AN ABSTRACT OF THE THESIS OF

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Title: INTRACELLULAR pH AND THE UPTAKE OF CARCINOSTATIC

DRUGS BY SELECTED EXPERIMENTAL TUMORS

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The relationship of intracellular drug levels and resultant drug

activity to intracellular pH (pHi) was studied in the experimental

tumors. Glucose (5 g/kg) and/or sodium oxamate (0.1 mmol) were

administered to Walker 256 ascites cells and Ehrlich ascites cells,

in an attempt to alter pHi as measured by the distribution of the weak

electrolyte, 5,5-dimethyl-2,4-oxazolidinedione (DMO), between

extracellular and intracellular fluids. Drugs employed included 5-

fluorouracil (FU), cytosine arabinoside (CA), 1-(2-chloroethyl)-3-

cyclohexyl-1-nitrosourea (CCNU), and procarbazine (PZ).

As measured by DMO distribution, pHi in the Walker 256 ascites

cells was significantly increased following prior glucose administra-

tion. This increase occurred both with and without concurrent sodium

oxamate pretreatment.

Intracellular FU concentrations in the Walker 256 ascites cells

were increased approximately two-fold when glucose was part of the

pretreatment regimen, again either alone or in combination with

oxamate. These increases were accompanied by an enhancement

of FU activity. The increases in Fu activity observed did not seem to be associated with a corresponding increase in FU toxicity; however, they were observed to be positively related to the dose of glucose employed as adjunct therapy. These results supported the concept that prior exposure of neoplastic tissues to glucose could enhance both the uptake and the resultant activity of FU.

In Ehrlich ascites tumor cells, pHi decreased following glucose pretreatment. This decrease was not evident when sodium oxamate was added to the pretreatment regimen. Following a glucose-oxamate pretreatment, the pHi of the Ehrlich cells remained at control levels.

The intracellular concentration of FU was always enhanced following the administration of glucose to Ehrlich ascites cells as part of the pretreatment regimen. Since the pHi of the cells was not significantly altered from controls when sodium oxamate was added to the pretreatment regimen along with glucose, the intracellular concentration of FU was more dependent upon the presence of glucose as part of the pretreatment than upon the pHi of the Ehrlich cells.

Enhancement of Fu activity in this model was only achieved by the prior administration of both glucose and sodium oxamate. This result indicated that although glucose increased FU levels within neoplastic tissues, the higher intracellular FU concentration might not lead to increased FU activity.

Procarbazine (PZ) concentrations within Ehrlich ascites cells were increased only by the prior administration of glucose and sodium

oxamate. The fluctuation in pHi due to the prior administration of glucose alone had no effect on intracellular PZ levels. The increased uptake following glucose-oxamate pretreatment was accompanied by an increase in PZ activity upon Ehrlich ascites tumor cells.

The intracellular levels and resultant activity of both CCNU and CA remained unchanged following any of the pretreatments despite the changes in pHi. The independence of CCNU uptake from alterations in pHi was probably a reflection of its non-ionized, neutral state within the physiological pH range, while the absence of any change in the intracellular levels of the acidic compound, CA, suggested that factors other than pHi were the major determinants of CA concentration within Ehrlich ascites cells.

These results indicated that in evaluating the utility of adjunct glucose and/or sodium oxamate therapy for the enhancement of drug effect, any changes in neoplastic pHi which are caused by the adminis- tration of either or both of these agents are neither the only, nor in some cases the main, factor which may influence drug uptake. More- over, the metabolic effects of either glucose or oxamate might affect the activity of specific drugs upon neoplastic tissues to a greater extent than their influence upon pHi. Thus, adjunct antineoplastic therapy with glucose and/or oxamate should not be considered in terms of general therapeutic application, but rather should be considered for each antineoplastic drug separately.

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INTRACELLULAR pH AND THE UPTAKE OF CARCINOSTATIC DRUGS BY SELECTED EXPERIMENTAL TUMORS

I. GENERAL INTRODUCTION

In all things there is a poison, and there is nothing without poison. It depends only upon the dose whether a poison is poison or not. . . That which redounds to the benefit of man is not poison; only that which is not of service to him, but which injures him, is poison. (Paracelsus, 1493-1541).

For these words Paracelsus is generally recognized as the "grandfather of pharmacology" because they represent the first written expression of the concept of dose-response. Although applicable to all of the therapeutic agents in the medical armamentarium, this passage acquires a special quality when it is applied to those chemical agents which are used in the treatment of neoplastic disease. Because of the very nature of the disease and the lack of knowledge about how to control or contain it, the available methods of treatment are at present all based upon the elimination of the offending neoplastic cells.

It is the lack of complete cytotoxic specificity for neoplastic cells which is one of the major problems in the chemotherapeutic approach to cancer therapy today. The toxicity of these agents to actively dividing cells of normal tissues is, in most instances, self-defeating since the physician must often remove the agent from therapy

before its work upon the neoplasm is complete. This is done in order to prevent the death of the patient due to the injurious effects of the chemical upon normal tissues. In short, cancer therapy usually involves a balance between efficacy and toxicity, the outcome depending upon whether the therapy invoked can eliminate the neoplastic cells before it eliminates the patient.

Thus, it is of interest to look at the words of Paracelsus, for antineoplastic agents are a group of substances which are not only of benefit to man, but which also injure him at the same dose, making them at once poison and non-poison. To state it in another form, this is a group of substances which is medicinally used at its poisonous dose.

How might this situation be altered? A number of investigators believe that the answer might lie in elucidating the physiological and biochemical differences between neoplastic and normal tissues. By either designing new chemical compounds or revising the way in which we utilize our present ones, it might be possible to exploit these differences.

One biochemical difference between neoplastic and normal tissues, first noted by Warburg et al. (1924), is the propensity of neoplastic tissue to use glycolysis as its primary energy source under both anaerobic and aerobic conditions. These workers based this conclusion, in part, upon the tremendous quantities of lactic acid

formed by neoplastic tissues. They further reasoned that this amount of lactic acid should render neoplastic tissues more acidic than normal tissues.

Using microelectrode techniques, various investigators found neoplasms to be more acidic, not only in animal tumor systems (Voegtlin et al., 1935; Kahler and Robertson, 1943; Naeslund and Swenson, 1953; Tagashira et al., 1953; Tagashira et al., 1954; Eden et al., 1955) but also in human tumors (Meyer et al., 1948; Naeslund and Swenson, 1953). Moreover, a number of these investigators discovered that the administration of glucose, usually in massive amounts, reduced the pH of neoplastic tissues to values which were approximately 0.5 pH unit below their previous pH. These values were 0.7-1.0 pH unit below the pH of 7.2-7.4 determined for both plasma and various normal tissues with the same microelectrode techniques.

Poole (1967), using the distribution of the weak electrolyte 5,5-dimethyl-2,4-oxazolidinedione (DMO), showed a similar reduction of intracellular pH (pHi) for Ehrlich ascites tumor cells in vitro upon the addition of glucose. Further work from the same laboratory suggested that the pHi of Ehrlich cells could also be depressed by the administration of either the sulfhydral (SH) inhibitor, iodoacetate, or the lactic dehydrogenase (LDH) inhibitor, oxamic acid (Poole and Butler, 1969). This reduction in pHi following ascites cell exposure to these inhibitors was due to the intracellular accumulation of the relatively more acidic phosphorylated glycolytic intermediates rather

than the freely diffusable lactic acid. As a result, the pHi became

relatively more acidic when compared to the extracellular pH (pHe)

since there was no diffusable acid to concurrently lower pHe.

Oxamic acid, an inhibitor of LDH, was reported to inhibit the

heart isoenzyme (H_4) up to 2.5 times more than the muscle isoenzyme

(M_4) when lactic acid was the substrate (Wilkinson and Walter, 1972).

Although LDH of both human and animal neoplasms was found to have

an isoenzyme spectrum characterized by a predominance of M_4

(Goldman et al., 1964; Massey et al., 1971; Schweitzer et al., 1973),

oxamate has been shown an effective inhibitor of LDH in HeLa cells

(Goldberg and Colowick, 1965) and Ehrlich ascites carcinoma cells

(Papaconstantinou and Colowick, 1961). Moreover, it was reported

that the resultant inhibition of HeLa cell growth could be intensified

by glucose (Goldberg et al., 1965). In addition, oxamic acid and

glucose were shown to inhibit the growth of Walker 256 carcinosar-

coma in vivo when administered together by regional arterial infusion

(Reynolds et al., 1963).

