleus and the external medium which differed from that between the cytoplasm and the external medium, the distribution of the PD measurements would be expected to fall into two distinct populations. This, however, is not observed (figure 2). Therefore, the membrane potential referred to in this study should be considered that PD which exists across the cell membrane; with present techniques, no distinction can be made as to whether or not it is between the external medium and the cytoplasm or the external medium and the nucleoplasm.

In all PD measurements careful selection of microelectrodes was made. Only those with tip potentials no larger than -3 mV were used. As defined in this study, the tip potential is the junction potential which exists at the tip of the microelectrode when it is immersed in a Ringer solution in which ordinarily, NaCl is in higher concentration than KCl. It is not known how this potential changes when the tip of the microelectrode penetrates a cell, where potassium is the dominant ion. When the microelectrode is withdrawn from the cell the PD returns to a level close to that of the base line. However, occasionally a change in the tip potential occurred. This is illustrated in the recording shown in figure 7. This recording begins while the microelectrode is already in the tissue but at the same starting level (tip potential) as it was in the Ringer solution, -2 mV. When the microelectrode was advanced, a PD of -6.5 mV was recorded from a cell. Then the microelectrode was withdrawn from the cell and the PD returned to a new level of about -3.5 mV instead of the original -2 mV. When such a change in tip potential occurred, the microelectrode was no longer used. This change may be due to clogging of the

tip with other ions or intracellular substances replacing the pure 3M KCl.

The results of the PD measurements on the Shay chloroleukemic tumor cell in external media containing increased concentrations of potassium indicate that the PD is relatively unaffected by the potassium ion distribution. In these experiments, it was found that at least 80% of the tissue sodium had exchanged with radioactive sodium in the medium in one hour. By equating exchangeability with equilibration it can be said that at least 80% of the tissue had equilibrated with the medium. This measure of equilibration was important because it serves as a reflection of the proportion of the tissue which was exposed to the outside medium. Since whole pieces of tumor were used it could not be tacitly assumed that the inner cells were equilibrated with the outside medium to the same extent as the peripheral cells.

Measurements of the PD of the Shay chloroleukemic cells bathed in media containing sulfate in place of chloride showed that the membrane depolarized to zero and reversed its sign when external chloride was decreased. Therefore, chloride was believed to play a major role in the maintenance of the PD. In these experiments, the control tissue, bathed in normal Ringer medium reached complete equilibration with radioactive chloride in the medium in the allotted two hours. However, the tissues bathed in the sulfate and high K-sulfate Ringer media reached only 40 to 44% equilibration. The following are some possible explanations for this observation. The first assumes that equilibration was really complete and a curve for the equilibration of tissues in the sulfate media with time would have shown a plateau betweeen 42 and 44%. This would indicate that approximately 56% of the chloride was non-exchangeable. This explanation implies that a reduction in external chloride or an increase in external sulfate alters the binding properties of the tissue, since at normal external chloride concentration, exchange is complete, but at low chloride concentrations, exchange is only about 50% complete. Non-exchangeable fractions of chloride have been found in other cells. Only about 70% of cell chloride was found to be exchangeable in Ehrlich ascites tumor cells (Aull, 1967). In the lobster walking leg muscle

about 30 of the 85 mM Cl/kg cells is nonexchangeable (Dunham and Gainer, 1968). The other possibility for the observed 42 to 44% equilibration in low chloride media assumes that equilibration was not complete and the kinetics of an uptake for Cl^{36} in these media would indicate a reduction in the flux and in the rate constant for exchange of chloride. The cells bathed in the low chloride media lost about 70% of their intracellular chloride. The following possibilities exist for a decreased rate constant for exchange resulting from decreased intracellular chloride: (i) The flux of chloride has a component of exchange diffusion in which the exit of chloride and consequently its exchangeability depends on its access to a carrier on the inner membrane, and external chloride is needed to transport the carrier to the inner membrane. (ii) Sulfate anions reduce the mobility of the returning carrier and its accessibility to internal chloride. In other words, sulfate reduces the conductance of chloride and conductance is directly related to the rate constant for exchange. In line with this explanation, a relationship between chloride and sulfate concentrations and fluxes has been examined in red blood cells. Passow (1969) found that the flux of sulfate increased when external chloride was reduced.

The kinetic analysis of the uptake data for Cl^{36} and Na^{22} was performed in order to find the rate constants for exchange and calculate the fluxes. This analysis is based on the assumption that the cells are in the steady-state and there are no net fluxes. As seen in table 4, the intracellular concentration of chloride for the tissues used in the Cl^{36} uptake experiments did not increase or decrease during the course of the experiment, i.e. no net change occurred. This implies that the cells were in the steady-state. However, in the case of the Na^{22} uptake experiments (table 5), the intracellular sodium concentration increased with time. The difference between the highest intracellular sodium (76.3 mEq/liter cell water at 120 minutes) and the lowest intracellular sodium (43.6 mEq/liter cell water at time zero) is 32.7 mEq/liter cell water. This difference, divided by two hours, gives the value of 16.4 mEq Na/liter cell water/hour for the net influx of sodium.

If a similar calculation is made for the total exchange flux based on the kinetic analysis of the uptake data, it is found that about 115.5 mEq Na/liter cell water/hour exchanges. Therefore, the net influx of sodium constitutes only about 14% of the total exchange flux for sodium. This calculation is as follows: (0.77 uM Na/minute)(60 minutes/hour)(0.4 ml cell water^{-1}) = 115.5 uM Na/ml/hour or 115.5 mEq Na/liter cell water/hour, where the flux of sodium across the membrane is 0.77 uM/minute and the cell water content is 0.4 ml. Therefore, in the Na^{22} uptake experiments, there was some net flux of sodium in the cells which constituted about 14% of the steady-state flux.

The results of the PD measurements of the Shay chloroleukemic tumor cell in external media containing decreased concentrations of chloride indicate that the chloride ion distribution plays a significant role in the maintenance of the PD. However, this PD is not entirely predicted by the Nernst equilibrium potential for chloride. Since the measured PD is lower, it was proposed that sodium ions were contributing to the PD. The data for the flux of sodium and chloride ions across the cell membrane show that these cells have a relatively high permeability to sodium. The theoretical PD of the cells based on the chloride ion distribution, sodium ion distribution and the relative permeability of the membrane to these ions can be calculated using the following form of the Goldman equation (Hodgkin and Katz, 1949):

$$E = -\frac{RT}{zF} \ln \frac{P_{Na}(Na)_i + P_{Cl}(Cl)_o}{P_{Na}(Na)_o + P_{Cl}(Cl)_i}$$

where E = membrane potential, or PD of the cell relative to the medium, and P = permeability coefficient. In order to calculate the theoretical PD from this relationship, the intra- and extracellular concentrations of sodium and chloride, as well as the fluxes of these ions across the cell membrane were used. Since the fluxes are determined from the kinetic analysis of the uptake data for Cl^{36} and Na^{22}, the intracellular concentrations of these ions were recalculated based on the results of the kinetic analysis. To express

these concentrations in units of uM/ml cell water or mEq/liter cell water, the cell water was recalculated on the basis of the "kinetic" ECS instead of the inulin water. The first step was to calculate the absolute number of micromoles of sodium or chloride in the intra- and extracellular compartments of each tissue from the following equations:

q = uM in ECS = $A_4(q + r)$(% equilibration)

r = uM in ICS = $A_2(q + r)$(% equilibration),

e.g. q = 0.442(15.01 uM Cl)(0.93) = 6.17 uM Cl in ECS, and r = 0.568 (15.01)(0.93) = 7.93 uM in ICS. These values were calculated for each tissue in each uptake experiment. Then the compartment size of q as calculated above was compared to the size of the ECS based on the inulin water to determine the percent difference between the two. The results of this comparison are given in table 9. By changing the value of the ECS according to the percentage indicated in the table, a new value for the ECS in terms of ml ECS/g wet wt was obtained. Then this value for the kinetic ECS was used in the following equation to obtain the ml cell water for each tissue: (ml ECS/g wet wt)(g wet wt) = ml ECS, and the ml water lost by the tissue in drying - ml ECS = ml cell water. The intracellular concentration of ion was then found by dividing the number of uM Na or Cl in compartment r, the ICS, by the ml cell water to give uM Na or Cl/ml cell water or mEq Na or Cl/liter cell water.

The relative permeability of sodium to chloride ions based on the fluxes between compartments 2 and 3, i.e. the cell membrane, were used as a measure of P, the permeability coefficient. The P_{Cl} was set equal to one, which was equal to the flux of chloride ions across the cell membrane, and the ratio of the flux of sodium to the flux of chloride was set as the P_{Na}. These were normalized to the external concentration of sodium and chloride. Since the values for the flux of sodium in the second sodium uptake experiment (II) were different from the values for the other two experiments, the Goldman equation was calculated twice, once using a mean sodium flux excluding the value from experiment II and then including this value. These

sodium fluxes are listed in table 7. The two calculations are as follows:

$$1) \quad E = -\frac{RT}{F} \ln \frac{P_{Na}(Na)_i + P_{Cl}(Cl)_o}{P_{Na}(Na)_o + P_{Cl}(Cl)_i}$$

$$= -0.0255 \ln \frac{2.18\,(56.6 \text{ mEq Na/liter}) + 1\,(154.0 \text{ mEq Cl/liter})}{2.18\,(162.4 \text{ mEq Na/liter}) + 1\,(45.1 \text{ mEq Cl/liter})}$$

$$= +9.3 \text{ mV}$$

where P_{Cl} = (0.33 uM/min)/(154.0 mEq/liter) = 1, then P_{Na} = (0.79 uM/min)/(162.4 mEq/liter) divided by (0.33 uM/min)/(154.0 mEq/liter); $(Na)_i$ = 56.6 ± 1.7 mEq/liter cell water (N = 27), $(Na)_o$ = 162.4 ± 1.3 mEq/liter (N = 27), $(Cl)_i$ = 45.1 ± 1.9 mEq/liter cell water (N = 24), and $(Cl)_o$ = 154.0 ± 0.8 mEq/liter (N = 24).

$$2) \quad E = -0.0255 \ln \frac{1.82\,(54.4) + 1\,(154.0)}{1.82\,(162.2) + 1\,(45.1)} = +8.7 \text{ mV},$$

where P_{Cl} = (0.33 uM/min)/(154.0 mEq/liter) = 1, then P_{Na} = (0.66 uM/min)/(162.2 mEq/liter) divided by (0.33 uM/min)/(154.0 mEq/liter). These predicted values for the membrane potential of the Shay chloroleukemic cell (+9.3 and +8.7 mV) are based entirely on the results of the kinetic analysis of sodium and chloride uptakes. However, the observed membrane potential is always negative under normal conditions. This may be due to the use of a value for the relative flux of sodium to chloride in the Goldman equation which is too great. Since the flux as calculated above does not distinguish between a leak flux and an exchange flux, the presence of a sizeable exchange diffusion flux for sodium would contribute to the value of the flux used to predict the PD while only the leak flux may be important in the PD. Only where sodium fluxes are measured in the absence of external sodium is it possible to determine the specific contribution of a sodium leak flux.

In order to determine what proportion of the total sodium flux constitutes the active transport, exchange diffusion and leak flux, further experiments are needed. Measurement of a ouabain-sensititve, K-sensitive sodium efflux would constitute the active transport process. The exchange diffusion component of sodium influx, while insensititve to cardiac glycosides, would depend

on a carrier mechanism and on the presence of intra- and extracellular sodium. That proportion of sodium influx which is abolished in the absence of external sodium and is independent of a carrier mechanism constitutes the sodium leak.

It appears that the membrane potential of the Shay chloroleukemic cell is in large part determined by the distribution of chloride and sodium ions across the membrane. With respect to potassium, only a slight depolarization of the membrane occurred when PD measurements were made in media containing 80- and 120 mM K. Therefore, the contribution of potassium to the membrane potential though observable, is not as great as that of chloride.

Measurements of the PD of other non-excitable cells have been made. It is evident that most of these cell types do not conform to the pattern of the resting potential of nerve and muscle in which potassium is primarily involved in the maintenance of the PD and the cells are relatively impermeable to sodium at high external potassium concentrations (Adrian, 1956; Hodgkin and Horowicz, 1959). In many non-excitable cells a high permeability to sodium which in turn contributes to the PD has been found. Aull (1967) found that the mean PD of Ehrlich mouse ascites tumor cells was -11.2 mV and increasing the external potassium concentration had no effect on this PD. However, when external chloride was replaced by sulfate the cells depolarized toward zero. Calculation of the Nernst equation for the chloride equilibrium potential gave a value of -33.5 mV, about three times greater than the measured PD. This calculation of the Nernst equation was based on an intracellular chloride concentration of 42 mEq/liter or 70% of the total chloride (60 mEq/liter) which was found to be exchangeable. With respect to sodium, a high passive permeability was found. The calculated unidirectional influx of sodium was 9.2 pmoles/cm^2second (or 33.1 umoles/cm^2hour) while the unidirectional chloride flux was 5.2 umoles/cm^2hour. It was hypothesized that sodium contributes significantly to the membrane potential of the Ehrlich ascites tumor cell.

Studies on the membrane potential of mammalian adrenal gland were

made by Matthews (1967). The mean PD of cortical cells from rabbit, rat and kitten adrenal glands was about -70 mV and was dependent on the external potassium concentration. However, the mean PD of cells in the medulla was only about -20 to -30 mV and was not significantly affected by changes in the external potassium concentration. It was suggested that high sodium permeability may underlie the low PD of the medullary cells. Schanne and Coraboeuf (1966) proposed that high sodium permeability may well be characteristic of all non-excitable cells. In their studies, increasing the external potassium concentration had little effect on the PD of rat liver cells. In other studies on rat liver cells, Beigelman and Schlosser (1969) found that variations in the external potassium concentration from 5 to 80 mEq/liter caused a decrease in the PD from -20 to -13 mV. The PD fell from -21 to -11 mV when external chloride was decreased from 128 to 7 mEq/liter. Good agreement between observed and calculated values was obtained with the Goldman equation using the values of Schanne and Coraboeuf (1966) for the liver intracellular ion concentrations and permeability ratios. The P_{Na} was 0.3 relative to the P_K which was taken as 1.

Woodbury and Woodbury (1963) found a mean PD for rat and guinea pig thyroid cells of about -50 mV. When potassium chloride was applied topically the membrane depolarized and the ratio of the sodium to potassium permeability was estimated to be 0.12.

Examination of the PD and permeability of mouse fibroblasts (L cells) in culture (Lamb et al., 1970) revealed that the PD of the cells was about -15 mV and the intracellular concentrations of sodium, potasssium and chloride were 10, 170 and 75 mM/liter water, respectively. Ion fluxes were measured using radioisotopes. The value for the sodium flux (3.6 pmoles/cm^2 second) was more than twice that for potassium (1.5 pmoles/cm^2 second) and the chloride flux was much greater than either of these (about 11 pmoles/cm^2 second). Since the value for the chloride equilibrium potential (-17.45 mV) and the measured membrane potential (-15.36 mV) were in close agreement it appeared that chloride was passively distributed.

Substitution of the observed PD and the intracellular concentrations of sodium and potassium in the Goldman equation gave a P_{Na}/P_K ratio of 0.66. Similarly, Borle and Loveday (1968) obtained a sodium to potassium permeability ratio of 0.57 for cultured HeLa cells. In Tris buffer, and with normal concentrations of potassium and sodium, the PD was -17.1 mV. Raising the extracellular potassium from 6 to 71 mM with an equivalent decrease of sodium in the buffer caused the PD to fall to -7.5 mV. A further increase in the external potassium caused an even greater depolarization of the membrane. On the basis of the permeability ratio of 0.57 and the intracellular concentrations of sodium and potassium given elsewhere (Wickson-Ginzburg and Solomon, 1963) for HeLa cells, the expected PD was calculated according to the Goldman equation. The PD calculated in this manner was very close to the measured PD. Similar sodium and potassium permeability ratios have been calculated for other cells. Tosteson and Hoffman (1960) found a value of 0.57 for high potassium sheep red cells. The value of this ratio was 0.34 for Ehrlich mouse ascites tumor cells (Aull, 1967). Borle and Loveday proposed that this high sodium permeability may be characteristic of cells which derive their metabolic energy from glycolysis. High aerobic glycolysis is a common feature of the red blood cell (Tosteson and Hoffman, 1960), the HeLa cell (Wickson-Ginzburg and Solomon, 1963), and the Ehrlich ascites tumor cell (Hempling, 1958). The Shay chloroleukemic cells have been shown to have a relatively high sodium permeability, however, no respiratory studies have been done on these cells. Human leukemic granulocytes exhibit low respiratory rates and high aerobic glycolysis (Laszlo, 1967).

Few studies have been made on the PD of leukocytes. Macrophages present in cultures of neuroglial cells were impaled with microelectrodes and the PD ranged from -5 to -15 mV (Wardell, 1966). In another study, Hild and Tasaki (1962) found a mean of -9.3 mV for macrophages under the same conditions. Beckmann et al. (1970) measured the PD of granulocytes taken from whole blood and from peritoneal exudates and found values of about -5 mV. Cultured human lymphocytes had about a -12 mV PD (Hause et al.,

1970). These values for the PD of white blood cells are in the same range as those observed for the Shay chloroleukemic cell.

More detailed studies on the PD of red blood cells have been made. Lassen and Sten-Knudsen (1968) found a range of values for the PD of human red blood cells from -6 to -14 mV. Jay and Burton (1969) found a mean for twenty human red blood cells of -8. 0 mV and a Nernst equilibrium potential of -10 mV for chloride when the intra- and extracellular concentrations of chloride are 73 and 110 mEq/liter, respectively. This indicates that chloride ions are passively distributed across the red blood cell membrane and are responsible for the observed membrane potential.

Table 10 lists the average PD value for a variety of normal and malignant cells. These measurements were made in intact animals, excised tissues or in culture, as indicated. In general, the cells in intact and excised normal tissues had a higher PD than normal cells maintained in culture. The malignant cells examined all have a relatively low PD, in the same range as cultured normal cells. Perhaps the low PD of granulocytes and red blood cells is related to the fact that these are circulating and not tissue cells. There is no clear-cut difference between normal and malignant cells based on their membrane potentials, however, malignant cells tend to have a relatively low PD. This may be indicative of a change in membrane structure and physiology which leads to higher sodium permeability.

Observations by Cone (1969) on mouse L (sarcoma) cells in vitro led to an hypothesis relating the membrane potential to the mitotic activity of the cell. A few hours before the morphological indications of prophase became apparent, a gradual thickening at the cell surface was seen and the cells became spherical in shape. An increase in cell volume occurred as well as an increase in the membrane potential from an interphase level of -10 mV to -16 mV at the beginning of prophase. Almost immediately after the cell became spherical, a rapid depolarization trend was initiated, returning the PD to the interphase level. Data on changes in cell volume were based on time-lapse cinematographic observations and actual measurements of the

PD of cells in known states relative to prophase were made. However, no description of the methods and instrumentation is given by the author. Based on these observations, Cone (1969, 1971) advanced the hypothesis that different PD levels play a role in the control of cell division. It was pointed out that cells which maintain a very high PD seldom if ever enter mitosis, e.g. nerve and muscle, while cells which have a small PD routinely divide.

