prevent removal of cholinesterase from red blood cells by lysolecithin. In an attempt to determine whether frog gastrocnemius loses cholinesterase on soaking in distilled water and whether chlorpromazine can block this loss, cholinesterase activity was determined manometrically on lyophilized, reconstituted water medium in which frog muscles had been immersed with and without chlorpromazine. The results demonstrated that if cholinesterase be leached out of the muscle, the amount is so small that it is impossible of determination by these technics. It was also found in characterizing the cholinesterase of frog muscle, that the absolute content of enzyme is exceedingly small.

Thus it would appear that no ready explanation is at hand for the ability of chlorpromazine and to a lesser extent certain other phenothiazines to inhibit water imbibition under these conditions, although correlation exists with increased cell membrane permeability to potassium and sodium.

No attempt has been made to determine the extent to which the drugs used are able to penetrate the frog muscle cell although differences in activity between such compounds as phentolamine, phenoxybenzamine, and azapetine suggest that permeability effects may have influenced these results to some extent.

Summary. 1. The chlorpromazine-induced inhibition of water imbibition by frog gastrocnemius is unrelated to tranquilizing ability, adrenergic blockade, and antihistaminic activity. 2. Selected compounds exhibiting carbonic anhydrase inhibition, anticholinergic activity, analgetic and antiinflammatory activity, adrenergic activity, monamine oxidase inhibition, metabolic uncoupling, cholinesterase inhibition and high energy phosphate activity produce only minor or no inhibition of water uptake. 3. Correlation exists between block of water imbibition and loss of sodium and potassium from the muscle.

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Effects of Chemotherapeutic Agents on Metabolism of Human Acute Leukemia Cells *in vitro*. (23666)

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Cancer chemotherapy is currently directed chiefly towards chemical synthesis, animal screening, and clinical trial of antimetabolites. Some of these antimetabolites are designed to act primarily against specific biosynthetic reactions with the thought that they might function as anti-tumor agents by interfering with nucleic acid or coenzyme production in neoplasms. None of the chemotherapeutic drugs employed has universal applicability to patients with cancer. Even within a single cancer type such as acute lymphatic leukemia, response of a particular patient to a given antimetabolite is unpredictable. This unpredictability is particularly serious in those patients with fulminating neoplasms for whom prompt selection of the most effective agent offers the only hope of remission.

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With these problems in mind, a method has been devised for screening chemotherapeutic drugs that depends upon an evaluation of their effects on the glycolysis of leukocytes taken from leukemic patients. To our knowledge there have been no published reports on the effect of chemotherapeutic agents on overall metabolism of leukemic cells. Glycolysis has been chosen as an indicator of metabolic anti-tumor activity for several reasons. Of these, the most important is the key role of aerobic glycolysis as an energy source for tumors(1). This is a universal property of all cancer cells thus far studied, which we have recently confirmed for leukemic cells(2). Secondly, there is an observed parallelism between inhibition of tumor growth *in vivo* and glycolytic inhibition *in vitro*(3-5). In addition, methods have been developed in our laboratories for selectively inhibiting glycolysis at the level of the hexokinase reaction. Acute leukemia is ideally suited for such study for several reasons: This neoplasm is readily accessible and can be serially sampled, and selected material is almost devoid of "contaminating" tissue cells. Much clinical experience is available in regard to the natural history and response to chemotherapy of acute lymphatic and acute myelogenous leukemias. *In vitro* studies of the metabolism of isolated leukemia cells enables one to measure the cells' metabolic sensitivity to a battery of usual chemotherapeutic agents. The effects of these anti-leukemic drugs can be used as a standard against which to evaluate new, clinically untested but theoretically interesting materials.