The addition of glucose, in massive quantities, to the treatment

schedule of several chemotherapeutic agents has been reported to

enhance antieoplastic activity and/or reduce toxicity in various animal

models. Included among these agents were a basic aromatic nitrogen

mustard (Ross, 1961), two epoxides (Connors et al., 1964), triethyl-

enemelamine (Connors et al., 1964), 5-fluorouracil (Kung et al., 1963),

and tetraazatricyclododecane (Stevens and Mosteller, 1969). All of these studies used some measurement of tumor growth inhibition as an indication of drug effectiveness. In addition, Woods et al. (1960) showed that the addition of glucose to a 5-fluouracil (FU) treatment schedule decreased the transplantability of both the Ehrlich ascites and Krebs-2 ascites carcinomas.

The theory used by many of these investigators to explain this enhancement of effect was first elaborated by Ross (1961). He envisioned basic drugs being selectively deposited in neoplasms after adjunct glucose therapy due to the decrease in pH that had been observed within neoplastic tissues after glucose administration. He further suggested that antineoplastic drugs should be designed which would be able to take advantage of this decrease in pH. This concept was broadened by his co-worker, Connors (et al., 1964), to also include the designing of drugs which would be activated in acidic media. In either case, the addition of glucose to the treatment schedule of one of these agents would be beneficial.

Kung et al. (1963), working with the weakly acidic FU, believed that its increase in activity after glucose administration was due to the more acidic environment producing more of the undissociated form of the fluoro-pyrimidine moieties, the form that had been shown to associate with enzymes (Goldberg et al., 1963). An increased amount of undissociated deoxyribonucleotide phosphate would have

led to a greater inhibition of the enzyme thymidylate synthetase.
These investigators also suggested that the decreased pHi might have
been either enhancing FU transport into the cell or altering the activity
of one of more of the enzymes involved in the lethal synthesis of FU
to the deoxyribonucleotide monophosphate. Any of these alternatives
could have led to the increased activity observed.

The concept of glucose enhancement of drug therapy received
clinical support from a study which reported a decrease in FU toxicity
when it was administered by slow intravenous (iv) infusion in 5% dex-
trose in water (D5W) as opposed to rapid iv injection in saline (Lemon,
1960; Lemon et al. , 1963). Another team of investigators showed no
difference in terms of patient toxicity between rapid and slow methods
of FU administration when the diluent was D5W (Moertel et al. , 1972).
Other clinical studies have appeared, however, which showed no
significant difference in drug effect or patient toxicity for FU when
it was administered in D5W as opposed to saline (Cressy and Schell,
1965; Hall et al. , 1966; Cressy and Schell, 1966). The amount of
glucose administered in these latter studies was only 25-50% of that
used in the positive clinical study. This fact became more important
when a later clinical study showed indications of improved therapy
when FU was given in 10 and 20% dextrose in water solutions rather
than D5W (Lahiri, 1968).

The amount of FU incorporation into ribonucleotides and RNA

in vitro was reported to be strongly correlated to drug response in certain murine leukemias (Kessel et al., 1966). A later study demonstrated the ability of inosine and deoxyinosine to increase the conversion of FU into nucleotides and thus increase the incorporation of FU into nucleic acids in Ehrlich ascites cells (Gotto et al., 1969). Glucose and inosine stimulated the rate of conversion of FU to ribonucleotides specifically in a study which appeared shortly thereafter (Kessel and Hall, 1969). This mechanism led to an increase in the amount of FU incorporated into RNA in a variety of tumor lines. The stimulation was not due to an increase in the rate of drug transport into the cells; therefore, the authors concluded that both compounds were acting as ribose donors, thereby stimulating the conversion of FU into ribonucleotides. These investigators, using the two tumor lines in which glucose stimulation of FU into RNA was greatest, could find no significant difference in in vivo effect (Kessel and Hall, 1969). Their conclusion was that the administration of glucose, while increasing FU intracellular concentrations, had no significant effect upon FU therapy. The glucose stimulated incorporation of pyrimidines into Ehrlich ascites tumor RNA was subsequently shown to be dose dependent using the natural base, uracil (Itzhaki, 1972). The dose dependence for this mechanism becomes interesting when viewed with the clinical evidence that indicates an importance for the amount of glucose used as an adjunct to FU therapy.

Woods and Burk (1969) reported a FU inhibition of glycolysis in Ehrlich ascites tumor cells both in vitro and in vivo. The presence of glucose was an absolute requirement for this inhibition. Moreover, for a series of halogenated pyrimidines, the inhibition showed a parallelism with earlier reports of antitumor and toxic activity. Further, these authors demonstrated that this FU inhibition of glycolysis was partially due to the trapping of inorganic phosphate, a mechanism of glycolytic inhibition previously suggested by Wu and Racker (1963).

It has also been reported that the amount of intracellular FU in intramuscular (im) Walker 256 carcinosarcoma slices was increased by the presence of glucose (Hult and Larson, 1976). A coordinated pHi study, using DMO distribution, demonstrated that the addition of glucose to these slices resulted in an elevation of pHi. This increase in pHi confirmed the work of Schloerb et al. (1965), which had shown an increase in the pHi of Walker 256 carcinosarcoma in vivo following administration of 6 g/kg glucose. This group of investigators suggested that this atypical increase in pHi for Walker 256 neoplastic tissue might be due to a large intracellular buffering capacity of the Walker 256 tumor.

Using 40 mM sodium oxamate and 5 mM glucose in the incubation medium, Hult and Larson (1976) showed a dissociation of FU intracellular concentrations from pHi in Walker 256 im tumor slices.

Under these conditions intracellular FU concentrations increased to the higher levels observed after incubation with glucose alone, while the pHi remained the same as control values (≈ 7.2). These authors concluded that the presence of glucose was more important than pHi in determining the amount of FU concentrating within the tumor cells.

The series of experiments reported upon herein was designed as an extension of the work which showed this dissociation of FU uptake from pHi. The main goal of these studies was to clarify whether adjunct glucose and/or oxamate therapy could influence the activity of antineoplastic agents, especially FU. If so, the question of dose dependence for this influence was to be investigated. Further, these experiments were intended to determine whether any shifts in pHi due to glucose and/or oxamate administration might lead to corresponding changes in drug uptake and resultant drug activity for a series of drugs with a broad spectrum of pK values.

It was initially realized that minor changes in one of the dependent variables (pHi, drug uptake, drug activity) which would be undetectable might lead to significant differences in another variable and conversely. It was hoped, however, that these studies would provide some initial answers which might lead to a more thorough understanding of the relationship among these three variables after either glucose or glucose and sodium oxamate pretreatment.

General Methods

Animals: Animals utilized in the following studies were either mice or rats bred and raised in the animal colony of the Department of Pharmacology and Toxicology. The mice were CF-1 males; the strain was originally obtained directly from Carworth Inc. The rats were Sprague-Dawley males derived from stock originally obtained from Simonsen Inc., Gilroy, CA. During all of these studies, the animals were given food and water ad libitum. All animals were kept at a constant temperature of 21-22°C with a 12 hour day and night cycle.

Rat tumor line: The rat tumor line used was the Walker 256 carcinosarcoma obtained from the National Cancer Institute (NCI), Bethesda, MD, and propagated weekly as an intraperitoneal (ip) ascites tumor using an NCI protocol (Geran et al., 1972). An animal which was ready to succumb to the tumor provided 8-12 ml of ascites fluid for serial transfer of the tumor. This fluid was diluted 1:100 with normal saline and 0.1 ml of the dilution provided an inoculation of approximately 10^5 Walker 256 ascites cells. This dose, when injected into a 100-125 g rat, proved lethal in 6-8 days.

Mouse tumor line: The Ehrlich ascites tumor line utilized in the mouse studies was obtained from Dr. M. Rittenburg at the University of Oregon Medical School, Portland, OR. This tumor was

propagated as the ascites tumor every 9-12 days using the following procotol. Two or more animals which showed definite signs of ascites formation (abdominal distention) were anesthetized with diethylether. The ascites fluid from each animal was then harvested by ip puncture using a 20 gauge needle. Following a thorough mixing of the ascites from all animals, the fluid was diluted 1:10 and 0.2 ml of the dilution was injected ip into the recipient animals. This protocol provided an inoculum of approximately 10^7 Ehrlich ascites cells which produced visible ascites formation in the recipient animals in 9-12 days.

Pretreatment regimens: Attempts to alter pHi and intracellular drug concentrations through the prior administration of either glucose, sodium oxamate, or both were a main feature of these studies. These three pretreatment possibilities, along with saline controls and non-injected controls, were listed as five different pretreatment regimens. Each regimen consisted of two ip injections and was defined as follows:

1) non-injected controls; these animals received no substances prior to drug exposure.