Cone and Tongier (1970) examined the effect of variation in the PD on the mitotic activity of naturally synchronized Chinese hamster cells in culture. To induce changes in the PD ranging from -10 to -90 mV, they used media designed to produce the corresponding intracellular ion concentrations and followed DNA synthesis with tritiated thymidine. Their results show that mitotic suppression was complete in cells incubated in a medium believed to impose a PD of -70 mV. Since completion of DNA synthesis was considered to be a prerequisite for mitotic induction, it was assumed that the observed mitotic blockage induced by a supposedly simulated high PD was a direct result of DNA synthesis blockage. However, the data obtained from this experiment are inadequate with respect to the hypothesis because no measurements of the actual ion concentrations or PD of the cells were made. It can only be said that a change in the external ionic and osmotic conditions effect the mitotic activity of the cells. Different levels of the membrane potential were proposed to be directly involved in mitotic regulation but the lack of data for the direct measurement of the membrane potential makes this experiment of questionable significance with respect to proving the hypothesis stated above. While Cone proposes an attractive hypothesis for the relationship of cellular metabolic activities to the membrane potential level, the experimental data upon which this hypothesis is based are incomplete. The low PD observed in malignant and cultured cells may not play a regulatory role in mitosis but may only be a reflection of the ionic conditions in the cell brought about by a regulatory change at some level other than the cell membrane. More definitive experiments are needed to establish the nature of the relationship between the membrane potential and the metabolic activities of the cell.

IV. Surface Properties of Malignant Cells

An important approach to the control of cancer is to elucidate the mechanisms controlling the proliferative and metastatic capabilities of malignant cells. Wallach (1968, 1969) proposed that an alteration in the membrane may be a common feature of all malignant cells. Some differences in the surface and membrane of malignant cells as opposed to their normal counterparts have been found.

Eagle (1968) reviewed the work on the growth-regulatory effects of cellular interactions in culture. With cultured cancer cells, cell growth is not sharply curtailed on the formation of a complete monolayer as in normal cells. Instead, the cells continue to grow and pile up on the surface of the glass, reaching maximum population densities of three to five times greater than those observed with most normal cells. Therefore, the ultimate cessation of growth involves processes other than simple contact inhibition. With some cell lines the entire multilayered cell sheet sloughs off the glass, with still others there exists a gradual diffuse degeneration of the whole population. An attractive theory results from these observations, i.e. that cancer cells have escaped from growth-regulatory processes in vivo because they are no longer subject to contact inhibition of growth and replication (Eagle, 1968; Abercrombie, et al., 1954, 1957). The term "contact inhibition" was applied by Abercrombie and co-workers to the behavior of freshly isolated chick embryo heart fibroblasts. Contact inhibition was defined as the stopping of locomotion of a cell in one direction by contact with another cell. This phenomenon occurred with chick and mouse fibroblasts but not with mouse sarcoma cells. However, Eagle (1968) suggests that a case could be made for the thesis that escape from contact inhibition is not the primary or perhaps even a major determinant of neoplastic behavior. It is not certain that the phenomenon of contact inhibition is basically concerned with growth regulation in the whole animal. The observation that some normal diploid cells, each of which is contact-inhibited in a crowded culture, do not inhibit each other, indicates that escape

from contact inhibition sometimes depends on the specific interacting cells and is not necessarily associated with neoplastic behavior. In addition, some cell lines which have been transformed in culture, continue to exhibit contact inhibition of growth. Sanford (1967) concluded that the increased rate of cell population growth induced in Syrian hamster embryo cells by polyoma virus appeared to be the cause of the morphologic response, i.e. multilayering growth, rather than loss of contact inhibition.

In attempts to relate the metastatic properties of cancer cells to their surface properties interest has focused on the surface electrical charge. Ambrose and co-workers (Lowick et al., 1961) found that cells derived from rat hepatomas and stilbestrol-induced hamster kidney tumors had higher electrophorectic mobilities than did their normal counterparts. Similarly, in human kidney fibroblasts transformed by polyoma virus in culture (Forrester et al., 1962), two types of cells resulted. One type had an electrophoretic mobility similar to normal cells and the other had about a 25% higher mobility. After incubation with neuraminidase, the mobility of both was reduced to the same value. This indicated that the 25% increase in mobility was due to sialic acid (neuraminic acid). Purdom et al. (1958) demonstrated a progressive change in electrophoretic mobility in sub-lines of a mouse sarcoma. The ease of producing the ascites form of the tumor, which leads to the appearance of early metastases, was correlated with a progressive increase in negative charge.

Burger and Goldberg (1967) isolated and characterized an agglutinin important in studies of the surface charge of malignant cells. This substance agglutinated baby hamster kidney fibroblasts (BHK) which were transformed with polyoma virus, but not normal cells. They demonstrated that the tumor-specific site that interacts with the agglutinin contains sialic acid. Based on the hypothesis that there might be a failure in the regulation of sialic acid metabolism in cancer cells, Ohta et al. (1968) examined the sialic acid content of non-transformed tissue cultures of BHK cells and their virally transformed counterparts. They found that the transformed cells had slightly

lower amounts of sialic acid than their respective normal lines. The transformed line which had lost its contact inhibition contained less sialic acid than its BHK21 mother line. They suggest that technical differences of cell growth, isolation, etc. could be responsible for conflicting results with other investigations. For example, in some work trypsin was used to detach cells from their surface and this enzyme releases some sialic acid.

In a recent study, Patinkin et al. (1970a, 1970b) showed that there were no differences in electrophoretic mobility between lymphatic leukemia cells or lymphosarcoma cells and the lymph node and thymus cells derived from apparently normal animals or the corresponding strains. In fact, leukemic cells of the AKR strain (lymphatic leukemia) had slightly lower mobilities than those of the normal lymph node cells of the same strain. Neuraminidase reduced the mobility of all the cells tested by about 20 to 40%.

Some evidence for a difference in the cell surface of tumor cells comes from another kind of experiment. Hause et al. (1970) studied the PD of various types of cells which were cultured on glass cover slips. They found a distinct positive pre-potential, preceding the normal PD, which ranged from 1 to 8 mV and was dependent on the pH (5 to 8 mV were recorded at pH 8). This pre-potential was found in all malignant cells studied which included malignant trophoblast cells, human laryngeal carcinoma, cervical cancer (HeLa) cells, choriocarcinoma, rat sarcoma (256 and L cells). It was also found in human trophoblast cells and lymphocytes whereas normal kidney, embryonic heart, and lung cells seldom gave prepotential records except in cases in which the cells were rounded just prior to mitosis. This may reflect an increase in cell surface chemicals during the G_2 phase of the cell cycle and the early mitotic process. It was suggested that the malignant cells have a thicker surface coating of mucopolysaccharides containing terminal groups of negatively-charged sialic acid moieties. The typical cell membrane forms an ionic double layer of positive and negative ions at its outer surface which establishes a potential continuum with the surrounding electrolytes. The sialomucin coating alters the ionic distribu-

tion at the surface possibly by displacing the normal bilayer of surface ions to the outer margin of the sialomucin layer. Similar to the normal cell surface, a reference potential is thus established between the negative terminal groups of sialic acid and the external electrolytes. With respect to this reference potential, an induced positive potential is recorded within the coating layer due to the positively charged outer bilipid cell membrane (Hause et al., 1970).

Surface sialic acid moieties may also be involved in adherence of tumor cells to other surfaces. Cormack (1970) found that adhesion of dissociated Walker 256 tumor cells to mesothelial tissue depends in part on the presence of sialic acid on the tumor cell surface. When the cells of tumors previously incubated in neuraminidase were injected into rats they survived longer than rats receiving untreated cells. Cormack ascribed two functions to surface sialic acid: (i) Its presence contributes to the antigenicity of the cells, for they become less antigenic when it is removed. (ii) It is implicated in the adherence of dissociated tumor cells to mesothelial membrane.

Another approach to the study of surface properties of normal and malignant cells has been taken by Loewenstein and co-workers, using electrophysiologic techniques. Studies on normal and malignant liver cells (Loewenstein and Kanno, 1967) showed that normal rat liver cells communicate rather freely with each other through permeable junctional membranes, while rat liver cancer cells show no communication at all. This was based on the measurement of the electrical resistance between adjacent cells; the resistance is relatively low between cells which communicate electrically. The surface membrane of the cancer cell was considered to be a strong barrier to diffusion all around the cells. Some evidence to suggest that cancer cells induce alterations in membrane permeability in normal liver cells was based on the observation that communication among the normal cells was much reduced when malignant cells grew near them. Therefore, the cells of normal liver form a functional continuum while the cells of cancerous liver behave like independent functional units. Jamakosmanović and Loewenstein

(1968) compared human, rat, mouse and hamster normal and malignant thyroid tissue. The resting potential of normal rat thyroid was about -47 mV while corresponding malignant cells had a range of -22 to -32 mV depending on the type. In addition, these investigators found that there was no communication between the malignant cells. These studies were extended to cells in culture by Borek et al. (1969). These investigators examined the pattern of communication between cancerous fibroblasts shortly after their isolation from animal tumors and their normal counterparts. Epithelial cells of normal rat liver and hamster embryo in tissue culture communicate through membrane junctions indicating that the membrane regions of cell contact are highly ion permeable. However, cancerous cells from liver tumors and X-ray transformed embryo cultures do not communicate with each other or with contiguous normal cells. However, some differences were found based on the culture conditions. Communication in some fibroblastic cells was sensitive to components of blood serum. Normal and transformed hamster embryo fibroblasts, which communicate when cultured in medium containing fetal calf serum, appear to lose communication in medium containing calf serum; the converse holds for adult hamster fibroblasts and 3T3 (mouse embryo) cells transformed by SV-40 virus.

It would be tempting to hypothesize that intercellular junctions permit cells to exchange control substances thereby influencing their activity and that the lack of intercellular communication through these electrically-coupled junctions would lead to a disruption of coordinated cell behavior as observed in malignant growth. However, Sheridan (1970) observed low-resistance junctions in four types of tumors. His experiments were performed on small tumor nodules which appeared growing on the mesentery after intraperitoneal injection of cells obtained from cultures or solid tumors of mouse sarcoma or rat hepatoma dissociated with trypsin or crudely minced. Sheridan (1970) also reports unpublished observations of Furshpan and Potter in which after a renewal of medium, sarcoma 180 cells in culture undergo a cycle of uncoupling followed by recoupling. Such changes observed in culture

might be related to the change in shape of the cells, i.e. the rounding up at the start of mitosis (Hause et al., 1970; Cone, 1969). These contradictory observations may be due to differences in technique and handling of the cells studied. The cells may be particularly sensitive to mechanical stress imposed by separation techniques. Impaling a cell with a microelectrode may lead to uncoupling depending upon the amount of stress imposed upon the cell. It is also possible that changes in the degree of electrical coupling between cells occur during different stages of the cell cycle.

There is some morphological evidence for intercellular junctions between adjacent tumor cells. Clarke (1970) examined mouse sarcoma tissue with the electron microscope and found that these cells are in relatively close approximation to one another with long segments of plasma membrane in parallel array. Two types of specialized junctions were seen. One type was characterized by a parallel approximation of cell membranes of adjacent cells to within 200 Å of one another. The intercellular space appeared to be filled with an amorphous material of greater density than that found within the contiguous intercellular spaces. The cytoplasm immediately adjacent was often more dense. In the second type, which was more complex, the adjacent plasma membranes were separated by a distance of about 400 to 600 Å. In the center of this intercellular space there was a dense plaque. An amorphous dense material filled the space and adjacent cytoplasm.

Well-defined "zipperlike" structural bonds were observed by electron microscopy in monocytic cells from patients with acute monocytic leukemia which were stimulated for phagocytosis. These structures connected the plasma membranes of adjacent cells and joined surfaces of approximating pseudopodia on the same cell (Sanel and Serpick, 1970). Preliminary examination of the electron micrograph of the Shay chloroleukemic cells (figure 1) shows that the membranes of adjacent cells are very closely apposed. In some short segments there is what resembles dense intercellular material.

Actual bridge formation between malignant cells in culture has been observed to arise from external merger of pseudopodia of neighboring cells

as well as by incomplete cytokinesis (Cone, 1968). Cone observed that groups of mouse sarcoma cells with time-lapse cinematography, become linked into syncytial networks by intercellular bridges. In such networks the division of any one cell was seen to initiate a mitotic stimulus which traveled outward via the bridge connections to the neighboring cells and induced them to divide. Proof of the cytoplasmic continuity of the bridges was demonstrated by direct hypotonic coalescence of connected cells through the bridge channel. Such a system can be inherently malignant (Cone, 1969). Wallach (1969) postulates that an oncogenic agent acts to introduce an inappropriate protein into cell membranes, either in replacement of or in addition to normal components. As a consequence, numerous membrane functions may be modified accounting for the membrane changes and pleiomorphism seen in neoplasia. It appears that alterations in the membrane and surface of malignant cells do occur but no regular pattern of change is obvious. Different neoplasms do not all have the same alterations. Therefore, it is difficault to propose an hypothesis for the regulation of cell division or metastatic behavior based on a universal change in the surface properties of malignant cells.

V. Concluding Remarks

It has been shown that the membrane potential of the Shay chloroleukemic cell is relatively low; probably a result of a high permeability to sodium. This may also be the case in other malignant cells. Ideally, the membrane potential and distribution of electrolytes in normal myeloblasts should be measured and compared to the findings discussed above for Shay chloroleukemic cells. However, the small number of myeloblasts in normal bone marrow do not make this kind of study feasible.

Experiments designed to test the hypothesis proposed by Cone, relating membrane potential and sodium permeability to cell proliferation would be desirable in examining the leukemic process. Attempts are being made to grow the Shay chloroleukemic cells in suspension culture. Such an in vitro

system would permit the study of the effects of changes in the electrolyte distribution and membrane potential on the proliferative activity of these cells. In addition, the response of these leukemic cells to various growth regulatory agents, e.g. hematopoietic stimulating factors, could be assessed.

The lymphocyte provides a good system for studying proliferative responses. It has been shown that transformation and proliferation in response to phytohemagglutinin (PHA) is a characteristic of normal lymphocytes. Chronic lymphocytic leukemic (CLL) lymphocytes respond to PHA but the magnitude of the response is reduced and the development of a peak proliferative activity is delayed (Havemann and Rubin, 1968). PHA is believed to affect the surface membrane of the lymphocyte and, acting primarily through gene activation, lead to cell enlargement, DNA replication and mitosis. The observation that the CLL lymphocyte is much less sensitive to this agent suggests that PHA may remove the regulating factors which inhibit mitosis in normal lymphocytes, leading to increased proliferation while the CLL lymphocyte has already been subjected to this altered regulation. This suggests that changes in the structure and/or function of the surface membrane, through which PHA is presumed to act, may be significantly involved in the neoplastic process.

Further studies of the kind discussed above on the Shay chloroleukemic cell would contribute to our understanding of the leukemic cell and perhaps more generally, elucidate mechanisms of the neoplastic process.

SUMMARY

The nature of the potential difference (PD) across the membrane of the Shay chloroleukemic tumor cell was examined. The mean PD was found to be -9.4 ± 0.4 (SE) mV, inside negative. The role of potassium and chloride ions in the maintenance of this PD was determined using the Nernst equation. It was found that increasing the external concentration of potassium had only a small effect causing the PD to shift from a mean of -9.2 ± 0.4 mV in normal Ringer medium to -6.7 ±0.4 mV in 80 mM K and -7.3 ± 0.4 mV in 120 mM K Ringer medium. Decreasing the external concentration of chloride caused a depolarization of the membrane to zero. However, the Nernst equilibrium potential for chloride (-21.3 mV) was greater than the measured PD in normal Ringer medium in these experiments (-7.4 ± 0.2 mV). If the Goldman equation is considered, the distribution of sodium ions across the membrane may play a role in "shunting" the chloride equilibrium potential, resulting in a lower PD. To investigate this possibility, the relative fluxes of chloride and sodium across the cell membrane were determined. The flux of sodium (0.4 to 0.8 umoles/minute) was almost twice as fast as that for chloride (0.3 umoles/minute). This makes the suggestion of a shunting role for sodium more feasible. Therefore, the membrane potential of the Shay chloroleukemic cell depends on the distribution of chloride and sodium ions in particular, while potassium ions seem to play a lesser role in this respect.

BIBLIOGRAPHY

Abercrombie, M. and J. E. M. Heaysman. Observations on the Social Behavior of Cells in Tissue Culture. II. Monolayering of Fibroblasts. Exp. Cell Res. 6: 293-306, 1954.

Abercrombie, M. and J. E. M. Heaysman. Social Behavior of Cells in Tissue Culture. III. Mutual Influence of Sarcoma Cells and Fibroblasts. Exp. Cell Res. 13: 276-291, 1957.

Adrian, R. H. The Effect of Internal and External Potassium Concentration on the Membrane Potential of Frog Muscle. J. Physiol. 133: 631-658, 1956.

Aull, F. Measurement of the Electrical Potential Difference Across the Membrane of the Ehrlich Mouse Ascites Tumor Cell. J. Cell Physiol. 69: 21-32, 1967.

Balitsky, K. P. and E. P. Shuba. Resting Potential of Malignant Tumor Cells. Acta Unio Intern. Contra Canc. 20: 1391-1393, 1964.

Baron, D. N. and P. M. Roberts. The Sodium, Potassium and Water Content of Isolated Normal Human Leucocytes. J. Physiol. 165: 219-226, 1963.

Beckmann, A., H. L. Jenssen, W. Kalkoff and K. Redmann. Das bioelektrische Potential an der zytoplasmatischen Membran der Granulozyten. Experientia 26: 186-187, 1970.

Beigelman, P. M. and G. H. Schlosser. Studies on Hepatic Cell Resting Membrane Potential: Report of in Vitro and in Vivo Experiments and Review of the Literature. Biochem. Med. 3: 73-83, 1969.

Bierman, H. R. Hypothesis: The Leukemias — Proliferative or Accumulative? Blood 30: 238-250, 1967.

Block, J. B. and S. L. Bonting. Sodium-Potassium Activated Adenosinetriphosphatase and Cation Transport in Normal and Leukemic Human Leukocytes. Enzymol. Biol. Clin. 4: 183-198, 1964.

Borek, C., S. Higashino and W. R. Loewenstein. Intercellular Communication and Tissue Growth IV. Conductance of Membrane Junctions of Normal and Cancerous Cells in Culture. J. Memb. Biol. 1: 274-293, 1969.

Borle, A. B. and J. Loveday. Effects of Temperature, Potassium, and Cal-

cium on the Electrical Potential Difference in HeLa Cells. Cancer Res. 28: 2401-2405, 1968.