Methods. Patients were selected who had high white blood cell counts, consisting chiefly of blast and abnormal cells. The blood sample was drawn in a heparinized syringe and transferred to a solution of heparinized fibrinogen in serum to increase the rate of sedimentation of the red blood cells(6). Using bovine fibrinogen (Armour), and banked human serum, the concentration of fibrinogen in the blood-serum mixture was raised to 8-9 mg/cc. This mixture was allowed to stand at room temperature for 15-45 minutes, and a favorable separation was achieved with 12-20 white blood cells per red blood cell in the supernatant. One cc aliquots of the supernatant were pipetted into Warburg manometer flasks, and glycolysis measured in the usual manner in the presence, and in some instances the absence, of air. The test vessels contained the white cell suspension to which had been added glucose at 2 mg/cc, sodium bicarbonate at 1.5 mg/cc and the chemotherapeutic drug under analysis. The anaerobic determinations measured only the process of acid formation from glucose. Summerson manometers were used to determine aerobic glycolysis, O_2 uptake, and CO_2 production by a single sample. In the presence of air, the observed manometric (h) readings during the experimental period represented the algebraic sum of gas pressure changes due to 3 processes: (1) CO_2 liberated from bicarbonate by the lactic and other acids formed in glycolysis, (2) CO_2 formed by respiration and (3) negative component due to O_2 uptake through respiration. These 3 components were quantitatively distinguished by means of the Summerson differential manometer. Thus far, 8 patients with acute lymphatic leukemia, 5 with acute myelogenous leukemia, and 4 non-leukemic patients with leukocytosis were studied, as well as several cases of chronic leukemias not to be detailed here. Several examples of the data obtained with acute leukemia cells are cited as representative of the general experience with such cases. Repeat experiments done when possible generally showed good reproducibility, provided that the clinical state remained relatively stable.

Results. Table I illustrates the effects of methotrexate (amethopterin, purified by Dr. M. C. Li) and 6-mercaptopurine on the metabolism of acute lymphatic leukemia cells, taken from a 4-year-old boy. The peripheral blood contained 163,000 white blood cells/cmm comprised of 96% blast cells. The bone marrow was almost totally replaced with leukemic cells.

Anaerobic glycolysis, expressed as cmm of acid (CO_2 equivalent) produced per mg dry weight‡ per hour ($Q_A^{N_2}$), was 28. Anaerobic glycolysis of cells from acute lymphatic and

‡ 1 mg dry weight averaged 14 million cells in numerous determinations made in the course of these studies.

TABLE I. Effects of 6-Mercaptopurine and Methotrexate on Metabolism of Acute Lymphatic Leukemia Cells.*

Metabolism (mm^3/mg dry wt/hr)		Control	6-MP (0.1 mg/cc)	MTX (0.1 mg/cc)
Anaerobic glycolysis	($Q_A^{N_2}$)	28 †	28	28
Aerobic "	($Q_A^{O_2}$)	9.8†	5.4	3.7
Oxygen consumption	(Q_{O_2})	−4.8	−5.6	−6.3
CO_2 production	(Q_{CO_2})	1.3	2.7	6.3
Respiratory quotient	(Q_{CO_2}/Q_{O_2})	− .27	− .48	−1.0
Aerobic glycolysis / Respiration	($-Q_A^{O_2}/Q_{O_2}$)	2.1	1.0	.6
"Metabolic type"		"Malignant"	"Benign"	"Normal"

* 4-year-old male. WBC 163,000/mm^3, 97% blast cells.

† Inhibited approximately 80% by hydrocortisone, triethylene thiophosphoramide and 6-azauracil (0.2-0.5 mg/cc).

Measurements in human serum with added heparin (8 mg %), bovine fibrinogen (6 mg/cc), 0.2% glucose, 0.15% $NaHCO_3$, 5% CO_2 in air or N_2, pH 7.4, 37°C, 3 hr.

acute myelogenous leukemias was found to range from 20-30. The anaerobic glycolysis of cells from this patient with acute lymphatic leukemia was unaffected by incubating with methotrexate and 6-mercaptopurine at a concentration of 0.1 mg/cc fluid. Aerobic glycolysis ($Q_A^{O_2}$), however, was lowered by 6-mercaptopurine from 9.8 to 5.4, and even more by methotrexate to 3.7. This has been the average pattern observed in our study to date with acute lymphatic leukemia cells, namely inhibition of aerobic glycolysis by 6-mercaptopurine and greater inhibition by methotrexate, the latter causing an inhibition by as much as 80%. The inhibitions obtained were relatively independent of concentrations varied from 0.1 to 0.5 mg/cc and more.