2) saline-saline; these animals received

   a) 1 ml/100 g body weight saline 90-120 min before drug exposure.

   b) 0.32 ml saline 30-45 min before drug exposure

3) saline-oxamate; these animals received

a) 1 ml/100 g body weight saline 90-120 min before drug

exposure

b) 0.32 ml of 35 mg/ml sodium oxamate (0.1 mmol) 30-45

min before drug exposure

4) glucose-saline; these animals received

a) 5 g/kg glucose (1 ml concentrate/100 g body weight)

90-120 min before drug exposure

b) 0.32 ml saline 30-45 min before drug exposure

5) glucose-oxamate; these animals received

a) 5 g/kg glucose (1 ml concentrate/100 g body weight)

90-120 min before drug exposure

b) 0.32 ml of 35 mg/ml sodium oxamate (0.1 mmol) 30-45

min before drug exposure

The dose of glucose employed (5 g/kg) and the timing for its

administration (2 hr prior to drug exposure) were similar to doses

in the literature for previous in vivo studies. The administration

of glucose at 6 g/kg had been shown to have maximum effect upon

pHi in various rat tumors at 2-4 hr (Voegtlin et al., 1935; Kahler

and Robertson, 1943; Eden et al., 1955). Those in vivo investigations

which reported positive effects for adjunct glucose therapy during

treatment with various antineoplastic drugs used doses of 5.0-7.5 g/kg

glucose delivered 1-2 hr prior to the drug (Ross, 1961; Kung et al.,

1963; Connors et al., 1964).

Poole and Butler (1969) had shown a maximal effect for 40 mM sodium oxamate upon the pHi of Ehrlich ascites cells 20-30 min following initial exposure. For the studies reported herein the dose of sodium oxamate was delivered in a saturated solution. When diluted by the 8-12 ml of ascites fluid usually present, it produced an initial oxamate concentration of approximately 10 mM.

Intracellular pH determination. The pHi of the tissues was determined by the distribution of the weak electrolyte, DMO, between extracellular and intracellular fluid. The original method, along with supportive arguments, was published by Waddell and Butler (1959). The daily working solution was made from a stock concentrate of ^{14}C-DMO (2.5 μCi/ml) in ethyl acetate. A portion of this concentrate was allowed to evaporate to dryness. The DMO was redissolved in normal saline (or ^{3}H-saline) to the desired radioactive concentration. This stock solution was refrigerated when not in use and was not kept for longer than seven days.

II. INFLUENCE OF GLUCOSE AND SODIUM OXAMATE PRETREATMENT ON ACUTE 5-FLUOROURACIL TOXICITY IN RATS

Introduction

Since the thrust of the studies described herein was to deter-
mine the utility of adjunct glucose and/or sodium oxamate in anti-
neoplastic treatment, it was important to observe the effects of these
substances upon drug toxicity. Adjunct therapy which enhanced drug
effect at the expense of equal or greater increases in drug toxicity
might very well be disadvantageous in terms of therapeutic benefit at
the clinical level.

Kung et al. (1963) suggested that the toxicity of FU in rats was
not significantly affected by concomitant glucose treatment at glucose
doses which enhanced FU activity. This conclusion was based upon
weight fluctuation data obtained from the treated animals in their
experimental study and average time until death data obtained from
non-tumor bearing animals. These non-tumor bearing animals were
given various doses of FU (40-100 mg/kg) daily with concurrent glu-
cose or saline treatment for one week. From this study these inves-
tigators said that the average times of death at the various FU doses
were similar in the saline and glucose-treated group pairs. Data
supporting this statement or the more general conclusion concerning
the absence of any change in FU toxicity with concurrent glucose

treatment were not given.

Connors et al. (1964) reported an increase in toxicity for the compounds that he was using following the addition of concurrent glucose treatment. These increases in toxicity usually paralleled the changes in drug activity upon the tumor model.

The purpose of the present study was to quantify any changes in FU toxicity which might occur following the pretreatment regimens which had been proposed. These regimens were formulated so that pretreatment with both glucose and oxamate before FU exposure was one of the treatment possibilities. The lack of substantive data from the work of Kung et al. (1963) concerning the effect of concurrent glucose treatment upon FU toxicity, the increase in drug toxicity reported by Connors et al. (1964) following the addition of glucose to antineoplastic treatment schedules, the addition of sodium oxamate as part of the pretreatment regimen, and the importance of toxicity data in determining the relative utility of adjunct therapy were points which indicated that a toxicity study to determine the effect of glucose and oxamate pretreatment upon FU toxicity should be the initial focus of attention.

Methods

The 60 rats selected for use in this study were six-week old males weighing 140-200 g. Each week for two weeks 30 animals were

randomly distributed into five groups of six animals each.

The first week each group of six animals received an ip injection of FU (Roche Laboratories, Nutley, NJ) at one of the following doses: 125, 200, 250, 300, or 500 mg/kg. The pretreatment for all of these animals was the saline-saline pretreatment described in General Methods. The first pretreatment injection was given 120 min prior to the FU exposure while the second injection was made 90 min later, i. e., 30 min prior to the FU injection.

The second group of 30 animals received the glucose (J. T. Baker Chemical Co., Phillipsburg, NJ)-oxamate (Aldrich Chemical Co., Milwaukee, WI) pretreatment regimen described in General Methods. The two pretreatment injections were administered at times corresponding to the two saline injections above. After the glucose-oxamate pretreatment each group of six animals was given an ip injection of FU at one of the following doses: 125, 150, 200, 250, or 300 mg/kg.

For all 60 animals the FU dose was delivered in a total volume of 1 ml/100 g body weight.

Following this acute exposure, the animals were checked at the same time daily (0900) for 14 days. During this daily examination, each animal was observed for any visible toxic signs. This information, as well as any deaths which had occurred, was recorded. Also recorded every 48 hr was the weight of each animal still alive. The

difference between the initial weight of an animal and the weight at each of these 48 hr weighings was calculated. Percent weight loss was figured from this difference and the numerical mean of these percent weight loss figures calculated for each FU dosage.

Seven and 14 day dose-response lines were plotted from the raw data by linear regression. Median lethal dose (LD50) values and their 95% confidence limits were calculated according to the method of Litchfield and Wilcoxon (1949). The slope function (S) and its 95% confidence limits were also determined in order to test for parallelism of the lines. Dose-response lines which did not deviate from parallelism were tested for significant differences in their LD50 values.

Results and Discussion

The first indication of toxicity was a weight loss which was noticeable at 48 hr after the acute dose of FU (Table 2-I, 2-II). This weight loss reached maximum values at 96 hr and remained near maximum values for an additional 48 hr at doses $\geq$ 200 mg/kg. At this time (144 hr or six days post-treatment) the great majority of animals began gaining weight at all doses of FU administered.

Severe gastrointestinal disturbances were noticeable at 60-72 hr post-treatment with diarrhea evident in all animals. This condition lasted until the seventh day following FU exposure. Then the stools

Table 2-I   Weight changes in rats following acute exposure to 5-fluorouracil.

FU Dose (mg/kg)	Initial Mean wt. (g)	Percent mean weight change (g) from initial value				
		48 hr	96 hr	144 hr	192 hr	240 hr
125	178	-4.78 (6)	-17.07 (6)	-11.32 (6)	-0.79 (6)	+6.33 (6)
200	155	-1.93 (6)	-10.96 (6)	-4.32 (6)	+7.09 (6)	+15.03 (6)
250	186	-4.83 (6)	-17.74 (6)	-13.17 (5)	-4.12 (4)	-5.32 (4)
300	178	-6.17 (6)	-18.37 (3)	-17.99 (3)	-10.46 (2)	-8.68 (2)
500	181	-6.35 (6)	-16.67 (1)	--	--	--

The figure in parentheses shows the number of animals out of six/dose surviving at the time of weighing.

Table 2-II   Weight changes in glucose and/or sodium oxamate pretreated rats following acute exposure to 5-fluorouracil (FU).

FU Dose (mg/kg)	Initial Mean wt. (g)	Percent mean weight change from initial value				
		48 hr	96 hr	144 hr	192 hr	240 hr
125	178	-8.13 (6)	-14.98 (6)	-8.08 (6)	00.00 (6)	+10.71 (6)
150	173	-5.10 (6)	-12.00 (6)	-6.78 (6)	-1.33 (6)	+8.98 (6)
200	168	-10.01 (6)	-20.56 (6)	-20.73 (6)	-7.01 (4)	+1.55 (4)
250	173	-9.93 (6)	-22.45 (6)	-21.02 (5)	-15.73 (5)	-3.01 (5)
300	164	-8.55 (6)	-21.29 (5)	-24.74 (3)	--	--

The animals were pretreated with 5 g/kg glucose 120 min prior to FU exposure and 0.1 mmol sodium oxamate 30 min prior to FU exposure. The figure in parentheses shows the number of animals out of six/dose surviving at the time of weighing.

became firmer, over a time course similar to that seen with the initiation of weight gain.

The final toxic symptom to appear was alopecia which became evident from days 5-10. Hair loss was predominantly from the head and dorsal thorax with the losses for those animals which received the higher doses of FU substantial.