Boyle, P. J. and E. J. Conway. Potassium Accumulation in Muscle and Associated Changes. J. Physiol. 100: 1-63, 1941.

Burger, M. M. and A. R. Goldberg. Identification of a Tumor-Specific Determinant on Neoplastic Cell Surfaces. Proc. Natl. Acad. Sci. 57: 359-366, 1967.

Clarke, M. C. Specialized Intercellular Junctions in Tumor Cells — An Electron Microscope Study of Mouse Sarcoma Cells. Anat. Rec. 166: 199-206, 1970.

Cone, C. D., Jr. Observations of Self-Induced Mitosis and Autosynchrony in Sarcoma Cell Networks. Cancer Res. 28: 2155-2161, 1968.

Cone, C. D., Jr. Electroosmotic Interactions Accompanying Mitosis Initiation in Sarcoma Cells in Vitro. Trans. N. Y. Acad. Sci. 31: 404-427, 1969 a.

Cone, C. D., Jr. Some Theoretical Aspects of Intercellular Bridges as a Potential Mechanism of Cancerous Proliferation. J. Theoret. Biol. 22: 365-379, 1969 b.

Cone, C. D., Jr. Control of Cell Division by the Electrical Voltage of the Surface Membrane. Presentation to the Twelfth Annual Science Writers Seminar. American Cancer Society, San Antonio, Texas, 1970 a.

Cone, C. D., Jr. Variation of the Transmembrane Potential Level as a Basic Mechanism of Mitosis Control. NASA pub. L-7164, 1970 b.

Cone, C. D., Jr. Unified Theory on the Basic Mechanism of Normal Mitotic Control and Oncogenesis. J. Theoret. Biol. 30: 151-181, 1971.

Cone, C. D., Jr. and M. Tongier, Jr. Control of Somatic Cell Mitosis by Simulated Changes in Transmembrane Potential Level. NASA pub. L-7126, 1970.

Cormack, D. Effect of Enzymatic Removal of Cell Surface Sialic Acid on the Adherence of Walker 256 Tumor Cells to Mesothelial Membrane. Cancer Res. 30: 1459-1466, 1970.

Dunham, P. B. and H. Gainer. The Distribution of Inorganic Ions in Lobster Muscle. Biochim. Biophys. Acta 150: 488-499, 1968.

Eagle, H. Growth-Regulatory Effects of Cellular Interaction in Vitro, and

and their Relevance to Cancer, in The Proliferation and Spread of Neoplastic Cells, pgs. 7-20. Williams and Wilkins Co., Baltimore, 1968.

Fawcett, P. R. W. An Electrophysiological Study of the Rat Adrenal Gland in Vivo. J. Neurol. Sci. 8: 381-383, 1969.

Forrester, J. A., E. J. Ambrose and J. A. MacPherson. Electrophoretic Investigations of a Clone of Hamster Fibroblasts and Polyoma-Transformed Cells from the Same Population. Nature 196: 1068-1070, 1952.

Girardier, L., J. Seydoux and T. Clausen. Membrane Potential of Brown Adipose Tissue. J. Gen. Physiol. 52: 925-940, 1968.

Goodford, P.J. and E. H. Leach. The Extracellular Space of Intestinal Smooth Muscle. J. Physiol. 175: 38-39P, 1964.

Handler, E. E. and E. S. Handler. Experimental Leukemias: Model Systems for the Study of Hematopoiesis, in Regulation of Hematpoiesis, A. S. Gordon, ed., pgs. 1273-1296. Appleton-Century-Crofts, New York, 1970.

Handler, E. E., E. S. Handler and A. E. Schaefer. Alterations in Marrow and Splenic Hematopoiesis in Acute Myelogenous Leukemia. J. Reticuloendothel. Soc. 5: 445-454, 1968.

Hause, L. L., R. A. Pattillo, A. Sances, Jr. and R. F. Mattingly. Cell Surface Coatings and Membrane Potentials of Malignant and Nonmalignant Cells. Science 169: 601-603, 1970.

Havemann, K. and A. D. Rubin. The Delayed Response of Chronic Lymphocytic Leukemic Lymphocytes to Phytohemagglutinin in Vitro. Proc. Soc. Exp. Biol. Med. 127: 668-671, 1968.

Hempling, H. G. Potassium and Sodium Movements in Rabbit PMN Leukocytes. J. Cell. Comp. Physiol. 44: 87-104, 1954.

Hempling, H. G. Potassium and Sodium Movements in the Ehrlich Mouse Ascites Tumor Cell. J. Gen. Physiol. 41: 565-583, 1958.

Hild, W. and I. Tasaki. Morphological and Physiological Properties of Neurons and Glial Cells in Tissue Culture. J. Neurophysiol. 25: 277-304, 1962.

Hodgkin, A. L. and P. Horowicz. The Influence of Potassium and Chloride Ions on the Membrane Potential of Single Muscle Fibres. J. Physiol.

148: 127-160, 1959.

Hodgkin, A. L. and B. Katz. The Effect of Sodium Ions on the Electrical Activity of the Giant Axon of the Squid. J. Physiol. 108: 37-77, 1949.

Jamakosmanović, A. and W. R. Loewenstein. Intercellular Communication and Tissue Growth III. Thyroid Cancer. J. Cell Biol. 38: 556-561, 1968.

Jay, A. W. L. and A. C. Burton. Direct Measurement of the Potential Difference Across Human Red Blood Cell Membrane. Biophys. J. 9: 115-121, 1969.

Lamb, J. F., J. R. Loudon and M. G. A. MacKinnon. The Permeability of the L Cell Membrane to Sodium, Potassium and Chloride. J. Physiol. 207: 25-26P, 1970.

Lassen, U. V. and O. Sten-Knudsen. Direct Measurement of Membrane Potential and Membrane Resistance of Human Red Cells. J. Physiol. 195: 681-696, 1968.

Laszlo, J. Energy Metabolism of Human Leukemic Lymphocytes and Granulocytes. Blood 30: 151-167, 1967.

Law, R. O. and C. F. Phelps. The Size of the Sucrose, Raffinose, and Inulin Spaces in the Gastrocnemius Muscle of the Rat. J. Physiol. 186: 547-557, 1966.

Levinson, C. and H. G. Hempling. The Role of Ion Transport in the Regulation of Respiration in the Ehrlich Mouse Ascites-Tumor Cell. Biochim. Biophys. Acta 135: 306-318, 1967.

Lichtman, M. A. and R. I. Weed. Cation and Membrane Characteristics of Human Leukocytes: Immaturity of the Small Lymphocyte. Clin. Res. 16: 307, 1968.

Lichtman, M. A. and R. I. Weed. The Monovalent Cation Content and Adenosine Triphosphatase Activity of Human Normal and Leukemic Granulocytes and Lymphocytes: Relationship to Cell Volume and Morphologic Age. Blood 34: 645-660, 1969.

Loewenstein, W. R. and Y. Kanno. Intercellular Communication and Tissue Growth. I. Cancerous Growth. J. Cell Biol. 33: 225-234, 1967.

Lowick, J. H. B., L. Purdom, A. M. James and E. J. Ambrose. Some Microelectrophoretic Studies of Normal and Tumor Cells. J. Roy.

Microscop. Soc. 80: 47-57, 1961.

Lubin, M. Intracellular Potassium and Macromolecular Synthesis in Mammalian Cells. Nature 213: 451-453, 1967.

Matthews, E. K. Membrane Potential Measurement in Cells of the Adrenal Gland. J. Physiol. 189: 139-148, 1967.

Michelsen, K. Determination of Inulin, Albumin, and Erythrocyte Spaces in the Bone Marrow of Rabbits. Acta Physiol. Scand. 77: 28-35, 1969.

Morrill, G. A., H. R. Kaback and E. Robbins. Effect of Calcium on Intracellular Sodium and Potassium Concentrations in Plant and Animal Cells. Nature 204: 641-642, 1964.

Ohta, N., A. B. Pardee, B. R. McAuslan and M. M. Burger. Sialic Acid Contents and Controls of Normal and Malignant Cells. Biochim. Biophys. Acta 158: 98-102, 1968.

Passow, H. Passive Ion Permeability of the Erythrocyte Membrane. Prog. Biophys. Molec. Biol. 19: 424-467, 1969.

Passow, H. and K. F. Schnell. Chemical Modifiers of Passive Ion Permeability of the Erythrocyte Membrane. Experientia 25: 460-468, 1969.

Patinkin, D., M. Schlesinger and F. Doljanski. A Study of Surface Ionogenic Groups of Different Types of Normal and Leukemic Cells. Cancer Res. 30: 489-497, 1970a.

Patinkin, D., A. Zaritsky and F. Doljanski. A Study of Surface Ionogenic Groups of Chick Embryo Cells Transformed by Rous Sarcoma Virus. Cancer Res. 30: 498-503, 1970b.

Pittman, J. A. and A. F. Debons. Thyroidal Extracellular Fluid Compartments. Am. J. Physiol. 210: 399-403, 1966.

Prieto, A., P. L. Kornblith and D. A. Pollen. Electrical Recordings From Meningioma Cells During Cytolytic Action of Antibody and Complement. Science 157: 1185-1187, 1967.

Purdom, L., E. J. Ambrose and G. Klein. A Correlation Between Electrical Surface Charges and Some Biological Characteristics During Stepwise Progression of a Mouse Sarcoma. Nature 181: 1586-1587, 1958.

Quastel, M. R. and J. G. Kaplan. Inhibition by Ouabain of Human Lymphocyte Transformation Induced by Phytohaemagglutinin in Vitro. Nature 219: 198-200, 1968.

Redmann, K. and W. Kalkoff. Elektrische Eigenschaften von Glasmikroelektroden nach Membranpotentialmessungen an Zellkulturen. Experientia 24: 975-976, 1968.

Rigas, D. A. Electrolyte, Nitrogen, and Water Content of Human Leukemic Leukocytes: Relation to Cell Maturity. J. Lab. Clin. Med. 58: 234-241, 1961.

Sanel, F. T. and A. A. Serpick. Plasmalemmal and Subsurface Complexes in Human Leukemic Cells: Membrane Bonding By Zipperlike Junctions. Science 168: 1458-1460, 1970.

Sanford, K. K. "Spontaneous" Neoplastic Transformation of Cells in Vitro: Some Facts and Theories. Nat. Cancer Inst. Monogr. no. 26: 387-418, 1967.

Schanne, O. and E. Coraboeuf. Potential and Resistance Measurements of Rat Liver Cells in Situ. Nature 210: 1390-1391, 1966.

Shay, H., M. Gruenstein, C. Harris and L. Glazer. Transfer of Myelogenous Leukemia Induced by Gastric Instillation of Methylcholanthrene in Wistar Rats. Blood 7: 612-622, 1952.

Shay, H., M. Gruenstein, H. E. Marx and L. Glazer. The Development of Lymphatic and Myelogenous Leukemia in Wistar Rats Following Gastric Instillation of Methylcholanthrene. Cancer Res. 11: 29-34, 1951.

Sheridan, J. D. Low-Resistance Junctions Between Cancer Cells in Various Solid Tumors. J. Cell Biol. 45: 91-99, 1970.

Solomon, A. K. Red Cell Membrane Structure and Ion Transport. J. Gen. Physiol. suppl. 43: 1-15, 1960.

Tosteson, D. C. Some Properties of the Plasma Membranes of High Potassium and Low Potassium Sheep Red Cells. Ann. N. Y. Acad. Sci. 137: 577-590, 1966.

Tosteson, D. C. and J. F. Hoffman. Regulation of Cell Volume by Active Cation Transport in High and Low Potassium Sheep Red Cells. J. Gen. Physiol. 44: 169-194, 1960.

Wallach, D. F. H. Cellular Membranes and Tumor Behavior: A New Hypothesis. Proc. Natl. Acad. Sci. 61: 868-874, 1968.

Wallach, D. F. H. Generalized Membrane Defects in Cancer. N. Eng. J. Med. 280: 761-767, 1969.

Wardell, W. M. Electrical and Pharmacological Properties of Mammalian Neuroglial Cells in Tissue-Culture. Proc. Roy. Soc. London, Ser. B 165: 326-361, 1966.

Wickson-Ginzburg, M. and A. K. Solomon. Electrolyte Metabolism in HeLa Cells. J. Gen. Physiol. 46: 1303-1315, 1963.

Wilson, D. L. and J. F. Manery. The Permeability of Rabbit Leukocytes to Sodium, Potassium and Chloride. J. Cell. Comp. Physiol. 34: 493-519, 1949.

Woodbury, D. M. and J. W. Woodbury. Correlation of Micro-electrode Potential Recordings with Histology of Rat and Guinea Pig Thyroid Glands. J. Physiol. 169: 553-567, 1963.

Table 1

Effects of Increased External Potassium on the PD and on the Potassium and Sodium Concentrations of Shay Chloroleukemic Tumor Cells

Ringer Medium	$(K)_i$	$(Na)_i$	% Cell Water
Normal	121.6 ± 9.2	48.4 ± 3.9	77.5 ± 0.5
20 mM K	115.5 ± 5.6	42.8 ± 7.7	78.1 ± 0.5
80 mM K	120.1 ± 8.7	32.9 ± 4.0	78.0 ± 0.3
120 mM K	137.7 ± 2.2	26.2 ± 4.5	78.5 ± 0.4

Ringer Medium	$(K)_o$	$(Na)_o$	% Equilibration	PD (-mV) (N)
Normal	7.0 ± 0.1	165.8 ± 4.9	82.3 ± 4.1	9.2 ± 0.4 (49)
20 mM K	20.8 ± 0.4	158.2 ± 7.2	89.3 ± 2.3	8.2 ± 0.4 (40)
80 mM K	80.7 ± 1.6	107.4 ± 5.0	81.3 ± 5.2	6.7 ± 0.4 (52)
120 mM K	119.2 ± 1.4	75.3 ± 3.3	82.0 ± 5.9	7.3 ± 0.4 (37)

In this and all subsequent tables, the values given are accompanied by the standard error (± SE). Intracellular concentrations (i) are given in mEq/liter cell water, and extracellular concentrations (o) are given in mEq/liter water.

Table 2

Effects of Decreased External Chloride on the PD and Chloride Concentration of Shay Chloroleukemic Tumor Cells

Ringer Medium	$(Cl)_i$	$(Cl)_o$	% Cell Water	% Equilibration
Normal	71.7 ± 6.7	163.1 ± 3.4	77.3 ± 0.6	96.9 ± 7.3
Sulfate	20.5 ± 1.7	10.0 ± 1.0	77.2 ± 0.6	44.1 ± 8.2
High K-Sulfate	19.3 ± 1.9	5.9 ± 0.3	76.1 ± 0.4	42.3 ± 3.8

Ringer Medium	Measured PD (mV) (N)	Calculated E_{Cl} (mV)
Normal	-7.4 ± 0.2 (46)	-21.3
Sulfate	+0.7 ± 0.4 (51)	+18.4
High K-Sulfate	+0.2 ± 0.2 (50)	+30.1

where $$E_{Cl} = \frac{RT}{zF} \ln \frac{Cl_o}{Cl_i}$$

Table 3

Sulfate and Inulin Spaces of Tumors Calculated on the Basis of the S^{35}-Sulfate and C^{14}-Inulin Uptake Experiments

Time (min)	Sulfate Space 23°	Sulfate Space 0°(1)	Sulfate Space 0°(2)	Inulin Space 23°	Inulin Space 0°
0	0.029	0.018	0.026	0.011	0.025
1	0.064	0.037	0.033	0.029	0.031
5	0.123	0.053	0.105	0.043	0.042
15	0.190	0.087	0.149	0.060	0.054
30	0.306	0.114	0.189	0.067	0.091
60	0.392	0.144	0.176	0.116	0.113
90	0.392	0.110	0.197	0.100	0.089
120	-	0.125	0.191	0.092	0.107
150	-	-	0.227	-	-

where

$$\text{Sulfate or Inulin Space} = \frac{\frac{\text{cpm } C^{14} \text{ or } S^{35}}{\text{g wet wt}}}{\frac{\text{cpm } C^{14} \text{ or } S^{35}}{\text{ml medium}}}$$

$$= \frac{\text{ml "inulin or sulfate water"}}{\text{g wet wt}}$$

Table 4

Chloride Concentrations of Tumor Cells and the Extent of Equilibration of Tissue Chloride with Cl^{36} in the Medium for Tumors Used in the Cl^{36} Uptake Experiments

Time (min)	$(Cl)_i$	$(Cl)_o$	% Cell Water	S. A. Tissue	S. A. Medium	% Equilibration
0	47.0 ±1.2	152.5 ±2.5	79.0 ±0.1	100 ±11	1171 ±52	8.5 ±0.7
5	58.1 ±6.9	151.7 ±0.8	79.6 ±0.5	512 ±52	1156 ±30	44.3 ±4.6
15	59.4 ±7.4	155.0 ±5.0	79.1 ±0.3	697 ± 5	1092 ±46	63.9 ±2.3
30	58.5 ±3.4	155.0 ±2.0	78.5 ±0.3	764 ±35	1093 ±27	70.1 ±5.0
60	56.9 ±4.5	156.7 ±0.8	78.7 ±0.3	887 ± 5	1078 ±41	82.4 ±3.1
90	62.5 ±1.9	151.7 ±0.8	78.9 ±0.2	1014 ± 7	1123 ±44	90.5 ±3.1
120	57.8 ±5.3	152.5 ±1.4	78.7 ±0.1	1022 ±52	1129 ±59	91.2 ±8.4
150	65.3 ±5.6	155.8 ±3.6	79.0 ±0.2	1017 ±57	1094 ±46	93.1 ±3.9

where S.A. = specific activity, in dimensions of cpm/micromole Cl.

Table 5

Sodium Concentrations of Tumor Cells and the Extent of Equilibration of Tissue Sodium with Na^{22} in the Medium for Tumors Used in the Na^{22} Uptake Experiments

Time (min)	$(Na)_i$	$(Na)_o$	% Cell Water	S. A. Tissue	S. A. Medium	% Equilibration
0	43.6 ±6.7	162.6 ±6.4	78.4 ±0.2	147 ±19	2028 ±120	7.3 ±0.9
1	46.4 ±5.9	160.7 ±3.3	79.1 ±0.1	426 ±18	1907 ±21	22.3 ±0.8
5	58.5 ±14.4	165.3 ±3.5	78.8 ±0.1	647 ±63	1817 ±69	35.9 ±4.7
15	61.9 ±3.1	159.0 ±0.8	78.8 ±0.5	929 ±32	1895 ±93	49.5 ±4.2
30	65.3 ±3.9	163.2 ±3.7	78.5 ±0.3	1288 ±96	1799 ±85	72.0 ±6.8
60	68.3 ±2.0	165.2 ±3.7	78.6 ±0.1	1544 ±37	1798 ±54	86.1 ±3.8
90	75.5 ±4.3	163.5 ±4.8	78.5 ±0.1	1491 ±114	1763 ±92	84.5 ±4.1
120	76.3 ±11.3	160.3 ±1.8	78.4 ±0.2	1664 ±56	1869 ± 4	89.0 ±2.8
150	68.8 ±2.5	159.6 ±2.6	78.0 ±0.2	1626 ±46	1792 ±109	91.3 ±5.0

where S. A. = specific activity, in dimensions of cpm/micromole Na.