The oxygen consumption (Q_{O_2}) of these cells was 4.8, and in other cases the general range was 4-8 in acute leukemias. Oxygen consumption was somewhat stimulated by 6-mercaptopurine and methotrexate, from 4.8 to 5.6 and 6.3 respectively. The respiratory carbon dioxide production (Q_{CO_2}) was low, 1.3, and was raised to 2.7 and 6.3 by 6-mercaptopurine and methotrexate respectively. It is of interest that the respiratory quotient (Q_{CO_2}/Q_{O_2}) of these lymphoblasts was of extremely low magnitude, −0.27. In other experiments the respiratory quotients varied widely from −0.8 to positive values.

The ratio of aerobic glycolysis to respiration ($Q_A^{O_2}/Q_{O_2}$) for these acute lymphoblasts was found to be 2.1, a value in the range typical of cancer tissues (>1-10) as described by Burk(7). In the presence of 6-mercaptopurine this ratio was lowered to that seen with benign tumors (~1), and with methotrexate it was decreased to values generally found with non-cancerous tissues (<1-0).

The first example of acute myelogenous leukemia cells presented here (Table II) is that of a 58-year-old woman with a white blood cell count of 264,000, 75% of which were blast and abnormal cells. Aerobic and anaerobic glycolytic and O_2 consumption rates were comparable to those of the lymphatic case. The chief difference was a 3-fold increase in respiratory CO_2 production which in turn was reflected by an R.Q. of −0.71. This R.Q. is in the normal range of mammalian cells and contrasts with the lower value found in the lymphatic case cited. No effects on glycolysis or respiration were noted with methotrexate at concentrations as great as 1 mg/cc, or 10 times the concentration used to obtain inhibitory effects in the previous case. Furthermore there was no inhibition of aerobic glycolysis with either 6-mercaptopurine or 6-mercaptopurine plus methotrexate. No change was observed in the R.Q. upon addition of methotrexate.

The second case of acute myelogenous leukemia presented in Table II is that of a 51-year-old male with a white blood cell count of 334,000/cmm. The figures for anaerobic glycolysis, aerobic glycolysis and respiration

TABLE II. Effects of Methotrexate on Metabolism of Acute Myelogenous Leukemia Cells.

Metabolism (mm^3/mg dry wt/hr)	Control*	MTX (1 mg/cc)	Control§	MTX (1 mg/cc)
Anaerobic glycolysis (Q^N_{λ})	21 †	21	20 ‡	20
Aerobic " (Q^O_{λ})	12 ‡	12	7.8	8.2
Oxygen consumption (Q_{O_2})	−5.6	−5.3	−6.3	−6.3
CO_2 production (Q_{CO_2})	4.0	3.7	4.9	4.9
Respiratory quotient (Q_{CO_2}/Q_{O_2})	− .71	− .69	− .78	− .78
Aerobic glycolysis / Respiration ($-Q^O_{\lambda}/Q_{O_2}$)	2.2	2.3	1.2	1.3
"Metabolic type"	"Malignant"	"Malignant"	"Malignant"	"Malignant"

* 58-yr-old female. WBC 264,000/mm^3, 75% blast and abnormal cells.
† *Not* inhibited by 6-MP or 6-MP and MTX.
‡ *Not* inhibited by 6-MP, hydrocortisone, triethylene thiophosphoramide or 6-azauracil (0.2-0.5 mg/cc).
§ 51-yr-old male. WBC 334,000/mm^3, 81% blast and abnormal cells.

are very similar to those observed in the previous patient. Furthermore, there was again no appreciable effect on these functions when the cells were incubated with methotrexate. These 2 studies are representative of the values seen with acute myelogenous leukemia.

The metabolism of leukocytes from 4 non-leukemic patients with leukocytosis was studied. These cells *in vitro* were found to have high rates of aerobic glycolysis, in agreement with previous reports(8-10) on human intact leukocytes and leukocyte homogenates, but different from rat leukocytes where aerobic glycolysis is quite small(11). Anti-leukemic agents such as methotrexate, 6-mercaptopurine, hydrocortisone and 6-azauracil had no significant effect on the *in vitro* aerobic glycolysis of cells taken from 3 of these patients. In respect to aerobic glycolysis and response to drugs, these cells were grossly indistinguishable from those of acute myelogenous leukemia, so far as studied.