All of these gross indicators of toxicity were observed in both the glucose-oxamate and saline-saline pretreated animals. Toxic signs appeared, reaching maximal values, and regressed along the same time course at corresponding dosage levels of FU following either pretreatment.

The LD50 determined for the saline-saline animals at seven days was 347 mg/kg (95% confidence limits, 269-448 mg/kg). This value decreased slightly at 14 days to 318 mg/kg (244-414 mg/kg). The LD50 for the glucose-oxamate pretreated animals decreased from seven to 14 days also, the seven day LD50 being 279 mg/kg (225-346 mg/kg) and the 14 day LD50 being 259 mg/kg (213-315 mg/kg). The LD50 values for the glucose-oxamate pretreatment were slightly lower than the LD50 values for the saline-saline pretreatment. The plots of the dose-response data are presented in Appendix I.

The test of parallelism established that for all possible pairs of the four lines, there was no significant deviation from parallelism. Because of this relationship, the difference between the LD50 values

for any pair of lines was tested for significance with the potency ratio test as described by Litchfield and Wilcoxon (1949). The results from this test showed that there was no significant difference ($P < .05$) between the seven and 14 day LD50 for either pretreatment. More importantly, there was also no significant difference ($P < .05$) between the glucose-oxamate pretreated and the saline-saline pretreated groups of animals at either seven or 14 days.

Thus, both the gross indicators of toxicity, including weight fluctuations, and the LD50 determinations indicated that acute pretreatment of rats with 5 g/kg glucose and 0.1 mmol sodium oxamate did not significantly affect the acute toxicity of FU. These results substantiated the conclusion reached by Kung et al. (1963) who reported no increase in FU toxicity following the concurrent administration of glucose alone.

In addition to the reasons enumerated in the introduction to this chapter, it was important to show no significant increases in FU toxicity following pretreatment with glucose and sodium oxamate because of the tumor model to be employed in the activity studies. The tumor model which had been selected was the measurement of the length of time until death of an animal after the ip inoculation of a standard number of Walker 256 ascites tumor cells. Preliminary work with the Walker 256 carcinosarcoma as an im tumor had shown the latter form of the tumor to be unsatisfactory for an in vivo study

of treatment effects. While precise values for pHi and FU uptake had been obtained following a regional arterial infusion of the im form of the tumor as advocated by Reynolds et al. (1963), no consistent measurement of tumor size (weight, length, or width) was found which would yield values demonstrating significant effects.

Skipper et al. (1964) had shown, using L1210 murine leukemia, that the virulence of that ascites tumor in terms of time until death was related to the number of cells inoculated. More recent experience by another investigator has shown this finding to be true for the transplantability of the Walker 256 tumor (Kirkvliet, 1975). The experience obtained in our hands from over two years of serial transfer of the tumor was also a convincing argument in favor of using the length of time until death as the measurement of treatment effect.

With death as the end-point it had to be shown that the glucose and oxamate pretreatments used in attempting to alter the pHi of the tumor would not unduly increase the toxicity of FU. Without this assurance, standard therapeutic doses of the drug might become lethal after glucose and/or oxamate pretreatment. Deaths due to the treatment would interfere with test results and might lead to erroneous conclusions. For this reason, as well as the therapeutic considerations stated in the introduction, the similarity in the toxic spectrum of FU with and without glucose and oxamate pretreatment was a favorable finding.

III. 5-FLUORUORACIL ACTIVITY UPON WALKER 256 ASCITES TUMOR AFTER GLUCOSE AND/OR SODIUM OXAMATE PRETREATMENT, IN VIVO

Introduction

The "potentiation" of FU effect upon Flexner-Jobling carcinoma by the prior administration of glucose was reported by Kung et al. (1963). These investigators suggested that the increase in FU effect was related to an assumed decrease in pH, an effect which had been previously observed in that tumor (Voegtlin et al., 1935). This same assumption has been used as a mechanism to explain the increase in antineoplastic effect of various other agents when glucose was added to their treatment schedule (Ross, 1961; Connors et al., 1964; Stevens and Mosteller, 1969). None of these studies measured pHi, however, but based their assumption of shifts in pH upon previous reports.

The purpose of this study was to determine whether the antineoplastic activity of a sub-acute dosage schedule of FU could be influenced by the prior administration of glucose and/or sodium oxamate. The in vivo results obtained from this model would then be compared to pHi and intracellular FU concentration data obtained from a later study.

The results from the previous study showed that the acute administration of 5 g/kg glucose and 0.1 mmol of sodium oxamate to

rats later exposed to an acute lethal dose of FU did not significantly alter the FU toxicity. Since the glucose-oxamate pretreatment had the greatest potential for influencing FU toxicity, these negative results were favorable to the use of the length of time until death after the inoculation of a known number of Walker 256 ascites cells as the in vivo model for monitoring changes in FU activity. It was felt that even though the negative results were obtained with an acute dose study, developing FU toxicity due to clinical, sub-acute doses of the drug would be influenced in the same manner. Careful monitoring of the saline-saline pretreatment group for those animals exposed to FU would detect developing FU toxicity so that FU therapy might be removed before lethalities due to FU toxicity in any of the pretreatment groups interfered with the length of time until death model.

Methods

One hundred sixty-three five-week old male rats were used for this study. The weight range for these animals was 80-130 g. To obtain this quantity of animals with both proper age and weight, it was necessary to conduct the study over a five week interval. Thus, an incomplete randomized block design with eight treatments was utilized. Each week, all five-week old animals within the indicated weight range were randomly distributed into groups of five or six animals each, yielding five or six groups per week. One of

these groups was used as a weekly control of tumor virulence (no injections = first treatment), while the other four or five groups of animals received one of the seven remaining treatments. Each of these seven treatments was administered to at least three different groups of animals, but to only one group per week.

Following the weekly distribution into groups, each animal was weighed and then inoculated ip with 10^5 Walker 256 ascites cells. The cells were given 72 hr to become a viable tumor within the new host before any treatment was initiated.

Each treatment consisted of one of the pretreatment regimens described in General Methods plus another ip injection of either 15 mg/kg FU (0.1 ml/100 g body weight) or an equivalent volume of saline. The first pretreatment injection was made 120 min prior to the FU exposure while the second pretreatment injection was given 90 min later, i. e. , 30 min prior to the FU (or saline).

The dose of FU used in this study was a standard clinical dosage for daily FU therapy which has been given either iv or ip (Clarkson et al. , 1964). In addition, Kung et al. (1963) showed a marked effect upon tumor growth with 15 mg/kg FU and 5 g/kg glucose when they were given daily.

The treatments in this study were administered daily from day 3-7 post-inoculation and then every other day for the balance of the experiment (through day 14). Before each series of injections, the

animal was examined for general condition and weighed.

Beginning at day five the animals were checked every eight hours (0800, 1600, 2400) and deaths were recorded. Animals still alive after 14 days were considered "cures" and the experiment for that week was terminated.

For each treatment the weights of all animals were pooled and the numerical means calculated for tabulation purposes. Three-day weight gain and/or loss for each animal was also calculated along with the means for each treatment group. Differences in the three-day weight gain or loss for each treatment were statistically analyzed by use of the Wilcoxon Rank Sum Test (Wilcoxon and Wilcox, 1964).

The mortality data from each treatment were analyzed according to Litchfield's Time-Percent Effect analysis (Litchfield, 1949). Linear regression was utilized to fit the probit line to the time-response plot of the raw data for this analysis. Median lethal time (LT50), slope function (S), and the 95% confidence limits for both parameters were determined. From these values tests for parallelism of the time-response lines and for significant differences among the LT50 values for the treatments were run.

Results and Discussion

During the first 72 hr following inoculation, all of the animals gained weight at the rate of 5-7 g/day (Table 3-I). For those animals

Table 3-I   Weight Changes in Rats Bearing Walker 256 Ascites Tumor During Daily Treatment with a Three Agent Regimen.

| Treatment Regimen[a] | | | Number of Animals | Mean Weight[b] (g) | | | Mean Weight Difference (g) | |
| Pretreatment | | Treatment | | | | | | |
-120 min (1 ml/100 g body wt.)	-30 min (0.32 ml)	time 0 (.1 ml/100 g body wt.)		Day 0	Day 3	Day 6	Days 3-0	Days 6-3
	CONTROL							
none	none	none	27	107.5[c]	124.0	141.4	16.5	17.4
saline	saline	saline	6	100.3	117.0	136.0	16.7	19.0
saline	35 mg/ml Oxamate	saline	21	106.0	123.8	139.5	17.8	15.9
5 g/kg Glucose	saline	saline	17	108.5	128.0	141.2	19.5	14.1
saline	saline	15 mg/kg FU	27	106.3	121.9	127.1	15.6	5.2[d]
saline	35 mg/ml Oxamate	15 mg/kg FU	18	102.5	119.9	124.4	17.4	4.5[d]
5 g/kg Glucose	saline	15 mg/kg FU	28	103.9	118.9	120.9	15.0	2.0[d]
5 g/kg Glucose	35 mg/ml Oxamate	15 mg/kg FU	17	102.9	120.8	124.7	17.9	3.9[d]

[a] Rats which had been inoculated with 10^5 ascites cells three days previously were given a daily series of three ip injections. The relative time of each injection and its volume are indicated. FU - 5-Fluorouracil

[b] The daily weight of each animal along with any change in each animal's weight were recorded.