Table 6

Rate Constants and Fluxes for Chloride Calculated on the Basis of the Kinetic Analysis of the Data From the Cl^{36} Uptake Experiments

Subscript	Rate Constant (k) (min^{-1})	Flux (uM Cl/min)
12	0.011	8.15
21	1.000	8.19
23	0.040	0.33
32	0.031	0.33

where Flux = Rate Constant x Compartment Size, and the compartment sizes are: p = 769.0 $\pm$ 3.8, q = 8.2 $\pm$ 0.4, and r = 10.5 $\pm$ 0.7 micromoles of chloride, for N = 24 pieces of tissue. The values of q and r were calculated for each piece of tissue from the following equations:

$q = A_4(q + r)$ (93% exchangeable chloride),

$r = A_2(q + r)$ (93% exchangeable chloride).

Table 7

Rate Constants and Fluxes for Sodium Calculated on the Basis of the Kinetic Analysis of the Data from the Na^{22} Uptake Experiments

Sub-script	Experiment I k	Experiment I flux	Experiment II k	Experiment II flux	Experiment III k	Experiment III flux
12	0.005	3.90	0.007	5.67	0.004	3.26
21	0.460	3.93	0.420	5.71	0.372	3.30
23	0.090	0.77	0.030	0.41	0.091	0.81
32	0.036	0.77	0.027	0.41	0.050	0.80

Compartment	Size	Size	Size
p	829.9 ±9.8	809.3 ±11.3	794.0 ±3.7
q	8.6 ±0.6	13.6 ±1.9	8.9 ±0.7
r	21.1 ±1.6	15.0 ±2.1	16.0 ±1.2
	(N = 9)	(N = 8)	(N = 9)

Table 8

Rate Constants Calculated from the Kinetic Analysis of the C^{14}-Inulin and S^{35}-Sulfate Uptake Experiments

	C^{14}-Inulin 23^{o}, 0^{o}	C^{14}-Inulin 23^{o}	S^{35}-Sulfate 0^{o} (1)	S^{35}-Sulfate 0^{o} (2)	S^{35}-Sulfate 23^{o}
k_{12}	0.003	0.004	0.003	0.012	0.008
k_{21}	0.353	0.184	0.220	0.620	0.167
k_{23}	0.035	0.020	0.060	0.060	0.025
k_{32}	0.027	0.038	0.060	0.060	0.021
A_4	0.440	0.653	0.500	0.500	0.464
A_2	0.565	0.346	0.498	0.500	0.535
$\frac{(q+r)}{p}$	0.022	0.029	0.028	0.040	0.102

Table 9

Comparison of Values for the Extracellular Space Based on the Kinetic Analysis and Based on the C^{14}-Inulin Distribution

	uM Cl	uM Na(I)	uM Na(II)	uM Na(III)
Kinetic ECS	8.2 ±0.4	8.6 ±0.6	13.6 ±1.2	8.9 ±0.7
Inulin ECS	6.0 ±0.2	11.8 ±0.6	10.8 ±0.6	8.1 ± 0.3
Difference	36%	28%	26%	10%

Table 10

Comparison of Membrane Potentials of Normal and Malignant Cells

Cell type	Condition	PD (-mV)	Reference
Normal Cells			
rat liver	intact	50; 43	Schanne and Corabouef, 1966; Beigelman and Schlosser, 1969
rat, guinea pig thyroid	excised	50	Woodbury and Woodbury, 1963, Jamakosmanovic and Loewenstein, 1968
rabbit, rat, kitten adrenal cortex	excised	70	Matthews, 1967; Fawcett, 1969
rabbit, rat, kitten adrenal medulla	excised	20 to 30	Matthews, 1967; Fawcett, 1969
rat adipose tissue	excised	51	Girardier et al., 1968
human embryonic heart	in culture	35	Hause et al., 1970
human embryonic kidney	in culture	20	"
human lung	in culture	22	"
monkey kidney fibroblasts	in culture	27	"
rabbit macrophages	in culture	5 to 15	Wardell, 1966; Hild and Tasaki, 1962
human lymphocytes	in culture	12	Hause et al., 1970
rabbit peritoneal, human peripheral granulocytes	excised	5	Beckmann et al., 1970
human peripheral red blood cells	excised	8	Jay and Burton, 1969

Table 10 (continued)

Malignant Cells			
rat thyroid	excised	22 to 32	Jamakosmanovic and Loewenstein, 1968
Ehrlich mouse ascites cells	excised	11	Auli, 1967
HeLa cells	in culture	17; 25	Borle and Loveday, 1968; Hause et al., 1970
KB cancer cells	in culture	12	Redmann and Kalkoff, 1968
human meningioma cells	in culture	10 to 20	Prieto et al., 1967
human choriocarcinoma	in culture	35	Hause et al., 1970
human laryngeal carcinoma	in culture	23	"
mouse L cells	in culture	15	Lamb et al., 1970
rat sarcoma 256	in culture	20	Hause et al., 1970

Figure 1

Electron Micrograph of the Shay Chloroleukemic Tumor Cells.
Magnification, 8000x.

Figure 2

Distribution of the Potential Difference of Shay Chloroleukemic Cells From Tissues Bathed in Normal Ringer Medium.

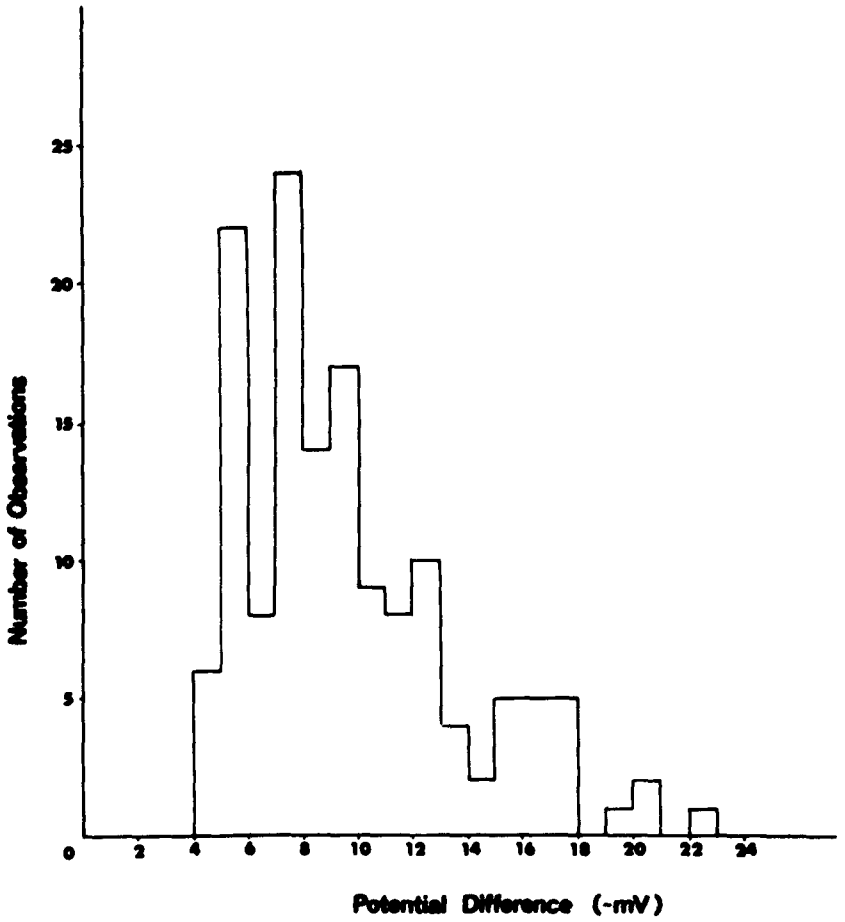

Number of Observations
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
22
24
Potential Difference (-mV)

Figure 3

The Effect of Increased External Potassium on the Distribution of the Potential Difference of Shay Chloroleukemic Tumor Cells.

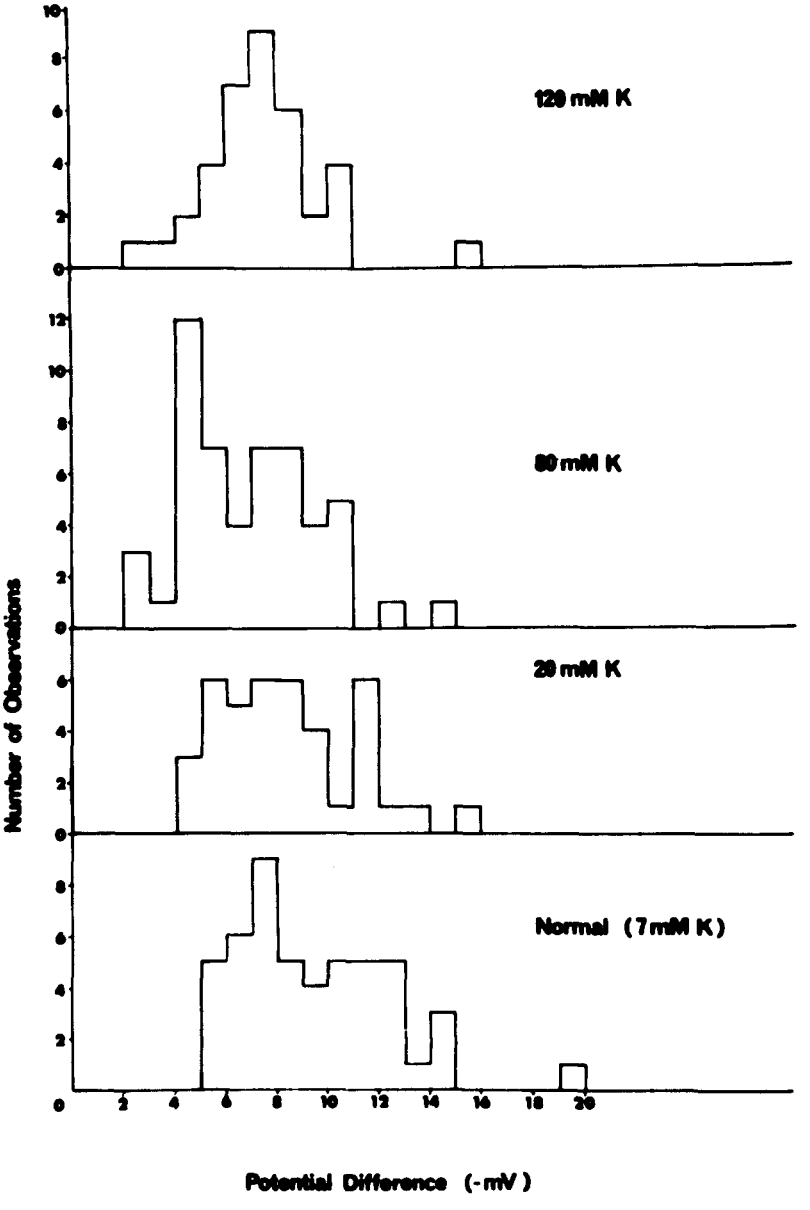

120 mM K
80 mM K
20 mM K
Normal (7 mM K)
Number of Observations
Potential Difference (-mV)

Figure 4

Typical Recording of the Potential Difference of Shay Chloroleukemic Tumor Cells in Normal (7 mM K) Ringer Medium. The recording was made from left to right as the microelectrode began penetrating the tissue (upward arrow). Movement was stopped when the -12 mV potential difference occurred and the microelectrode was slowly withdrawn (downward arrow) about five seconds later.

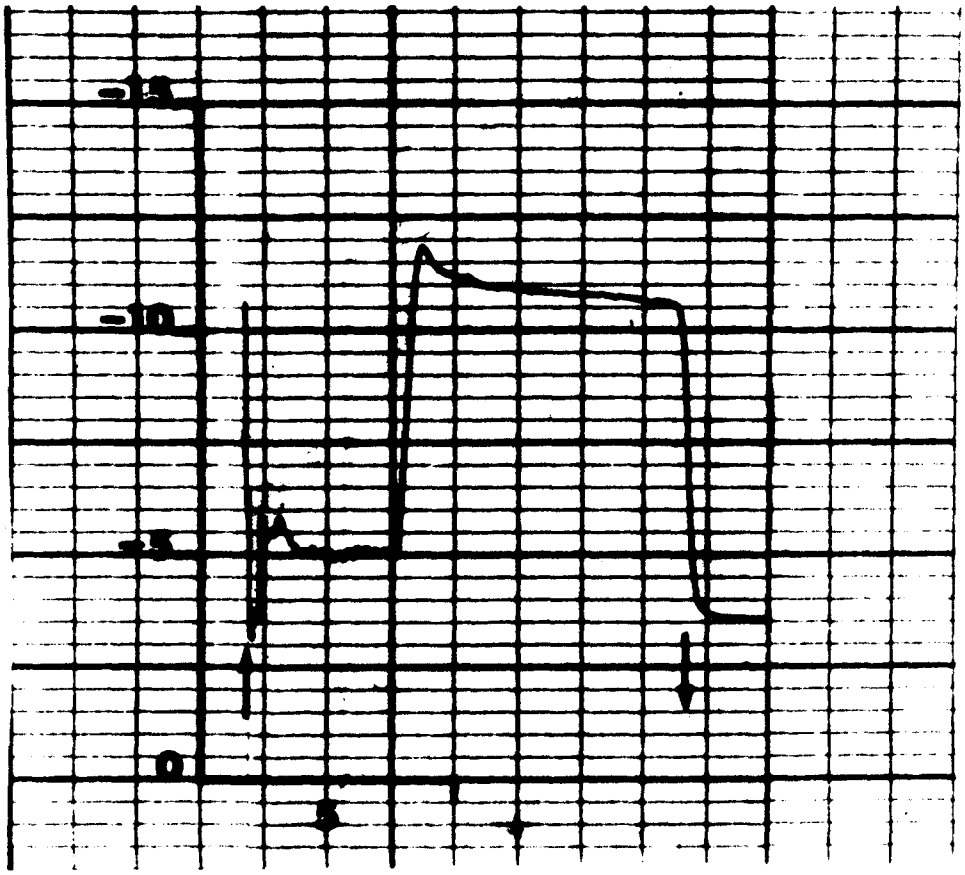

-15
-10
-5
0

Figure 5

Typical Recording of the Potential Difference of Shay Chloroleukemic Tumor Cells in 20 mM K Ringer Medium. This potential difference is -8 mV. The upward arrow indicates penetration of the tissue by the microelectrode. Movement was stopped when the 8 mV deflection occurred. The microelectrode was withdrawn almost five seconds later (downward arrow).

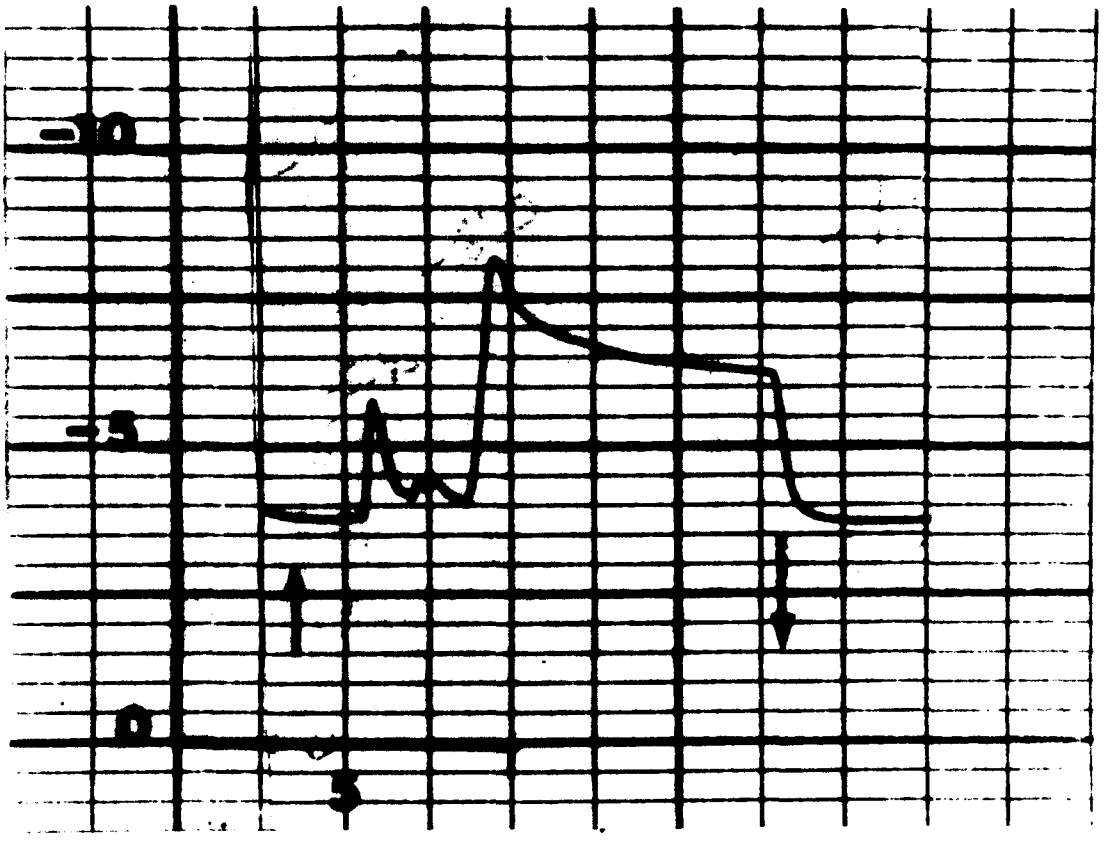

10
5
0
5

Figure 6

Typical Recording of the Potential Difference of Shay Chloroleukemic Tumor Cells in 80 mM K Ringer Medium. The first upward arrow indicates the penetration of the tissue by the microelectrode followed by the recording of a -7 mV potential difference. Further penetration led to the recording of a -8.5 mV potential difference (second upward arrow). Then the microelectrode was withdrawn from the tissue (downward arrow).