Discussion. It is believed that experiments of the type described here represent the first systematic study of the metabolic effects of chemotherapeutic agents on relatively pure human leukemic cells *in vitro*. The question of whether or not leukemic cells are cancer cells in the metabolic sense seems to be resolved by the observation that these acute leukemic blast cells exhibit significant amounts of aerobic glycolysis if incubated under "physiological" *in vitro* conditions. This question will be covered more fully in a later publication.

It has been indicated that reproducible metabolic changes occurred when leukemic cells were incubated with certain chemical agents. The metabolic response appeared to be a function of the cell type. Thus it was observed that agents such as methotrexate, 6-mercaptopurine, hydrocortisone, triethylene thiophosphoramide, and 6-azauracil (1,2,4 triazine-3,5-dione)§ usually inhibited aerobic glycolysis of acute lymphatic leukemia cells. The latter 3 drugs also inhibited glycolysis of cells under anaerobic conditions. On the other hand, blast and other abnormal cells from acute myelogenous leukemic cases were particularly resistant to glycolytic inhibition by these same anti-leukemic agents. None of the 5 patients with acute myelogenous leukemia studied showed any appreciable metabolic effect with methotrexate. These observations are consistent with clinical impressions on the value of methotrexate in inducing remissions in acute lymphatic leukemia and the rarity of its beneficial effects in acute myelogenous leukemia. 6-mercaptopurine, and 6-mercaptopurine plus methotrexate, have shown occasional inhibitory effects on aerobic glycolysis in the cases of acute myelogenous leukemia studied. This finding is consistent with the use of 6-mercaptopurine as the "drug of choice" in treatment of this disease. Extensive correlative studies must necessarily await careful follow-up evaluations with a large group of such patients.

§ 1,2,4 triazine-3,5-dione was kindly supplied by Dr. Arnold Welch.

Preliminary studies with leukocytes from 3 non-leukemic patients with leukocytosis indicated that these cells were similar to those of acute myelogenous leukemia with regard to lack of glycolytic inhibition by anti-leukemic agents.

The respiratory quotients of acute lymphatic cells were often unlike those of any other known human cells, in that carbon dioxide production in relation to oxygen consumption was extraordinarily low, indeed at times CO_2 was apparently consumed (positive R.Q.). It can be seen that the effect of 6-mercaptopurine was to alter the R.Q. in the direction of normalization, and that methotrexate effected a similar and greater change in R.Q., to values as high as –1.0.

Despite the demonstrated lack of glycolytic inhibition of acute myelogenous leukemia cells by drugs currently used in therapy of this disease, it is desirable to point out that we have found compounds that do profoundly depress glycolysis of such drug resistant cells (2,5,14-16). Certain glucose analogues, of which 2-desoxyglucose and 2-desoxygalactose are examples, are very potent glycolytic inhibitors. These are not only effective against leukemic cells(12) but also against K-2 carcinoma ascites cells and other tumor cells (12,13). It has been observed that folic acid, teropterin, methotrexate, and 6-mercaptopurine may also be very active glycolytic inhibitors of leukemia cells and ascites cells. Hochstein in our institute has demonstrated(14) that a profound glycolytic inhibition can be obtained in mouse melanoma mitochondria with methotrexate or 6-mercaptopurine, and that this can be overcome by increasing the concentration of ATP in the media, or by substituting glucose-6-phosphate for glucose as substrate. The localization of primary site of action on the mitochondrial hexokinase reaction in this non-ATP synthesizing system, together with evidence found on the similarity of action of folic acid and methotrexate in inhibiting glycolysis in certain cancer cell types, throws a new light on the question of the mode of action of these "anti-metabolites." We have proposed that all of these structurally related compounds act by interfering competitively with ATP in glucose phosphorylation by the hexokinase reaction.

Summary. 1. A method for studying the effects of known anti-leukemic agents on the glycolysis of intact leukemic leukocytes is reported. The possible applications of this method in evaluating other new and theoretically promising agents, and in selecting the most active agent in a particular clinical case, are indicated. 2. Methotrexate and 6-mercaptopurine inhibited the aerobic glycolysis of acute lymphatic leukemia cells. Methotrexate did not inhibit the equally large aerobic glycolysis of acute myelogenous leukemia cells, and 6-mercaptopurine did so only rarely. These metabolic findings parallel general clinical experience. 3. The bearing of these findings on the mechanism of action of certain cancer anti-metabolites (anti-folics and anti-purines) is pointed out.

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