[c] Numerical means for all parameters shown were calculated for display purposes.

[d] Indicates a group of animals whose distribution of weight gain was significantly different ($P < .05$) from the weight gain of the control group using the Wilcoxon Rank Sum Test.

not exposed to FU as part of their treatment protocol, weight gain continued at the same rate (5-7 g/day) until just before their death from the tumor. However, those treatment groups exposed to FU as part of their treatment, showed a noticeable reduction in the rate of weight gain to less than 2 g/day (Table 3-I). This decrease in rate of weight gain was significant at the 95% confidence level.

There was no difference in rate of weight gain among the groups whose treatment regimens included FU. This result was consistent with the results obtained during the acute FU toxicity study. For both the sub-acute and the acute exposure to FU, there was no significant shift in weight gain or loss between those FU treated animals receiving a saline-saline pretreatment and those FU treated animals receiving a glucose-oxamate pretreatment. This fact served to substantiate the conclusion of the previous study that there was no appreciable increase in FU toxicity following glucose and oxamate pretreatment.

The LT50 for those animals serving as the controls for tumor virulence (non-injected) was determined with excellent precision (Figure 3-1). The low variance with which this LT50 (7.3 days) could be determined verified the expectation that length of time until death would provide an in vivo model that was capable of discriminating among treatment effects. Time-response plots of the data for all pretreatment groups are presented as Appendix II.

Figure 3-1   Median Lethal Time (LT50) for rats bearing Walker
256 ascites tumor following treatment with different
combinations of glucose, sodium oxamate, and
5-Fluorouracil (FU).   A treatment regimen which
consisted of three ip injections was administered on
days 3-7, 9, 11, and 13.   Each of the three injections
was a choice as follows:
    1) 5 g/kg glucose or an equivalent volume of
        saline (1 ml/100 g body weight) 120 min prior
        to injection #3.
    2) 0. 32 ml of 35 mg/ml sodium oxamate (0. 1 mmol)
        or 0. 32 ml saline 30 min prior to injection #3.
    3) 15 mg/kg FU or an equivalent volume of saline
        (0. 1 ml/100 g body weight).
LT50s were obtained from a time-percent effect line
plotted from the raw data by linear regression.   The
method of analysis used was that of Litchfield (1949).
LT50s are shown with their 95% confidence limits in
brackets.

Superscript letters which are NOT alike designate those
groups whose LT50s are significantly different (P <. 05)
from one another.

The figure in parentheses indicates the number of
animals used for the determination of that LT50.

PRETREAT-MENT	None	Saline	Saline	Glucose	Saline			Glucose
TREATMENT	None	Saline	Oxamate	Saline	Saline			Oxamate
	None	Saline	Saline	Saline	FU		FU	FU
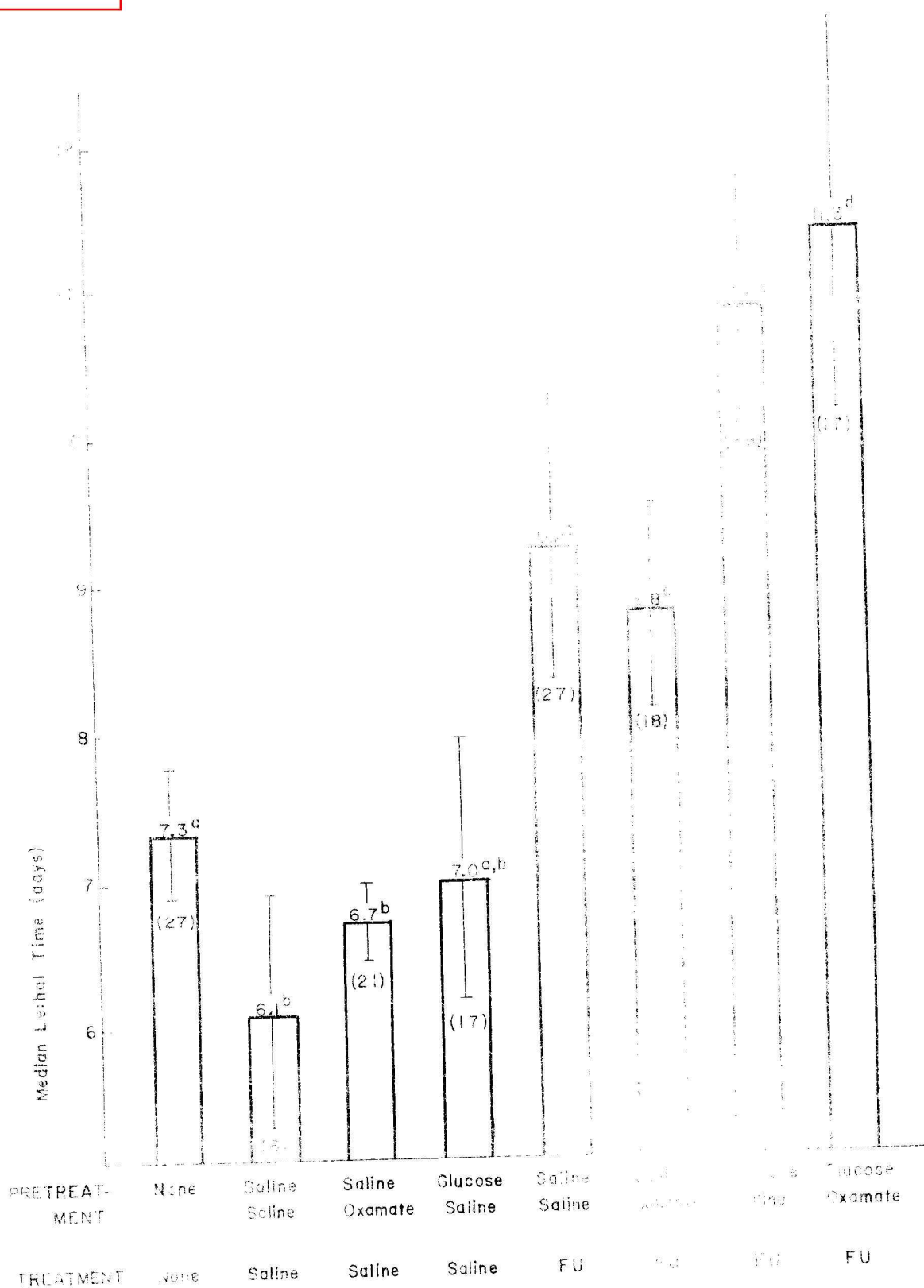
The treatment regimens which utilized the saline-saline, saline-oxamate and glucose-oxamate pretreatments in the absence of FU reduced the LT50 by at least eight hours (Figure 3-1). A comparison of the LT50 between each of these three treatments and the control (non-injected animals) indicated that both the saline-saline and saline-oxamate pretreatments had significantly ($P < .05$) reduced the LT50. The apparent increased virulence of the ascites tumor that resulted after each of the treatments not utilizing FU might have been related to injection stress; however, this hypothesis was not tested.

The addition of FU to the treatment schedule significantly ($P < .05$) increased the LT 50 over the control value (Figure 3-1), even though it caused some degree of toxicity in terms of weight loss (Table 3-I). This increase was a reversal of the reduction in LT50 observed following those treatments which did not include FU. The use of the saline-oxamate pretreatment or the saline-saline pretreatment in a treatment regimen which contained FU resulted in similar increases in the LT50. These LT50s (8.8 and 9.2 days respectively) were an increase in life span of 20-25% over the non-injected controls.

Further increases in the LT50 were observed when glucose was a part of the pretreatment before FU therapy. The LT50s for the glucose-saline (10.8 days) and the glucose-oxamate (11.3 days)

pretreatments were significantly (P <. 05) different from the LT50s

obtained following the saline-saline and the saline-oxamate pretreat-

ments. Thus, the prior administration of glucose to animals being

treated with FU significantly increased the activity of the FU as

measured by the length of time until death of the animal after inocu-

lation of Walker 256 ascites tumor.

Kung et al. (1963) showed an increase in FU activity upon

Flexner-Jobling carcinoma when glucose was added to the treatment

schedule. The pH in this tumor had previously been shown to de-

crease upon the administration of glucose (Voegtlin et al., 1935).