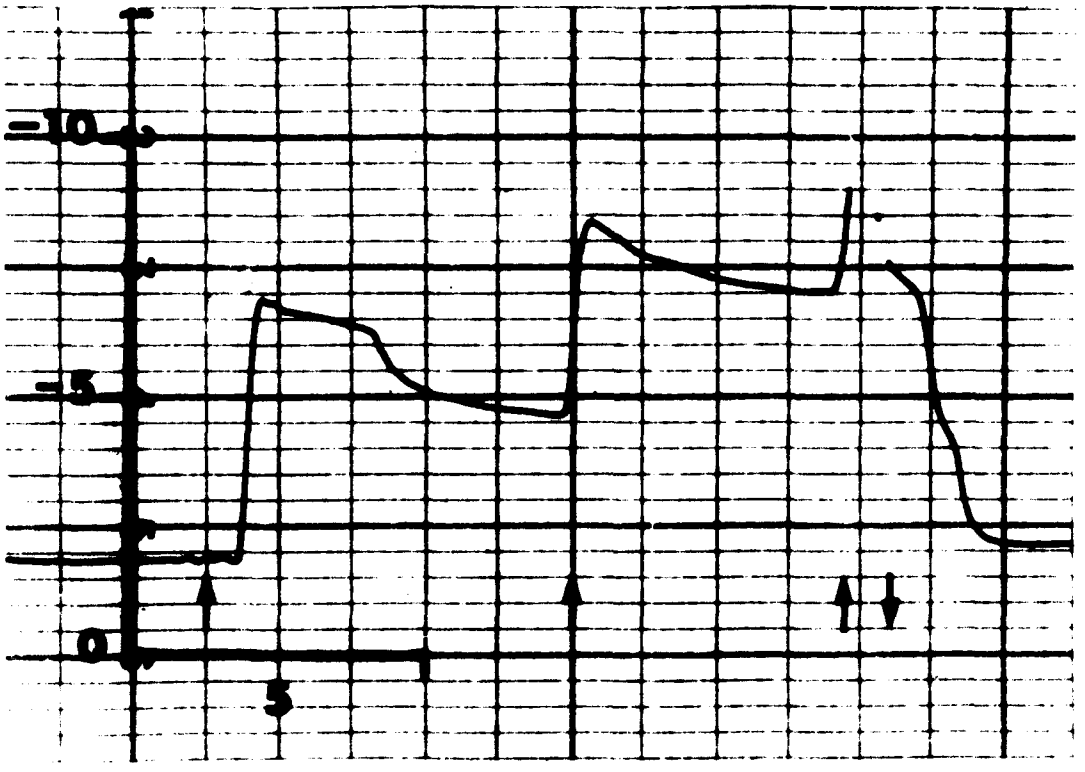

-10
-5
0
5

Figure 7

Typical Recording of the Potential Difference of Shay Chloroleukemic Tumor Cells in 120 mM K Ringer Medium. The potential difference is -6.5 mV (upward arrow). After about 8 seconds the microelectrode was slowly withdrawn (downward arrow).

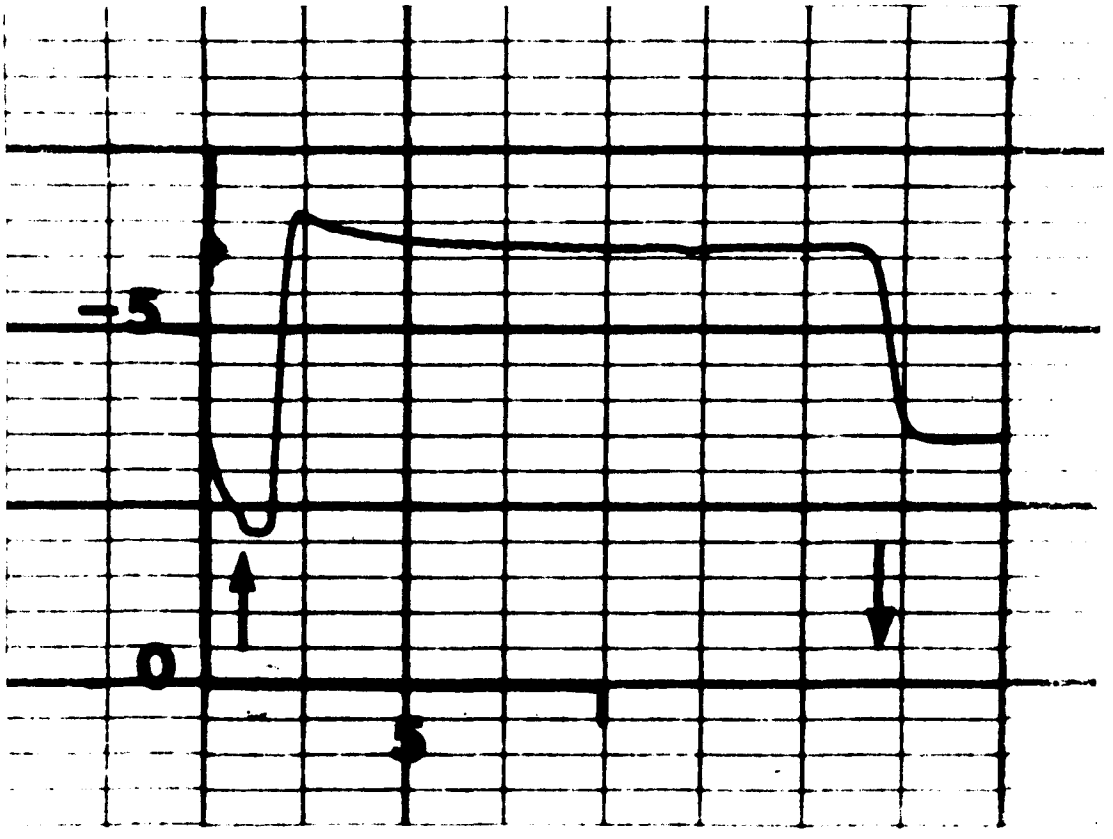

-5
0
5

Figure 8

The Effect of Sulfate and High Potassium - Sulfate Ringer Medium on the Distribution of the Potential Difference of Shay Chloroleukemic Tumor Cells.

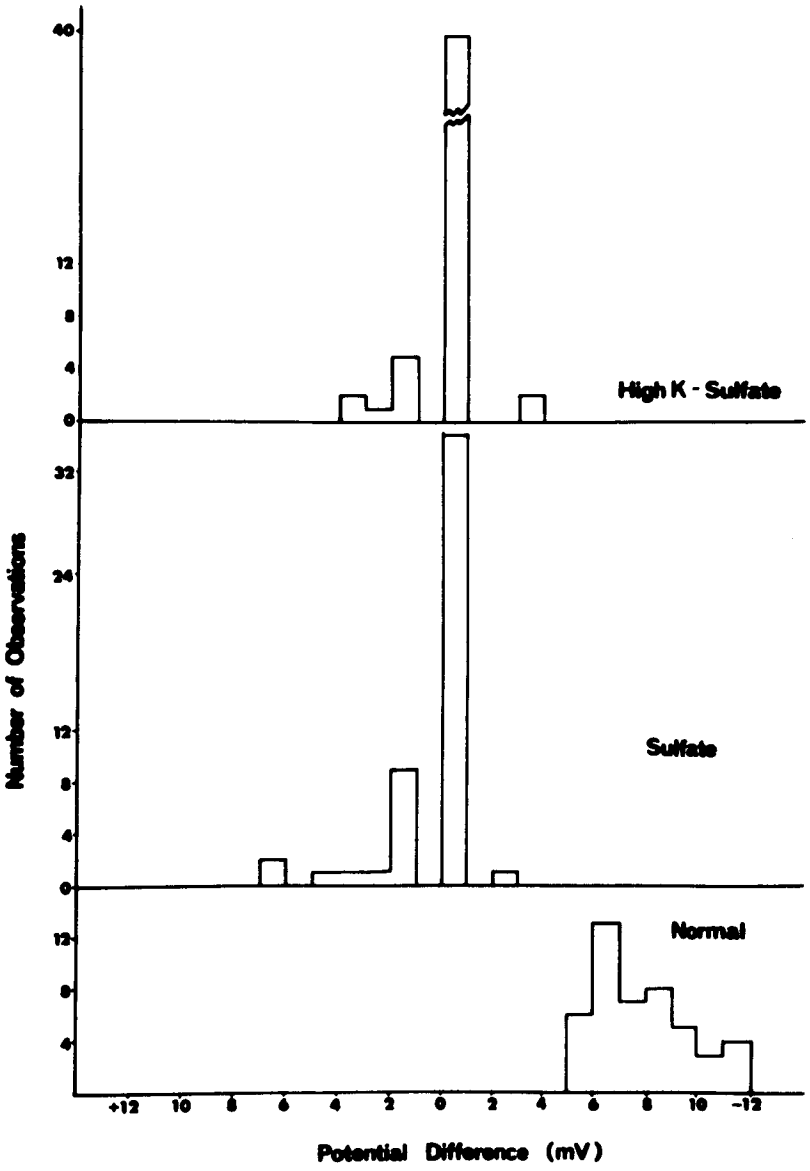
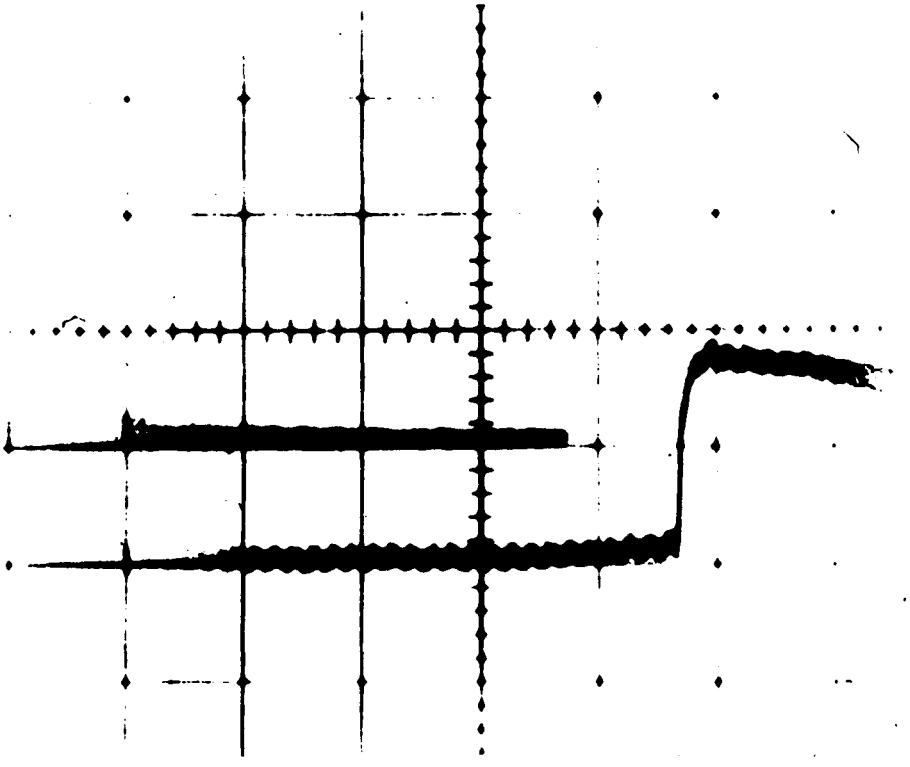

40
12
8
4
0
High K - Sulfate
32
24
12
8
4
0
Sulfate
12
8
4
Normal
Number of Observations
+12
10
8
6
4
2
0
2
4
6
8
10
-12
Potential Difference (mV)

Figure 9

Typical Recordings of the Potential Difference of Shay Chloroleukemic Tumor Cells in Normal Ringer Medium. The dimensions for these oscilloscope tracings are 100 msec/cm horizontally and 5 mV/cm vertically. When a potential difference occurred (upward deflection), the oscilloscope beam was stopped after a few seconds and the picture was taken.

a. A -9 mV potential difference.

b. A -10 mV potential difference.



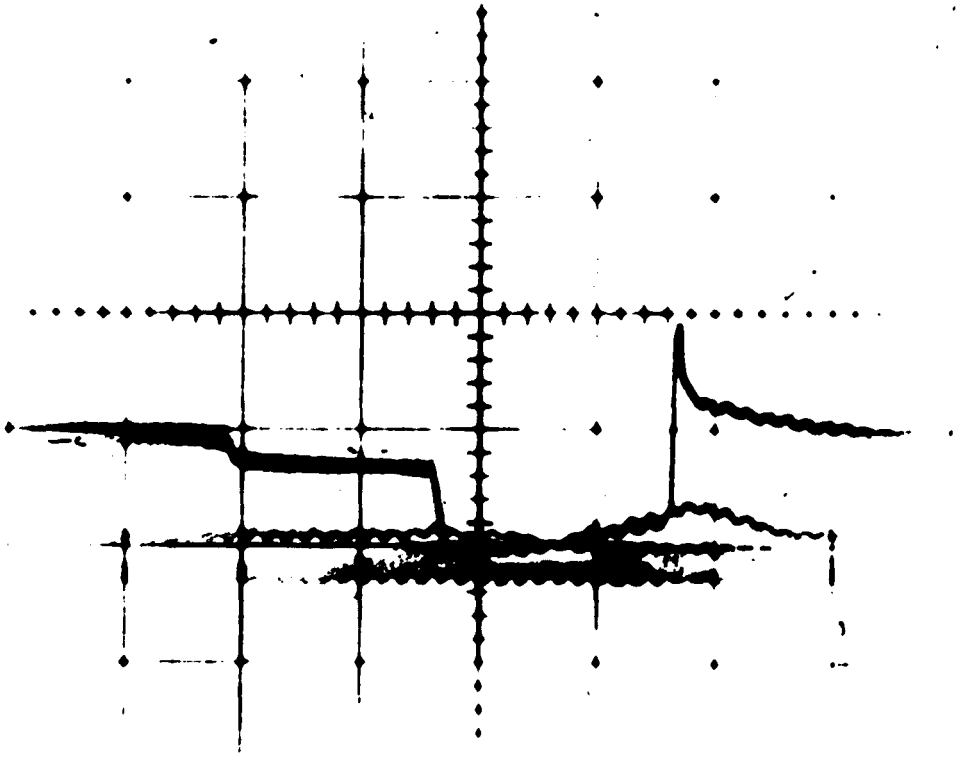
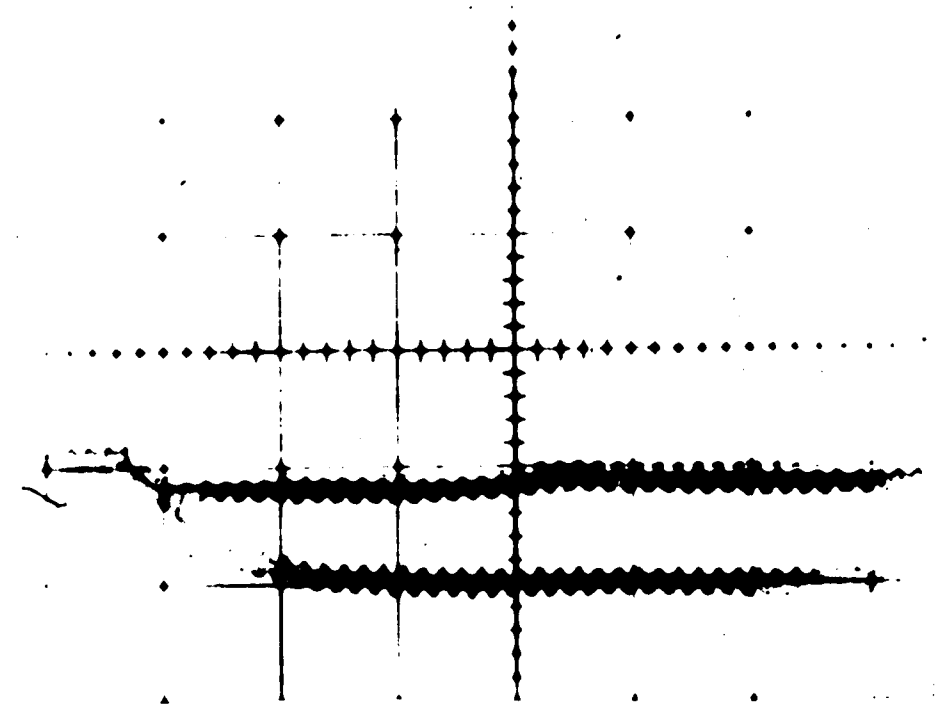


Figure 10

Typical Recordings of the Potential Difference of Shay Chloroleukemic Tumor Cells in Sulfate and High Potassium-Sulfate Ringer Medium. The dimensions for these oscilloscope tracings are 100 msec/cm horizontally and 5 mV/cm vertically. The downward deflection indicates a positive potential difference.

a. A +5 mV potential difference of a cell in sulfate Ringer medium.

b. A positive deflection of about 2 mV recorded from a tissue in high K-sulfate Ringer medium.



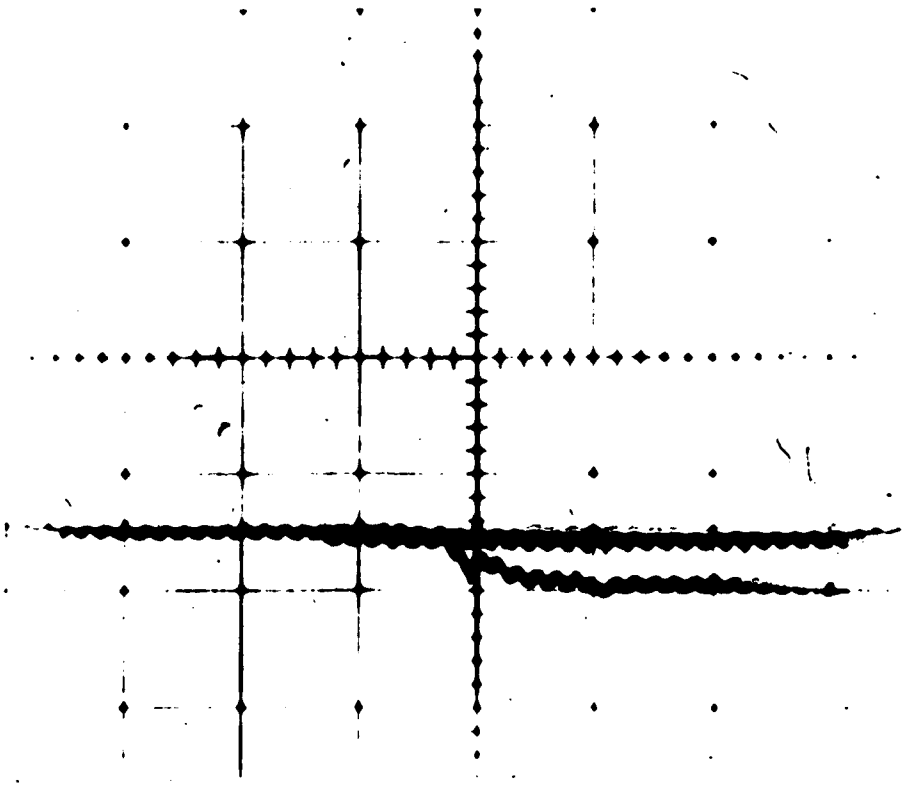


Figure 11

Curve Fitted to the C^{14}-Inulin Uptake Data at 0^{o} and 23^{o} by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.155, P2 = 0.353, P3 = 0.035, P4 = 0.027, A2 = 4.40, A4 = 5.65.