The study reported herein has also shown an increase in FU effect

after the addition of glucose to the treatment regimen. In this in-

stance, however, the tumor utilized (Walker 256 ascites) has been

reported to increase in pHi (Schloerb et al., 1965; Hult and Larson,

1976) when the pHi was measured in another form of the tumor

(Walker 256 im tumor). Therefore, it would appear that any pH

changes within a tumor due to glucose administration might not be

the major factor influencing FU activity.

Whatever the mechanism by which FU activity was increased,

it did not lead to a corresponding increase in FU toxicity as meas-

ured by weight fluctuations. This dissociation of changes in activity

from changes in toxicity has been noted previously (Kung et al.,

1963). It would thus appear that when glucose effectively increases

FU activity within a tumor system, the increased activity is not accompanied by an increase in host toxicity. This set of circumstances would result in an increase in the therapeutic index for FU following adjunct glucose therapy.

IV. GLUCOSE DOSAGE AS A DETERMINANT OF
5-FLUOROURACIL ACTIVITY UPON
WALKER 256 ASCITES TUMOR
IN VIVO

Introduction

Results from clinical studies designed to examine the therapeutic possibilities of concurrent glucose administration in FU therapy have been reported as both positive (Lemon, 1960; Lemon et al., 1963; Lahiri, 1968) and negative (Cressy and Schell, 1965; Hall et al., 1966; Cressy and Schell, 1966). Two factors could account for this discrepancy, the disparity in the neoplastic diseases utilized for each study and the amount of glucose used in each study. Those studies which showed positive results with adjunct glucose therapy used either a greater amount of the same concentration of glucose solution (D5W [Lemon, 1960; Lemon et al., 1963]) or approximately the same amount of a more concentrated glucose solution (Lahiri, 1968) when compared to the studies which produced negative results.

Itzhaki (1972) reported that the glucose stimulated incorporation of uracil into Ehrlich ascites tumor RNA was dose-dependent upon glucose. A previous study had shown that the incorporation of FU into ribonucleotides and RNA in vitro was strongly correlated to drug response in certain murine leukemias (Kessel et al., 1966).

Thus, the dose of glucose as an adjunct to FU treatment might be important in those neoplasms which might be susceptible to a toxic mechanism based upon FU incorporation into RNA.

In the preceding study the addition of glucose to an FU treatment schedule improved the antineoplastic activity of FU upon Walker 256 ascites tumor cells. The following study was designed to determine whether the amount of stimulation of FU activity by glucose was dependent upon the dose of glucose utilized.

Methods

The animals used for this study were five-week old male rats. Seventy-two animals weighing 75-135 g were used over a two week period. Each week all the animals of correct age and within the proper weight range were pooled and six groups of six animals each randomly selected from this pool. After formation of the groups, each animal was weighed and then inoculated with 10^5 Walker 256 ascites cells by ip injection.

Treatment regimens were initiated following 72 hr. This delay before the initiation of treatment insured tumor viability in the new hosts. Animals which were treated received two ip injections. The initial injection was either a selected dose of glucose delivered in a volume of 1 ml/100 g of body weight or an equivalent volume of saline. The doses of glucose used were: 0.25, 0.50, 1.0, 3.0, 5.0,

and 10. 0 g/kg.   A second injection of 15 mg/kg FU was administered

to all treatment animals 120 minutes following the first injection.   As

in the previous study, one group of animals each week served as a

control for tumor virulence and received no treatment.

Each animal received its specified treatment daily from day 3-7

post-inoculation, and then every other day during a second week of

observation (days 9, 11, and 13).   Before each treatment the weight

and general condition of each animal was recorded.

Beginning with day five, the animals were checked for mortali-

ties every 12 hr (at 1200 and 2400).   Two weeks following inoculation,

those animals which remained were considered "cures" and the experi-

ment was terminated.

The daily weights for the animals in each treatment group were

pooled and the numerical mean calculated for tabulation purposes.

Three-day weight gain or loss was calculated for each animal and the

mean gain or loss for all animals in each treatment group was also

calculated for display purposes.   The Wilcoxon Rank Sum Test

(Wilcoxon and Wilcox, 1964) was used to determine differences among

the pretreatment groups for all weight data.   The weight data were

also compared with the data obtained from the previous study.

The mortality data were analyzed by use of Litchfield's Median

Lethal Time analysis (1949), with the time-response line plotted by

linear regression of the raw data.   For those points where more than

one group of animals was used, the time-response line was plotted to the pooled data. The resultant LT50s along with their 95% confidence limits were linearly plotted against their corresponding glucose doses and a "best fit" regression line determined. Significant differences between LT50 values were determined by an analysis of the potency ratio (Litchfield, 1949).

Results and Discussion

The weight changes for these animals were similar to those found for the animals used in the previous study (Table 4-I, Table 3-I). The rate of weight gain (5-7 g/day/animal) seen during the first 72 hr following the inoculation of the Walker 256 ascites cells continued in those control animals not exposed to FU. Exposure to FU significantly (P <.05) decreased the rate of weight gain to less than 2 g/day/animal. This reduction in rate of weight gain was not affected by any of the doses of glucose utilized as a pretreatment. When compared to previous weight data, these results suggested that none of the doses of glucose altered the toxicity of FU to the extent that the median lethal time analysis would be influenced.

The LT50 determined from the plot for the non-injected controls (7.3 days) was exactly the same as the LT50 for the non-injected animals in the previous study (Figure 4-1, Figure 3-1). This LT50 was again determined with excellent precision. The plot for the

time-response curve from which this LT50 was determined is presented in Appendix III along with the plots of the data obtained from the treatment animals.

Treatment of the animals with 15 mg/kg FU (saline-saline pretreatment) resulted in an increase in the LT50 to 8.5 days. Although it was not as great as the corresponding LT50 in the previous study (9.2 days, Figure 3-1), this increase in LT50 was still significant (P < .05) when compared to the non-injected controls.

Pretreatment of the animals with selected doses of glucose increased the LT50 to even higher levels than that seen with only FU treatment (Figure 4-1). Based upon an analysis of potency ratios, this increase became significantly different from the saline pretreatment - FU treatment LT50 when the glucose pretreatment was 3 g/kg or higher. At 10 g/kg of glucose pretreatment the LT50 had increased to almost 12.5 days. The fact that significant changes in FU activity were not apparent until the dose of glucose reached 3 g/kg underscored the possible importance of glucose dosage as a factor for the contrasting results noted in the aforementioned clinical studies.

A correlation (R=.84) was found between the pretreatment dose of glucose and the resultant LT50. That this was a positive correlation (increase in one variable increases the other) was indicated by the positive slope of the "best fit" regression line. These results suggested a strong positive relationship between the amount of glucose

Table 4-I   Weight Changes in Rats Bearing Walker 256 Ascites Tumor During Daily Treatment with Selected Doses of Glucose and 15 mg/kg 5-Fluorouracil (FU).

Treatment Regimen[a]		Number of animals	Mean Weight[b] (g)			Mean Weight Difference (g)	
Pretreatment -120 min (1 ml/100 g body wt.)	Treatment (.1 ml/100 g body wt.)		Day 0	Day 3	Day 6	Days 3-0	Days 6-3
CONTROL							
none	none	12	109.3[c]	129.8	145.8	20.5	16.0
saline	15 mg/kg FU	12	106.0	126.0	130.3	20.0	4.3[d]
.25 g/kg Glucose	15 mg/kg FU	6	108.2	128.2	131.8	20.0	3.6[d]
.50 g/kg Glucose	15 mg/kg FU	6	102.0	119.8	125.0	17.8	5.2[d]
1.0 g/kg Glucose	15 mg/kg FU	6	112.5	130.2	134.8	17.7	4.6[d]
3.0 g/kg Glucose	15 mg/kg FU	6	105.3	124.5	134.7	19.2	10.2
5.0 g/kg Glucose	15 mg/kg FU	18	111.0	128.1	130.3	17.1	2.2[d]
10.0 g/kg Glucose	15 mg/kg FU	6	103.7	123.3	123.0	19.6	-0.3[d]

[a] Three days following the ip inoculation of 10^5 ascites cells, the animals were given a daily set of two ip injections, a selected dose of glucose (0.25-10 g/kg) or saline, 120 min prior to 15 mg/kg FU.

[b] Animal weights were recorded daily and three day changes in weight calculated.

[c] Numerical means for all parameters for each pretreatment group were calculated for display purposes.

[d] Indicates weight change in a pretreatment group which is significantly different ($P < .05$) from the corresponding weight change in the control group by the Wilcoxon Rank Sum Test.

Figure 4-1   Median lethal time (LT50) for Walker 256 ascites tumor bearing rats pretreated with selected doses of glucose (.25-10 g/kg) prior to the administration of 15 mg/kg 5-fluorouracil (FU).   Both compounds were administered ip, the glucose 120 min prior to the FU.   This treatment protocol was begun three days after the animals had been inoculated with 10^5 ascites cells and was administered on the following days: 3-7, 9, 11, and 13.   LT50 values and their 95% confidence limits (indicated by brackets) were calculated according to the method of Litchfield from time-percent effect lines fitted to the data by linear regression.   Linear regression was also used to calculate the dose-response line showing the relationship between the dose of glucose and the increase in LT50.

a indicates an LT50 significantly different (P $<$ .05) from the LT50 for the group of animals receiving FU alone.

The figure in parentheses indicates the number of animals used for that determination.

y = .6401(x) + 17.9510

R = .8400

Median lethal time (days)

Dose of Glucose (mg/kg)
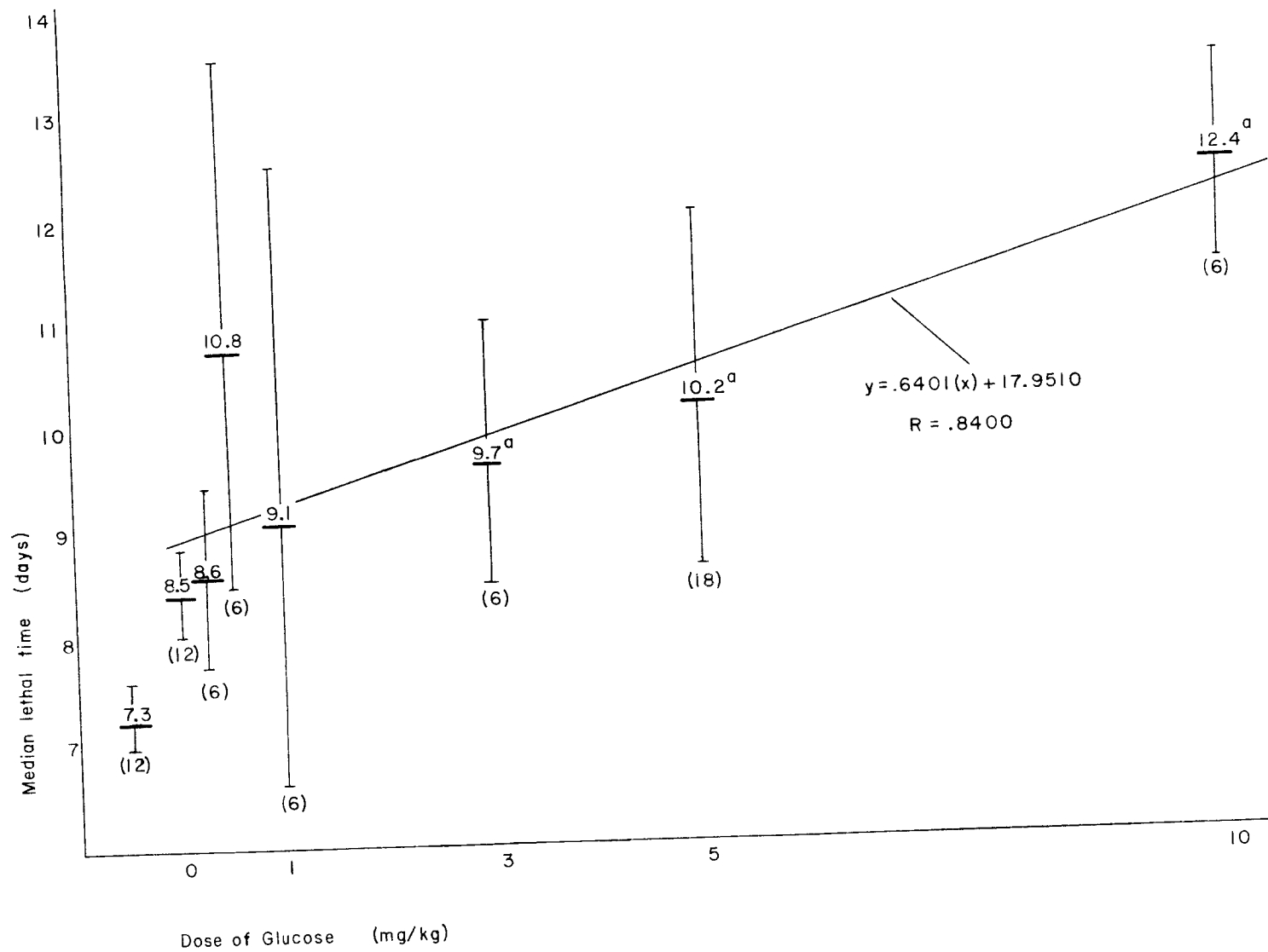
administered prior to FU therapy and the activity of that FU therapy upon the Walker 256 ascites tumor. Whether the glucose dose dependent stimulation of FU activity observed in this study is related to the glucose dose dependent stimulation of pyrimidine incorporation into RNA which has been reported (Itzhaki, 1972) deserves further investigation.

V. INTRACELLULAR pH AND 5-FLUOROURACIL UPTAKE INTO WALKER 256 ASCITES TUMOR AFTER GLUCOSE AND/OR SODIUM OXAMATE PRETREATMENT

Introduction

A previous study demonstrated an increase in FU effect upon the Walker 256 ascites tumor attributable to the prior exposure of the tumor cells to glucose. This increase in FU activity appeared to be unaccompanied by a corresponding increase in drug toxicity. Further, the magnitude of this increase in FU activity was found to be dependent upon the dose of glucose used.

The administration of a massive dose (6 g/kg) of glucose has been reported to increase the pHi of Walker 256 carcinosarcoma, in vivo (Schloerb et al., 1965). This finding was confirmed by a subsequent in vitro study which demonstrated an increase in pHi for Walker 256 tumor slices exposed to 5 mM glucose (Hult and Larson, 1976). Moreover, the in vitro study showed an increased FU uptake into the tumor slices exposed to glucose in the incubation medium. These results could be explained by the hypothesis that since FU is weakly acidic (pKa = 8.0±0.1 (Florey, 1973), drug uptake was influenced by pHi. The further addition of sodium oxamate to the incubation medium, however, resulted in an apparent dissociation of FU uptake from pHi. FU levels remained as high as those obtained when only glucose was provided while pHi was not increased, but remained

at control levels (Hult and Larson, 1976).

The following study was designed to determine whether the dissociation in FU uptake from pHi seen in the _in vitro_ study with Walker 256 tumor slices could be replicated in the Walker 256 ascites tumor model. In addition, a comparison of the results from this study with the FU activity results obtained in the earlier study would be utilized for formulating some initial conclusions concerning the relationship between pHi, FU uptake, and resultant FU activity.

Methods

Thirty male rats six weeks of age were used. These animals had been injected seven days previously with 10^5 Walker 256 ascites cells. Previous experience with the tumor during several months of serial transfer had shown that at approximately 2-3 hr before death the animal would begin to show signs of developing stress. These signs would include blanching of the ears and tail, piloerection, and fatigue. They were considered indicators of large numbers of red blood corpuscles (RBC) being lost to the abdominal cavity by internal hemorrhage. At this time abdominal swelling became quite evident. When these signs appeared, the initial injection of a specified pre-treatment regimen was administered to the animal. It was necessary to wait until this point to insure the presence of enough ascites fluid for the experimental procedure.

Six animals were used for each of the five pretreatment regimens listed in General Methods; ten experiments were run each week for three weeks. As an animal began to display the aforementioned signs, it was assigned to the next pretreatment from a randomized list of all 30 pretreatment possibilities.

The first pretreatment injection was given 90 min prior to harvesting of the ascites fluid and the second injection was made 45 min later, i. e. , 45 min prior to harvesting of the ascites fluid. At zero time the animal was killed by cervical separation. The ascites fluid was harvested and 2. 5 ml was placed into each of three culture tubes. Each of these tubes contained 0. 3 ml of solution that had been composed from the following three components:

1) 0. 1 ml of 2. 8 M 2-amino-2-(hydroxymethyl)-1, 3-
propanediol (TRIS)-Hydrochloride buffer at pH 7. 2.
(J. T. Baker Chemical Co. , Phillipsburg, NJ)

2) 0. 1 ml of heparinized saline (one I. U. of heparin)
(Eli Lilly and Co. , Indianapolis, IN)

3) 0. 1 ml (0. 25 μCi) of either
   a) ^{14}C-DMO (New England Nuclear Corp. , Boston, MA)
   b) ^{14}C-FU (Schwarz/Mann, Orangeburg, NY)
   c) ^{14}C-Inulin (New England Nuclear)

After addition of the ascites fluid to tube a (which contained DMO), the tube was stoppered and mixed thoroughly on a Vortex

mixer. Immediately following mixing, a 0.1 ml sample was drawn from the tube into a 1.0 ml disposable tuberculin syringe and injected into the sample chamber of a Radiometer Copenhagen Model BMS3 Mk 2 blood micro system (Copenhagen, Denmark). This sample was analyzed for pH, P_{O_2}, and P_{CO_2} within the DMO culture tube. No more than two minutes elapsed from the time of sacrifice until all three readings had been taken (15 sec for each reading in the order P_{O_2}, P_{CO_2}, pH). While these determinations were being made, the other two samples of ascites fluid were drawn and mixed with the contents of the other two culture tubes, b and c.