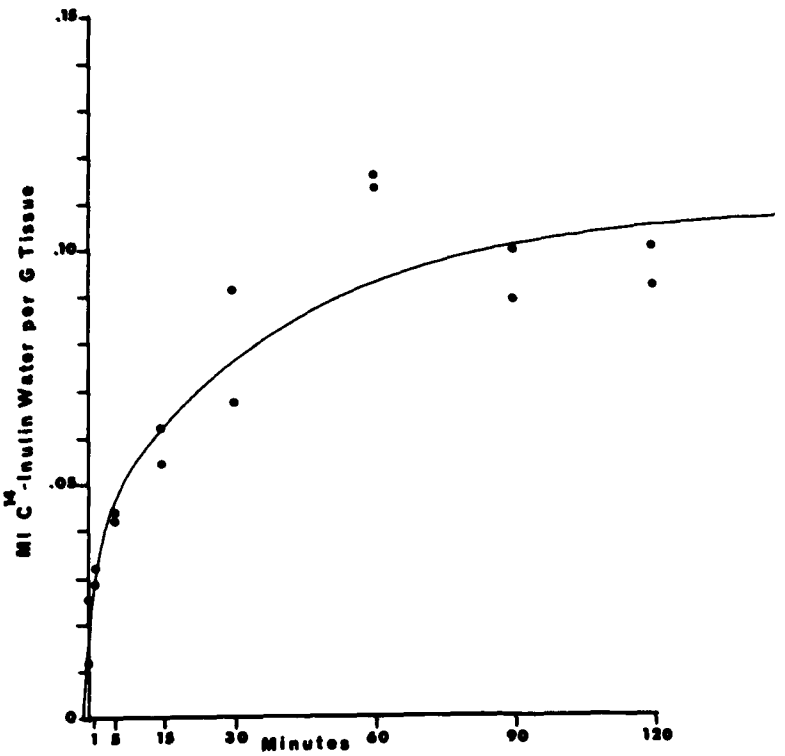

.15
.10
.05
0
Ml C^{14}-Inulin Water per G Tissue
1
5
15
30
Minutes
60
90
120

Figure 12

Curve Fitted to the C^{14} -Inulin Uptake Data at 23^{o} by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.120, P2 = 0.184, P3 = 0.020, P4 = 0.038, A2 = 6.53, A4 = 3.46.

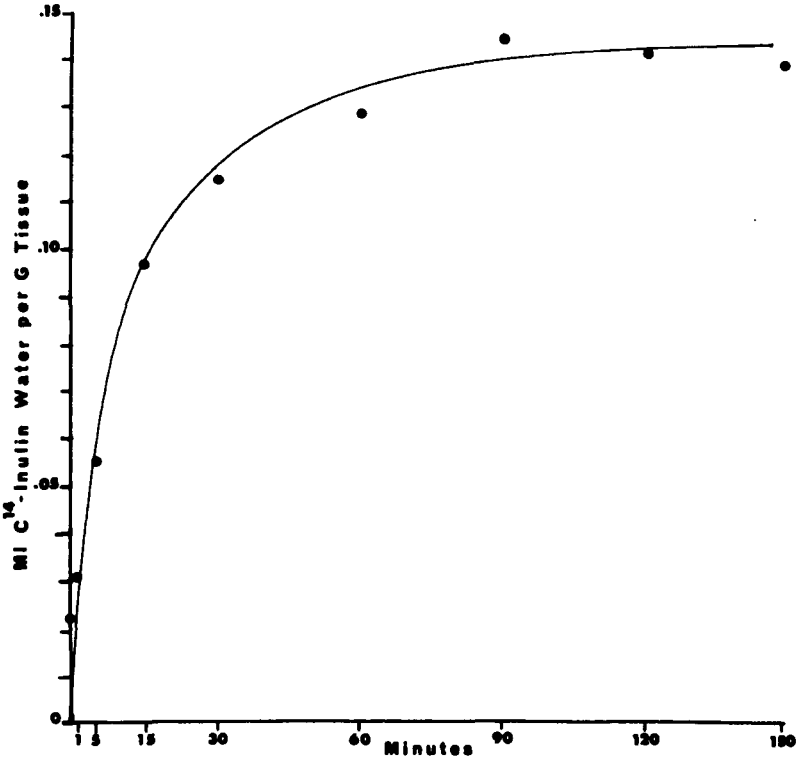

.15
.10
.05
0
Ml C^{14}-Inulin Water per G Tissue
1
5
15
30
60
90
120
180
Minutes

Figure 13

Curve Fitted to the S^{35} -Sulfate Uptake Data at 0^{o} (1) by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.110, P2 = 0.220, P3 = 0.060, P4 = 0.060, A2 = 5.00, A4 = 4.98.

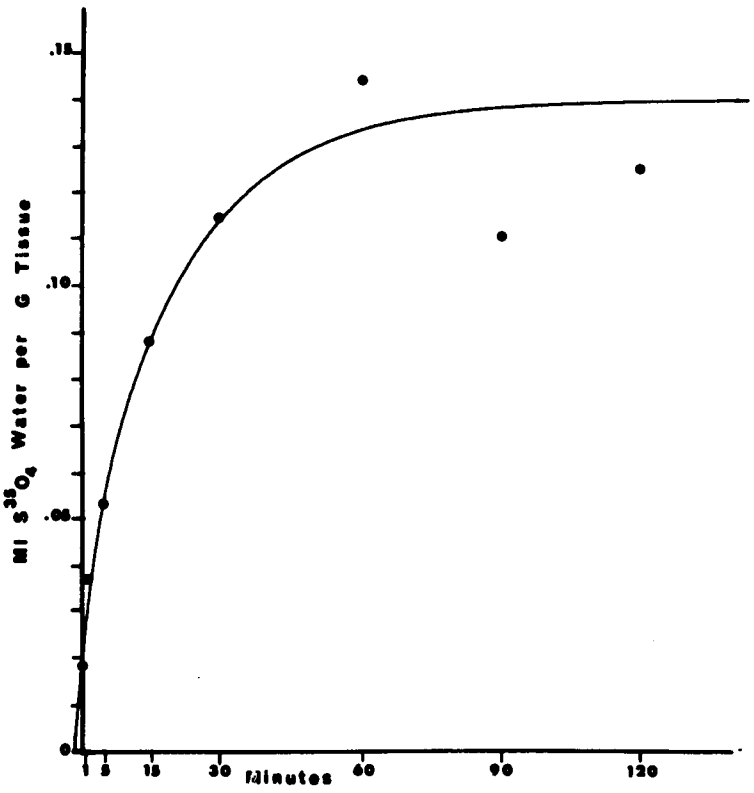

.15
.10
.05
0
Ml $S^{35}O_4$ Water per G Tissue
1
5
15
30
Minutes
60
90
120

Figure 14

Curve Fitted to the S^{35} -Sulfate Uptake Data at 0^{o} (2) by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.310, P2 = 0.620, P3 = 0.060, P4 = 0.060, A2 = 5.00, A4 = 5.00.

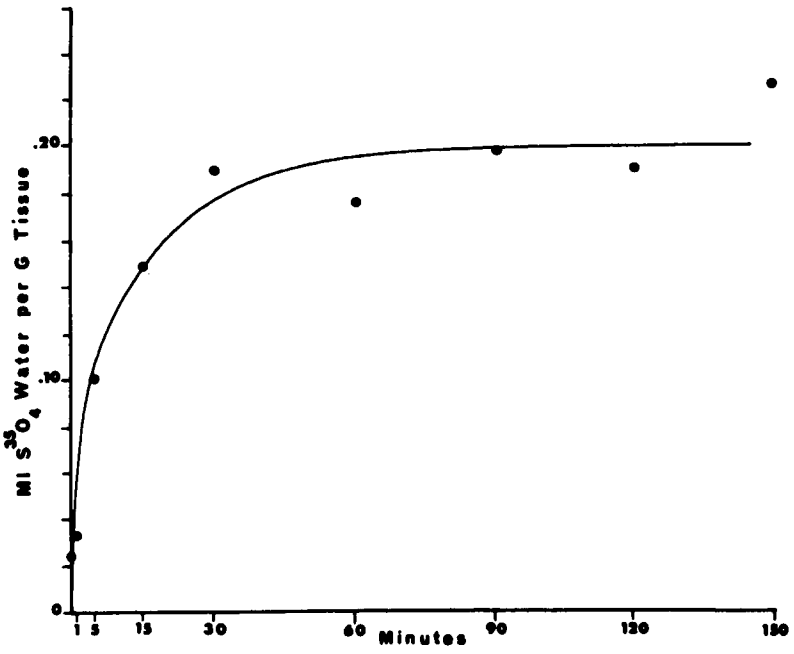

Ml $S^{35}O_4$ Water per G Tissue
.20
.10
0
1
5
15
30
60
90
120
150
Minutes

Figure 15

Curve Fitted to the S^{35} -Sulfate Uptake Data at 23^{o} by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.075, P2 = 0.167, P3 = 0.025, P4 = 0.021, A2 = 4.64, A4 = 5.35.

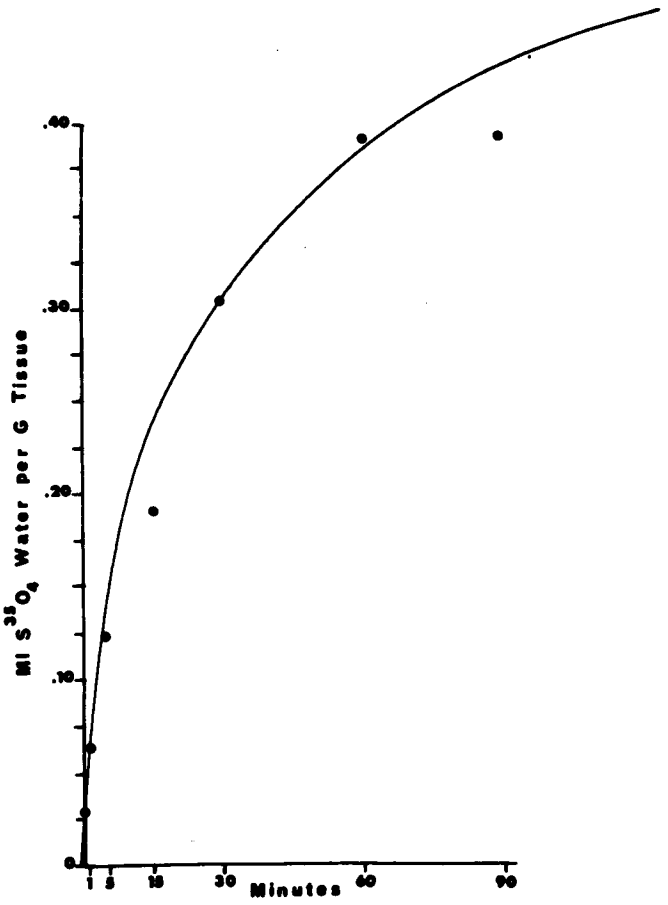

.40
.30
.20
.10
0
Ml $S^{35}O_4$ Water per G Tissue
1
5
10
30
40
90
Minutes

Figure 16

Curve Fitted to the Mean Cl^{36} Uptake Data by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.440, P2 = 1.000, P3 = 0.040, P4 = 0.031, A2 = 5.68, A4 = 4.42.

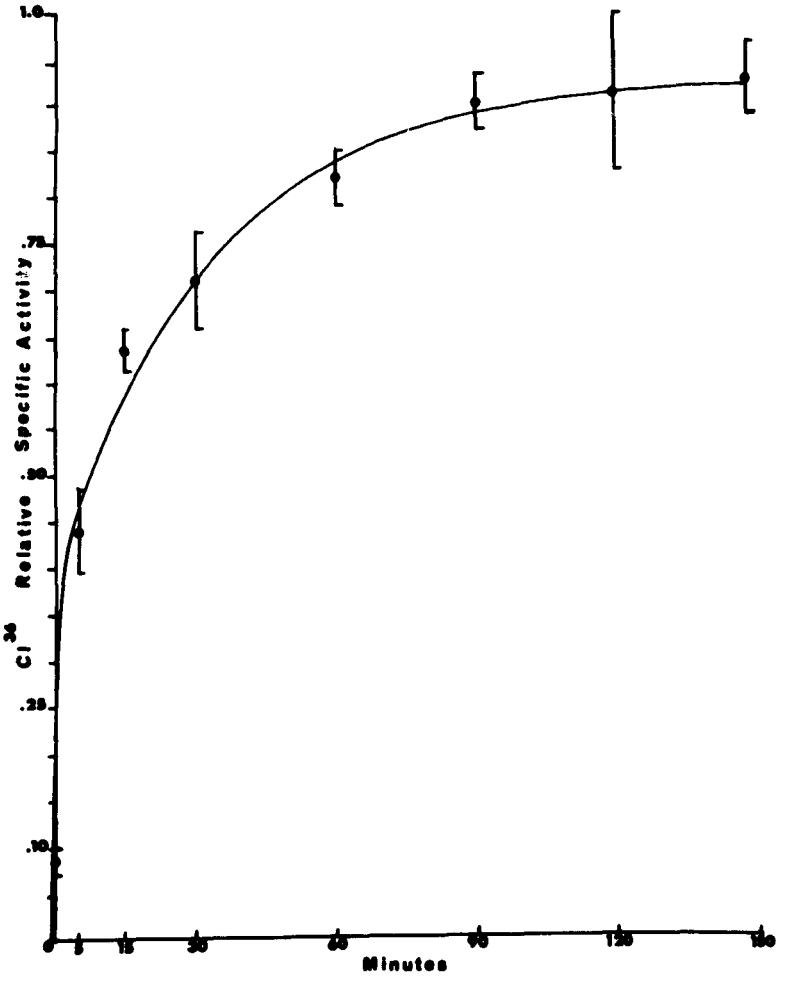

Relative Specific Activity
Cl 36
1.0
.75
.50
.25
.10
0
5
15
30
60
90
120
180
Minutes

Figure 17

Mean Na^{22} Uptake by Pieces of Shay Chloroleukemic Tumor.

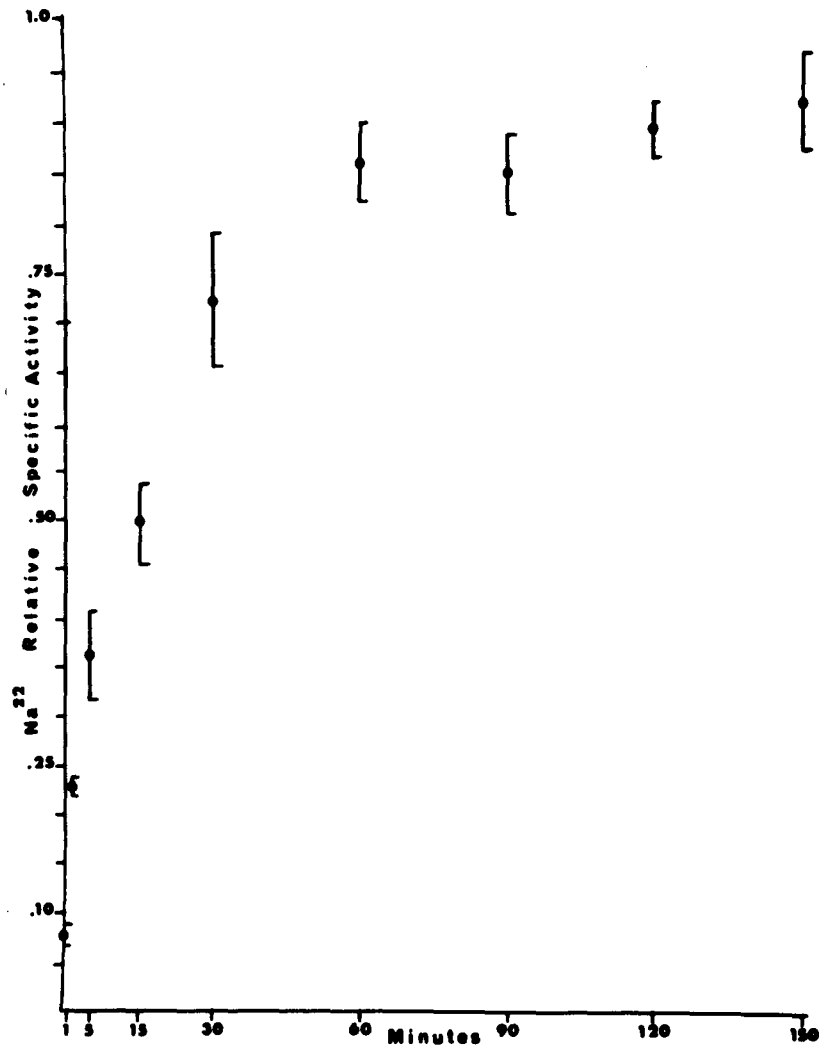

1.0
.75
.50
.25
.10
Na22 Relative Specific Activity
1
5
15
30
60
90
120
150
Minutes

Figure 18

Curve Fitted to the Na^{22} Uptake Data (I) by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.133, P2 = 0.460, P3 = 0.090, P4 = 0.037, A2 = 7.12, A4 = 2.88.

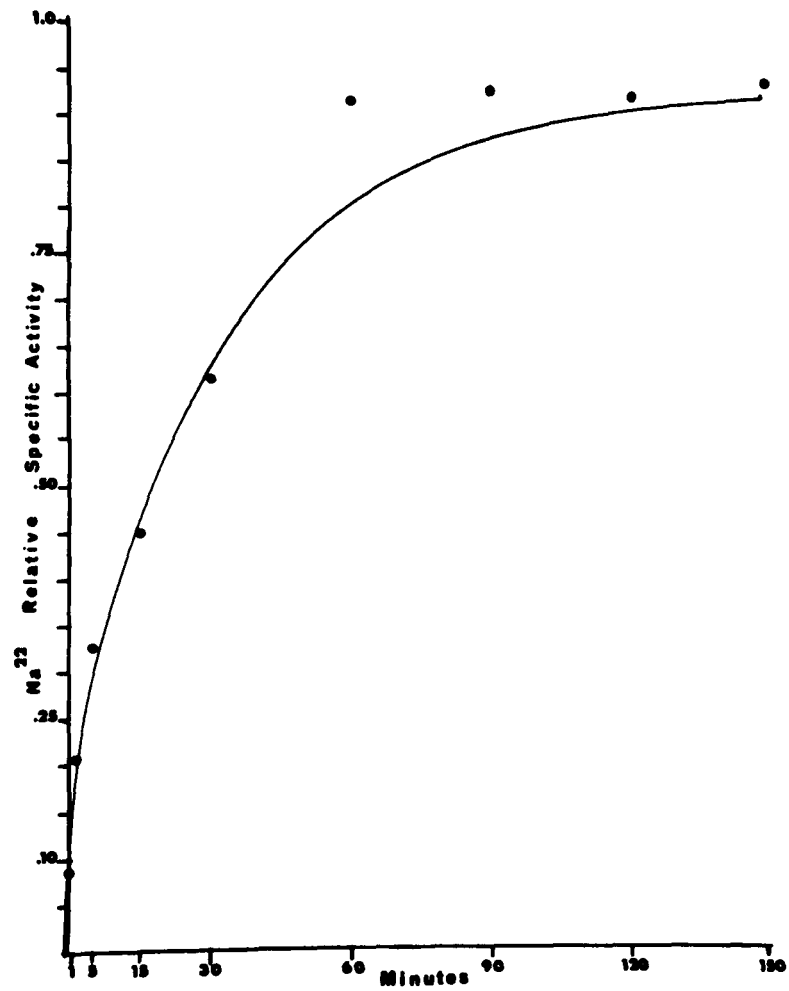

1.0
.50
.25
.10
Na22 Relative Specific Activity
1
5
15
30
60
90
120
180
Minutes

Figure 19

Curve Fitted to the Na^{22} Uptake Data (II) by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.200, P2 = 0.420, P3 = 0.030, P4 = 0.027, A2 = 5.25, A4 = 4.77.

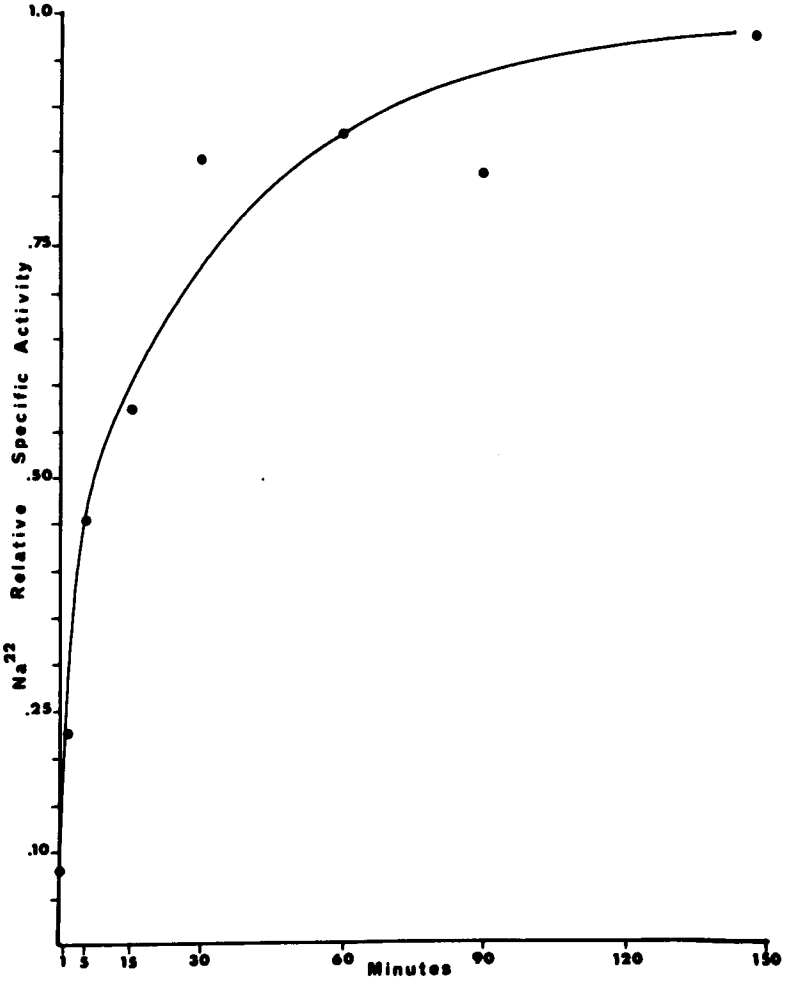

1.0
.75
.50
.25
.10
Na22 Relative Specific Activity
1
5
15
30
60
90
120
150
Minutes

Figure 20

Curve Fitted to the Na^{22} Uptake Data (III) by the Analog Computer. The following potentiometer and amplifier settings were used: P1 = 0.132, P2 = 0.372, P3 = 0.091, P4 = 0.050, A2 = 6.43, A4 = 3.57.

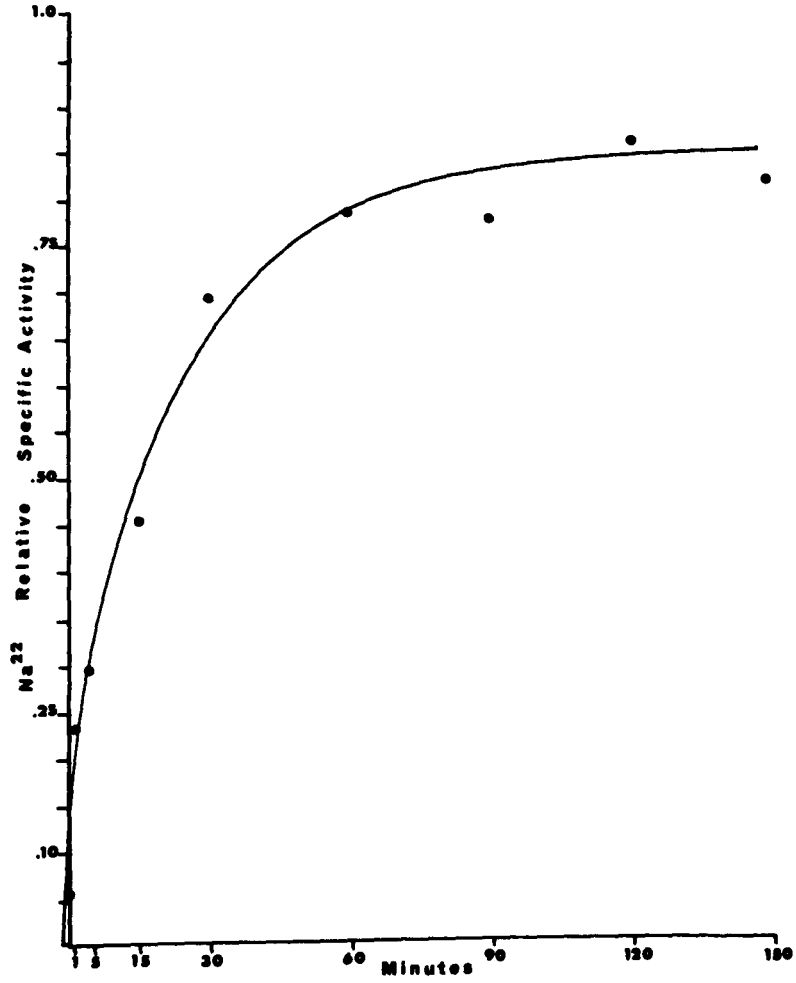

1.0
.75
.50
.25
.10
Na22 Relative Specific Activity
1
5
15
30
60
90
120
180
Minutes

Figure 21

Model of the Shay Chloroleukemic Tumor Based on the Distribution of Cl^{36}. The compartment sizes are: 1 = 769.0 uM Cl for the medium; 2 = 8.2 uM Cl for the fast exchanging compartment or ECS; 3 = 10.5 uM Cl for the slow exchanging compartment or ICS. The rate constants are: $k_{12} = 0.011$, $k_{21} = 1.000$, $k_{23} = 0.040$, $k_{32} = 0.031$ $minutes^{-1}$. Fluxes are calculated as, flux = (rate constant)(compartment size). The mean wet weight of the tissues was 0.35 ± 0.02 grams (N = 24). The volumes of water in the compartments are: 1 = 5 ml, 2 = 0.039 ± 0.001 ml, and 3 = 0.24 ± 0.01 ml.

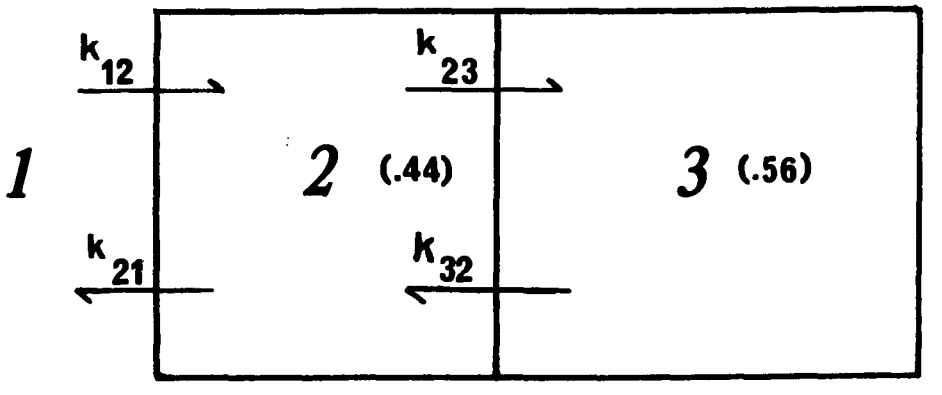
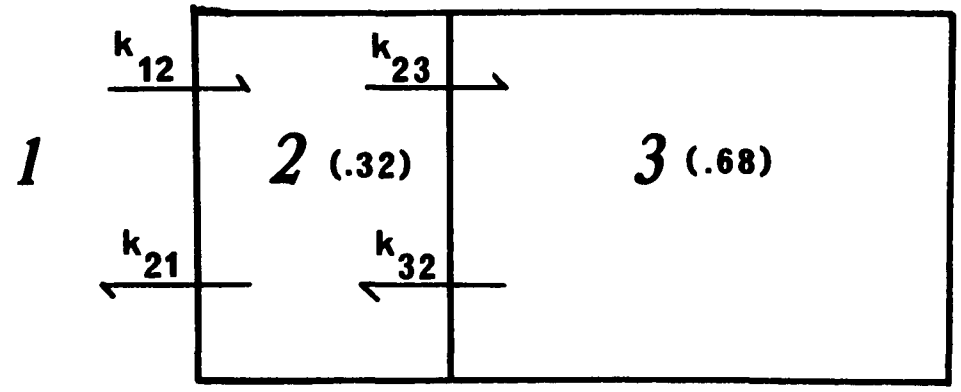

k12
k23
1
2 (.44)
3 (.56)
k21
k32

Figure 22

Model of the Shay Chloroleukemic Tumor Based on the Distribution of Na^{22}

a. Mean Data from Experiments I and III. The compartment sizes are: 1 = 812.0 uM Na for the medium; 2 = 8.7 uM Na for the fast exchanging compartment or the ECS; 3 = 18.6 uM Na for the slow exchanging compartment or the ICS. The rate constants are: k_{12} = 0.005, k_{21} = 0.416, k_{23} = 0.091, k_{32} = 0.043 $minutes^{-1}$. Fluxes are calculated as flux = (rate constant)(compartment size). The mean wet weight of the tissue was 0.46 $\pm$0.02 grams (N = 18). The volume of water in the compartments was: 1 = 5 ml, 2 = 0.061 $\pm$0.003 ml and 3 = 0.32 $\pm$0.01 ml.

b. Mean Data from Experiments I, II and III. The compartment sizes are: 1 = 811.1 uM Na for the medium; 2 = 10.4 uM Na for the fast exchanging compartment or the ECS; 3 = 17.4 uM Na for the slow exchanging compartment or the ICS. The rate constants are: k_{12} = 0.005, k_{21} = 0.417, k_{23} = 0.070, k_{32} = 0.038 $minutes^{-1}$. Fluxes are calculated as flux = (rate constant)(compartment size). The mean wet weight of the tissue was 0.47 $\pm$0.01 grams (N = 27). The volume of water in the compartments was: 1 = 5 ml, 2 = 0.064 $\pm$0.003 ml and 3 = 0.32 $\pm$0.01 ml.


k12
k23
1
2 (.32)
3 (.68)
k21
k32

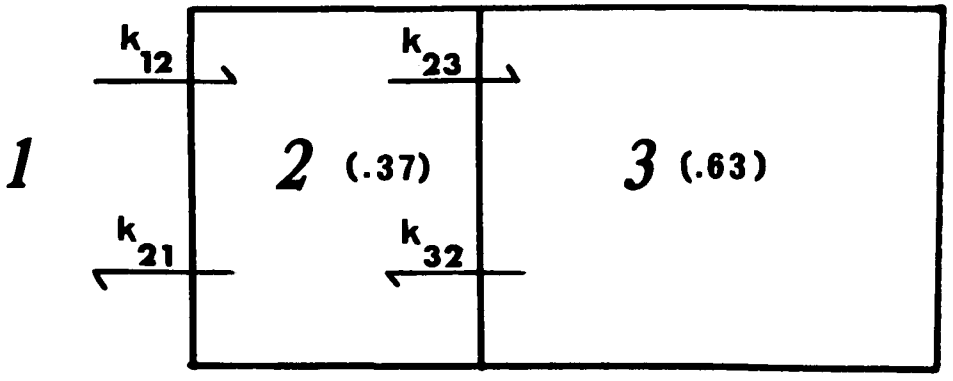
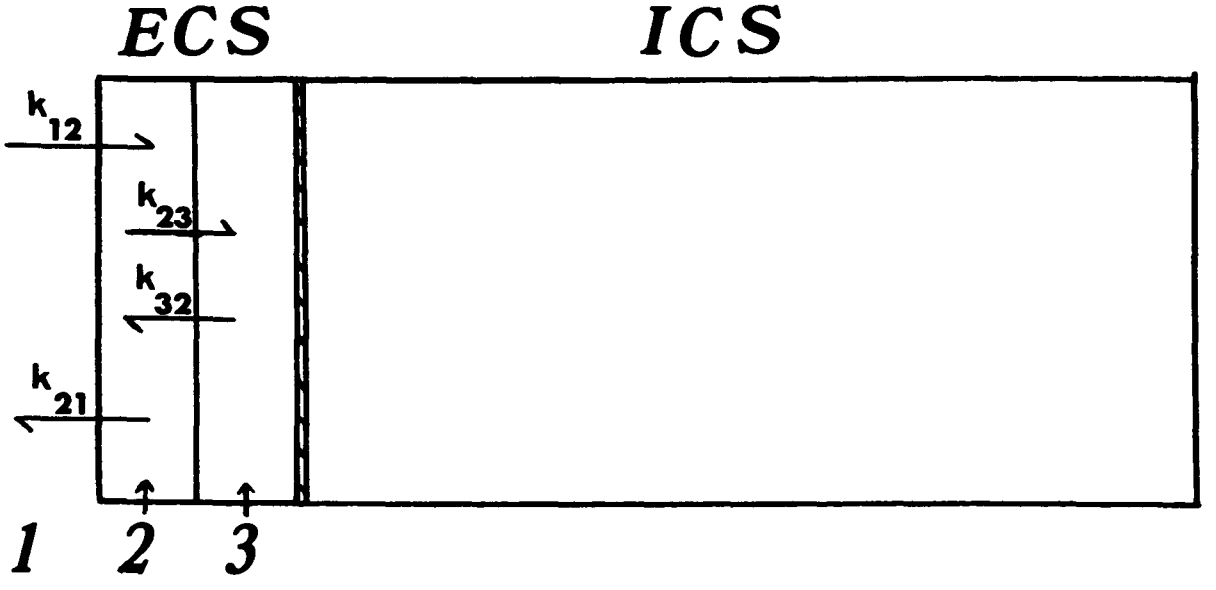

k12
k23
1
2 (.37)
3 (.63)
k21
k32

Figure 23

Model of the Shay Chloroleukemic Tumor Based on the Distribution of C^{14} - Inulin. The extracellular space consists of two compartments, 2 and 3, constituting about 14% (ml inulin water/g wet wt) of the tissue; compartment 1 represents the external medium.


ECS
ICS
k12
k23
k32
k21
1
2
3

APPENDIX

I. Statistical Analysis

The following equation was used for calculating the standard error (SE) of a mean: $SE = S/\sqrt{N}$, where $S = \sqrt{\leq(Y - Y)^2/N-1}$.

The student "t" test was used to determine the significance of the difference between two means, where

$$t = \frac{(\overline{Y}_1 - \overline{Y}_2)}{\sqrt{SE_1^2 + SE_2^2}}$$

and the degrees of freedom $= N_1 + N_2 - 2$.

II. Calculations and Equations

A. Extracellular Space (ECS)

The ECS was calculated from the S^{35}-sulfate or C^{14}-inulin uptake data according to the following equation:

$$ECS = \frac{\frac{cpm_{extract}}{g\ wet\ wt}}{\frac{cpm_{medium}}{ml\ medium}} = \frac{ml\ medium}{g\ wet\ wt}$$

e.g.

$$ECS = \frac{\frac{593\ cpm_{extract}(5\ ml\ dilution/0.2\ ml\ counted)}{0.3524\ g\ wet\ wt}}{\frac{60256\ cpm_{medium}}{0.2\ ml\ medium\ counted}}$$

$$= 0.140\ ml\ medium/g\ wet\ wt.$$

B. Cell Water Content

$$\%\ cell\ water = \frac{ml\ cell\ water}{g\ cells} \times 100$$

where ml cell water = ml water lost by tissue in drying - ml ECS, g cells = g wet wt of tissue - ml ECS, and ml ECS = (ml C^{14}-inulin water/g wet wt)(g wet wt).

C. Intracellular Ion Concentrations

The basic equation for calculating the intracellular concentration of an ion was:

$$(ion)_i = \frac{uM_{tissue\ extract} - uM_{ECS}}{ml\ cell\ water}$$

The following are examples for each ion:

1. Sodium, for extraction by ashing,

$$(Na)_i = \frac{0.50\ uM\ Na/ml\ (5.2\ ml)(10\ ml/1\ ml\ dilution) - 0.06\ ml\ ECS\ (155\ uM\ Na/ml\ medium)}{0.35\ ml\ cell\ water}$$

= 47.7 uM Na/ml cell water or 47.7 mEq Na/liter cell water.

For acetic acid extraction, the 5.2 ml dilution factor was replaced by 5.0 ml.

2. Potassium

$$(K)_i = \frac{0.40 \text{ uM K/ml (5.2 ml)(25 ml/1 ml dilution)} - 0.05 \text{ ml ECS (7 uM K/ml medium)}}{0.45 \text{ ml cell water}}$$

= 114.8 uM K/ml cell water or 114.8 mEq/liter cell water.

3. Chloride

$$(Cl)_i = \frac{0.75 \text{ uM Cl/ml (5 ml)(10 ml/2 ml dilution)} - 0.03 \text{ ml ECS (160 uM Cl/ml medium)}}{0.25 \text{ ml cell water}}$$

= 55.8 uM Cl/ml cell water or 55.8 mEq Cl/liter cell water.

D. The equations used to calculate the specific activity (s.a.) of an isotope in the tissue, cells or in the medium were:

$$s.a._{tissue} = \frac{\frac{cpm_{extract}}{ml\ extract}}{\frac{uM_{ion}}{ml\ extract}} = \frac{cpm_{tissue}}{uM_{ion}}$$

$$s.a._{medium} = \frac{\frac{cpm_{medium}}{ml\ medium}}{\frac{uM_{ion}}{ml\ medium}} = \frac{cpm_{medium}}{uM_{ion}}$$

The equilibration of the tissue with the medium was taken as:

$$\% \text{ Equilibration} = (s.a._{tissue})/(s.a._{medium}) \times 100.$$

Examples of these calculations are the following:

1. Radioactive Sodium

$$s.a._{tissue} = \frac{18400 \text{ cpm(5 ml dilution/4 ml counted)}}{0.31 \text{ uM Na/ml (5 ml)(10 ml/1 ml dilution)}}$$

= 1484 cpm/uM Na

$$s.a._{medium} = \frac{45350 \text{ cpm (25 ml/1 ml dilution)/4 ml counted}}{160 \text{ uM Na/ml medium}}$$

= 1771 cpm/uM Na

Equilibration = (1484/1771) x 100 = 83.8%.

2. Radioactive Chloride

$$s.a._{tissue} = \frac{540 \text{ cpm (5 ml dilution/0.2 ml counted)}}{0.625 \text{ uM Cl/ml (5 ml) (10 ml/2 ml dilution)}}$$

$$= 864 \text{ cpm/uM Cl}$$

$$s.a._{medium} = \frac{32570 \text{ cpm/0.2 ml counted}}{155 \text{ uM Cl/ml medium}}$$

$$= 1051 \text{ cpm/uM Cl}$$

Equilibration = (864/1051) x 100 = 82.2%

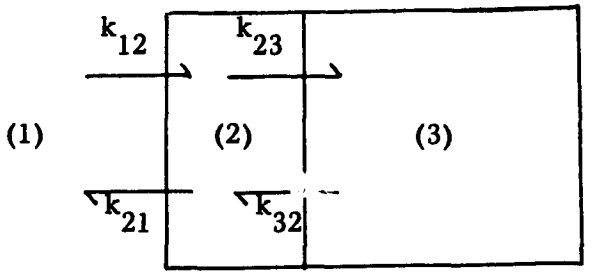
$$s.a._{cells} = \frac{cpm_{extract} - cpm_{ECS}}{uM\ Cl_{extract} - uM\ Cl_{ECS}}$$

$$s.a._{cells} = \frac{1800 \text{ cpm (5 ml dilution/0.2 ml counted)} - 0.064 \text{ ml ECS } (32300 \text{ cpm}_{medium}/0.2 \text{ ml counted})}{1.34 \text{ uM Cl/ml (5 ml dilution)} - 0.064 \text{ ml ECS (10.7 uM Cl/ml medium)}}$$

$$= 5763 \text{ cpm/uM Cl}$$

III. Theoretical Considerations

Proposed model of three compartments:



where the numbers 1, 2 and 3 refer to the external medium, tissue ECS, and ICS respectively, and k represents the rate constant for fluxes into or out of each compartment, i.e. $k_{12} = k_{\text{medium}\rightarrow\text{ECS}}$, $k_{21} = k_{\text{ECS}\rightarrow\text{medium}}$, $k_{23} = k_{\text{ECS}\rightarrow\text{ICS}}$, and $k_{32} = k_{\text{ICS}\rightarrow\text{ECS}}$.

The following equations describe the change in specific activity (s.a.) with time ($ds.a./dt = \dot{s.a.}$) in each tissue compartment in the model:

$$\dot{s.a.}_{ECS} = k_{12}(s.a._1) - k_{21}(s.a._2) - k_{23}(s.a._2)$$

$$\dot{s.a.}_{ICS} = k_{23}(s.a._2) - k_{32}(s.a._3).$$

The specific activity of the medium remains essentially constant. The kinetics of isotope distribution among the components of a three compartment system can be solved by analog computation. The following is an unpublished analysis provided by H. G. Hempling:

1) $\dot{Q} = k_{12}P - k_{21}Q - \dot{R}$

$\dot{R} = k_{23}Q - k_{32}R$

where P = external medium, Q = ECS, and R = ICS, in dimensions of cpm, and k = rate constant in the dimension of reciprocal time. If P, the radioactivity in the medium, remains constant because the compartment is so much larger than Q or R, then at isotopic equilibrium (∞):

2) $P/p = (Q+R)/(q+r)$ or $P = (Q+R)\left[\frac{p}{(q+r)}\right]$

where p = medium compartment size, q = ECS compartment size, and r =

ICS compartment size in dimensions of uM (micromoles). (Q + R) was scaled to be equivalent to the maximal output of the analog computer (EAI TR20, Electronic Associates Inc.) of 10 volts. Then P was scaled as $\frac{P}{\frac{p}{(q+r)}}$ so that the scaled variables of P and (Q + R) are equal in volts at ∞. The original equation was: $\dot{Q} = k_{12}P - k_{21}Q - \dot{R}$

$$\dot{R} = k_{23}Q - k_{32}R$$

and the scaled equation is then:

3)
$$[\dot{Q}] = k_{12}\frac{p}{(q+r)}\left[\frac{P}{\frac{p}{(q+r)}}\right] - k_{21}[Q] - [\dot{R}]$$

$$[\dot{R}] = k_{23}[Q] - k_{32}[R] .$$

If we use as the scaled variable not radioactivity, but specific activity, normalized to the total content of the tissue (q + r), we can divide both sides of both equations by (q + r).

4)
$$\left[\frac{\dot{Q}}{(q+r)}\right] = k_{12}(p/(q+r))\left[\frac{P}{(p/(q+r))(q+r)}\right] - k_{21}\left[\frac{Q}{(q+r)}\right] - \left[\frac{\dot{R}}{(q+r)}\right]$$

$$\left[\frac{\dot{R}}{(q+r)}\right] = k_{23}\left[\frac{Q}{(q+r)}\right] - k_{32}\left[\frac{R}{(q+r)}\right]$$

Scaling is completed by the insertion of B (Beta) into the denominator of all terms in order to convert machine time (T) to real time (t) according to the equation: $T = B\,t$.

The circuit diagram for this analog program is shown in figure II. Table IV defines the potentiometers and lists the amplifier outputs. Note that amplifier 6 gives $\frac{Q}{(q+r)} + \frac{R}{(q+r)} = \frac{Q+R}{(q+r)}$ or the specific activity of the tissue as a function of time. The output from this amplifier was used to fit the computer solution to the experimental data.

The selection of a scale factor for P such that this compartment would equal (Q + R) at ∞ made easier the task of fitting the machine solution to the

experimental data to arrive at values for the rate constants and fluxes.

Consider that at equilibrium, $[\dot{Q}]$ and $[\dot{R}] = 0$, then:

5) $$k_{12}\frac{p}{(q+r)}\left[\frac{P}{\frac{p}{(q+r)}}\right] = k_{21}[Q], \text{ and}$$

6) $$\frac{k_{12}\frac{p}{(q+r)}\left[\frac{P}{\frac{p}{(q+r)}}\right]}{k_{21}} = [Q]$$

(since $[\dot{R}] = 0 = k_{23}[Q] - k_{32}[R]$, and $k_{23}[Q] = k_{32}[R]$) then,

7) $$\frac{k_{23}}{k_{32}}[Q] = [R].$$

The total radioactivity, T, properly scaled, is defined as

8) $[Q] + [R] = T.$

Since from equation (7), $[R] = \frac{k_{23}}{k_{32}}[Q]$, then

9) $$[Q] + \left[\frac{k_{23}}{k_{32}}\right][Q] = T, \text{ factor out } [Q], \text{ to give}$$

10) $$[Q]\left[1 + \frac{k_{23}}{k_{32}}\right] = T.$$

Substitution of the value of $[Q]$ from equation (6) in equation (10) gives,

11) $$\frac{k_{12}\frac{p}{(q+r)}}{k_{21}}\left[\frac{P}{\frac{p}{(q+r)}}\right]\left[1 + \frac{k_{23}}{k_{32}}\right] = T.$$

Since the scaling was arranged to have $[Q] + [R] = T = P = \frac{P}{\frac{p}{(q+r)}}$

12) $$\frac{T}{\left[\frac{P}{\frac{p}{(q+r)}}\right]} = 1 = \frac{k_{12}\frac{p}{(q+r)}}{k_{21}}\left[1 + \frac{k_{23}}{k_{32}}\right], \text{ and}$$

$$13)\quad \left[1 + \frac{k_{23}}{k_{32}}\right] = \frac{k_{21}}{k_{12}\frac{p}{(q + r)}}$$

This relation, by defining the constraints on the rate constants, simplifies the selection of the proper rate constants for fitting the machine solution to the experimental data.

From amplifiers 2 and 4 of the computer, the fraction of total chloride, for example, in compartments q and r were obtained. From amplifier 4, $Q/(q + r)$, was gotten. Since at equilibrium, the following relationship holds:

14) $Q/q = (Q + R)/(q + r)$, and $Q = (q)(Q + R)/(q + r)$, then divide through by $(q + r)$ to get,

15) $Q/(q + r) = (Q + R)/(q + r)\ (q)/(q + r)$.

Since $(Q + R)/(q + r)$ equals the s.a. of the total tissue, when normalized to the value of 1, the equation becomes,

16) $Q/(q + r) = q/(q + r)$.

Therefore, amplifier 4 is equivalent to $q/(q + r)$ or the fraction of the tissue chloride in compartment q, which is the ECS. In the same manner, from amplifier 2, $R/(q + r)$ was obtained. The fraction of the tissue chloride in compartment r, the ICS, is equivalent to $R/(q + r)$ which is equal to $r/(q + r)$.

Table I. Solutions and Mixtures Used for Embedding Tissues for Electron Microscopy.

Solutions: 2.5% glutaraldehyde in 0.1M cacodylate
1% osmium tetroxide in 0.1M cacodylate
0.25% uranyl acetate in 0.1M acetate buffer

Fixation Mixture: 1 part glutaraldehyde solution to 2 parts osmium tetroxide solution at pH 7.4

Epon Mixture A: 62 ml of Epon 812 plus 100 ml of DDSA (dodecenyl succinic anhydride

Epon Mixture B: 100 ml of Epon 812 plus 82 ml of MNA (methyl nadic anhydride)

Complete Resin Mixture: 5 ml of Epon Mixture A, plus 5 ml of Epon Mixture B, plus 1 ml of DMP-30 (2,4,6-tri-dimethylamino-methylphenol)

Table II. Preparation of Ringer Solutions

A. Volumes of Salt Solutions

Ringer	NaCl	KCl	Na_2SO_4	K_2SO_4
Normal	9 grams	40 ml of 0.154M	-	-
20 mM K	145 ml of 1M	20 ml of 1M	-	-
80 mM K	85 ml of 1M	80 ml of 1M	-	-
120 mM K	45 ml of 1M	120 ml of 1M	-	-
Sulfate	-	40 ml of 0.154M	860 ml of 0.13M	-
K-Sulfate	-	-	400 ml of 0.14M	500 ml of 0.14M

In addition to the ingredients listed above, all solutions contained 1 ml of 1M $CaCl_2$, 85 ml of 0.11M Na_2HPO_4, 15 ml of 0.11M NaH_2PO_4, and were brought to 1 liter. The pH was 7.4 and the osmolarity was 310-320 mOsm/liter.

B. Concentrations of Major Ions (in mEq/liter)

Ringer	Na	K	Cl
Normal	180	7	160
20 mMK	165	20	160
80 mM K	105	80	160
120 mM K	65	120	160
Sulfate	244	7	7
K-Sulfate	132	140	0

Table III. Dilutions for Measuring Sodium and Potassium Concentrations in the Ringer Solutions.

Solution	Na Analysis	K Analysis
Normal	(5.2ml/0.2ml)(10ml/1ml)	(10ml/1ml) including 2 ml of 250 ppm Li
20 mM K	(5.2ml/0.2ml)(10ml/1ml)	(25ml/1ml) including 5 ml of 250 ppm Li
80 mM K	(5.2ml/0.2ml)(10ml/2ml)	(10ml/1ml)(10ml/1ml) including 2 ml of 250 ppm Li
120 mM K	(5.2ml/0.2ml)(10ml/2ml) all including 2 ml of 1250 ppm Li	(10ml/1ml)(25ml/1ml) including 5 ml of 250 ppm Li

Table IV. Definition of the Potentiometers and the Amplifier Outputs for the Analog Computer Program.

Potentiometer		Amplifier	
1	$k_{12}p/B(q + r)$	1	$-Q/(q + r)$
2	k_{21}/B	2	$R/(q + r)$
3	k_{23}/B	3	$-R/(q + r)$
4	k_{32}/B	4	$Q/(q + r)$
		5	$-R/(q + r)$
		6	$(Q - R)/(q + r)$

Figure I

Schematic Diagram of the Electrical Recording Set-Up.

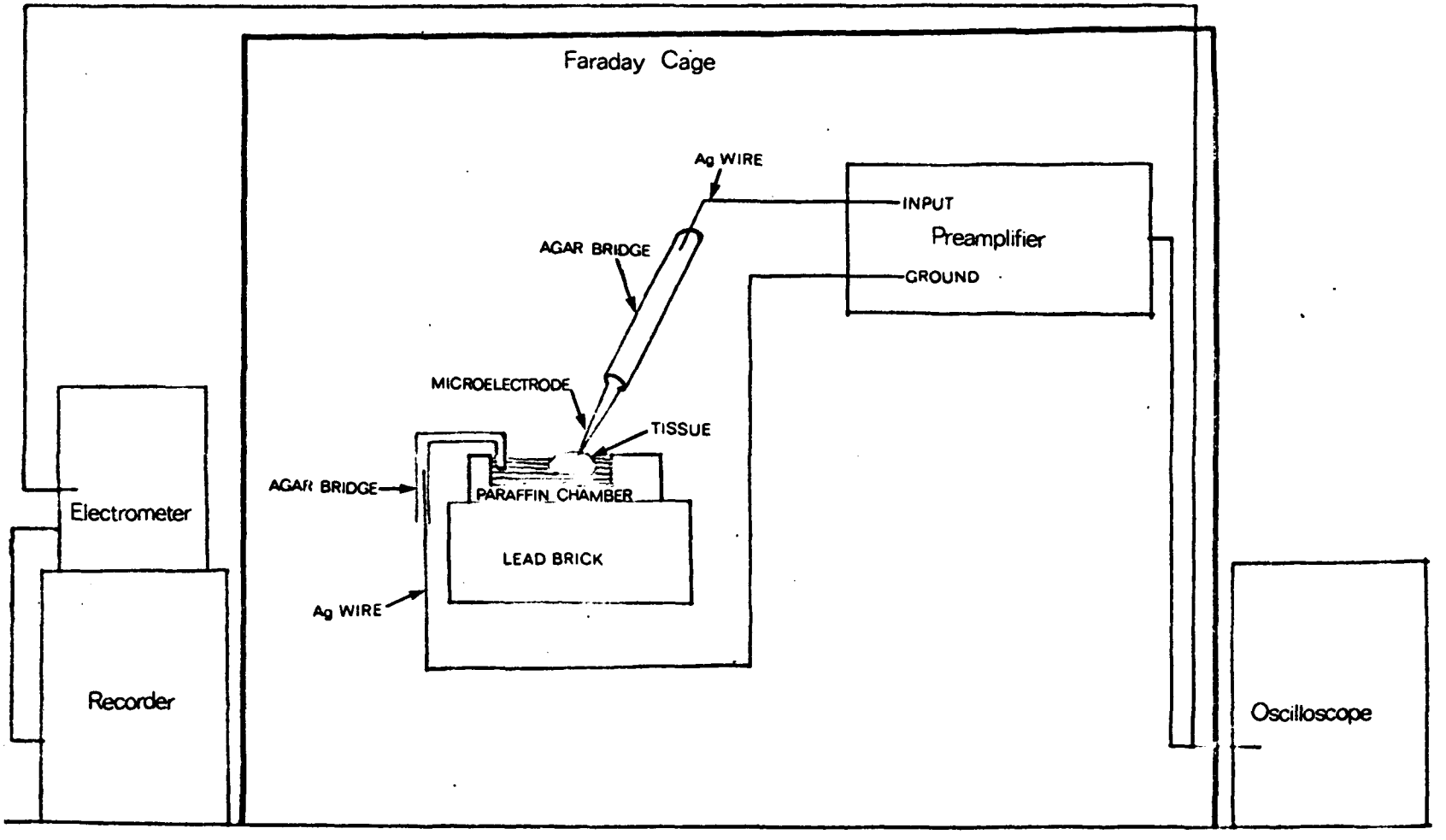
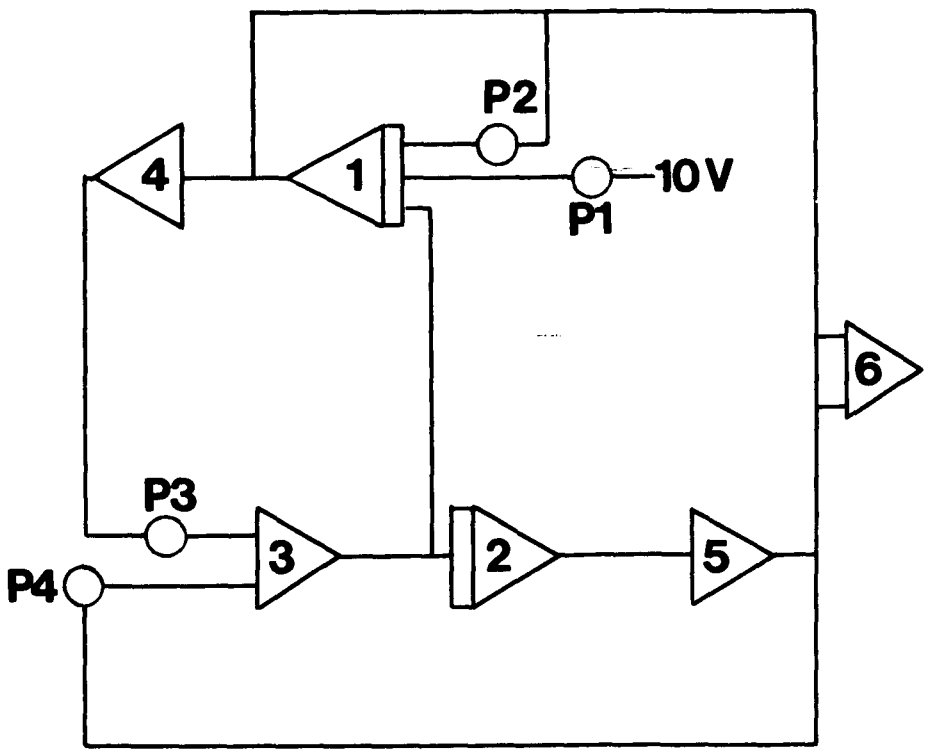

Faraday Cage
Ag WIRE
INPUT
Preamplifier
AGAR BRIDGE
GROUND
MICROELECTRODE
TISSUE
AGAR BRIDGE
PARAFFIN CHAMBER
LEAD BRICK
Ag WIRE
Electrometer
Recorder
Oscilloscope

Figure II

Circuit Diagram for the Analog Computer Program.


P2
4
1
10V
P1
6
P3
3
2
5
P4