At five minutes after ascites harvesting all three culture tubes were centrifuged at 800 x g for three minutes in an IEC Model UV centrifuge (International Equipment Corp., Needham Heights, MA). The resultant supernatant was removed from all three tubes until the packed cell volume was 60% (initial value was 25-35%). The cells were then resuspended in the remaining supernatant.

Ten minutes after the initial harvesting of the ascites fluid, three heparinized microhematocrit tubes (Kimble #73810) were filled from culture tube b. This procedure was then repeated for each of the other two tubes, first a and then c. After preparing these nine samples, two drops of a 1:100 dilution of Siliclad®(Clay Adams, Parsippany, NJ) were added to culture tube c. The tube was remixed and three samples were drawn into microhematocrit tubes

which had been previously coated with the 1:100 solution of Siliclad®

according to the manufacturer's directions.

Following the preparation of these samples (25 minutes after

harvesting), the hematocrit tubes were placed in an IEC clinical

centrifuge (International Equipment Corp., Needham Heights, MA)

and centrifuged for 10 min at 1500 x g. After this centrifugation three

distinct zones had appeared in the hematocrit tube; a supernatant, a

buffy layer which consisted of approximately 50% RBC - 50% Walker

ascites cells by count, and a RBC layer which contained less than

0.5% ascites cells by count.

Thirty-five minutes after harvesting the ascites fluid, 10 mm

sections were cut from each zone of the three microhematocrit tubes

containing ^{14}C-FU. As each 10 mm section was cut from a zone, it

was placed in a scintillation vial marked for that particular zone of

the FU microhematocrit tubes. All scintillation vials contained 1 ml

of the digestant, Soluene®, (Packard Instrument Co., Inc., Downers

Grove, IL). The DMO and inulin microhematocrit tubes were handled

in the same manner. By 50 min after harvesting, the contents of

three 10 mm sections of microhematocrit tube were digesting in each

of nine scintillation vials, one vial for each of the three zones which

had appeared in each of the three sets of microhematocrit tubes.

The three Siliclad®- coated microhematocrit tubes containing

the Siliclad® ^{14}C-Inulin mixture were then sectioned in the same

manner and the 10 mm sections for each zone were placed in a separate 10 ml beaker which contained 1-2 ml normal saline. Using a small glass stirring rod, all sections of hematocrit tube in these three beakers were crushed. Siliclad®️ was used to minimize the tendency of the tumor cells to adhere both to the wall of the tube and to each other; thus, crushing of the tube liberated individual cells into the contents of the beaker.

The contents of each beaker were poured into a separate 100 ml volumetric flask. Several saline rinses from each beaker were also added to the contents of the flasks, which were then filled with saline and mixed. Depending upon which zone was involved (total cell concentration), 0.1, 0.2, or 1.0 ml of this 100 ml dilution was removed and added to 20 ml of the filtered electrolyte solution, Isoton®️, (Coulter Diagnostics Inc., Hialeah, FL) in a 30 ml beaker.

A thorough stirring prepared this sample for analysis by a TA II Coulter Counter fitted with a Population Accessory (Coulter Electronics Inc., Hialeah, FL). One-half milliliter was passed through a 100 μm aperture tube which had previously been calibrated so that a separation of the RBC from the Walker 256 ascites cells by volume was possible. After the contents of each beaker had been counted and the counts for each channel printed out, a blank consisting of 1.0 ml of saline in 20 ml of Isoton®️ was counted to determine an average background count.

Upon complete digestion of the contents of the three micro-hematocrit tube sections, 10 ml of the counting fluor, Dimilume®, (Packard Instrument Co.) were added to each scintillation vial. The radioactivity in each vial was then determined by a Packard Tricarb Model 3385 liquid scintillation spectrometer (Packard Instrument Co.) using the ^{14}C quickset. Disintegrations per minute (dpm) were calcu-lated from the resultant counts per minute (cpm), using the ^{14}C quench curve for this particular digest. From the dpm and the Coulter counter printouts both the pHi and the FU uptake for the RBC and the Walker 256 ascites cells were determined using the method outlined in the following paragraphs.

The amount of extracellular space, as measured by the ^{14}C-Inulin distribution, was calculated for both the buffy and RBC zones of the microhematocrit tubes. Then, for each of the two cell types in each zone of the microhematocrit tubes, the counts obtained from the Coulter counter were multiplied by the dilution factor for that zone to determine the total number of cells of each type in 30 mm of hematocrit tube of that zone.

Fluorouracil uptake into both cell types was then determined as follows:

1) since the RBC layer was essentially completely RBC

FU uptake by RBC = dpm in FU RBC layer - (RBC layer inulin space)

x (dpm in FU supernatant)

2)

FU uptake by ascites cells = dpm in FU buffy

- (FU in RBC) x (RBC in buffy layer)

- (buffy layer inulin space) x

(dpm in FU supernatant)

The uptake of DMO into both cell types was also determined in the same manner.

When the distribution of the weak electrolyte, DMO, between the intracellular and the extracellular water of a sample is used for the determination of pHi, three other pieces of data are needed for the determination. These three items are: the concentration of DMO in the extracellular water, the concentration of DMO in the intracellular water, and the pHe. In this case the pHe was determined directly and the extracellular concentration of DMO was found by dividing the dpm of DMO in the supernatant layer by the volume of 30 mm of microhematocrit tube. This volume was easily obtained since the average diameter of the tubes was 1.15 mm.

The intracellular concentration of DMO was calculated by dividing the dpm of DMO within the Walker 256 ascites cells of the buffy layer (which had just been determined) by the volume of the ascites cells counted in that layer. As they were counted, the ascites cells were sorted into separate channels of the Coulter counter according to their volume. Preliminary work had shown that for

multiple samples from the same animal, this volume distribution of cells remained constant within the counting capability of the instrument. Once the number of cells in each channel was determined it could be multiplied by the average volume of the particles counted by that channel. The sum of these volumes for all channels used to count ascites cells became the total volume of the ascites cells in which the calculated dpm were isolated.

Intracellular pH was now calculated by solving the following equation:

$$pHi = \underset{\text{(6.13 for DMO)}}{pKa} + \log \left\{ \frac{dpm\text{-}intracellular}{dpm\text{-}supernatant} (1 + 10^{pHe-pKa}) - 1 \right\}$$

This same procedure was utilized in calculating the pHi of the RBC.

For both cell types the FU uptake and pHi values for each pretreatment were pooled and the mean calculated for display purposes. Significant differences in FU uptake and pHi among the pretreatments were evaluated by means of the Wilcoxon Rank Sum Test (Wilcoxon and Wilcox, 1964).

This same statistical procedure was used for both display and analysis of the pHe, Po_2, and Pco_2 data.

Results and Discussion

As measured by DMO distribution, the pHi for both Walker 256 ascites cells and RBC was determined with excellent precision; the

range of values for any pretreatment group rarely exceeded 0.2 pH

unit (Table 5-I). The control (no injection) pH for Walker 256 ascites

cells (6.84) was 0.3-0.4 pH unit below the previously published pHi

values for Walker 256 im tumor (Schloerb et al., 1965; Hult and

Larson, 1976). The same method of pHi determination, however,

yielded RBC pHi values which were similar to previously reported

physiological values. Walker 256 ascites cell pHi was not affected

by pretreatment with the saline-saline or saline-oxamate injections

(Table 5-I).

Intracellular concentrations of FU in Walker 256 ascites cells

were not influenced by either the saline-saline or saline-oxamate

pretreatments either (Table 5-I). Uptake for the animals which re-

ceived these pretreatments (22.54 and 21.97 dpm of $FU/10^5$ ascites

cells respectively) was not significantly different from the uptake for

the control (no injection) animals (18.62 dpm of $FU/10^5$ cells).

The administration of the glucose-saline pretreatment resulted

in a significant (P <.05) increase in pHi of 0.18 pH unit (to pH of 7.02)

which corresponds to a 34% decrease in $[H^+]$ activity. This change

was comparable to that seen in previous studies (Schloerb et al.,

1965; Hult and Larson, 1976). The significant increase in pHi due

to the glucose-saline pretreatment was accompanied by a significant

(P <.05) increase in the amount of intracellular FU. This increase

in intracellular FU levels could be explained by the corresponding

Table 5-I   Intracellular pH (pHi) and 5-Fluorouracil (FU) Uptake in Walker 256 Ascites Cells and Red Blood Cells Following _in vivo_ Treatment with Glucose and/or Sodium Oxamate.

Treatment[a]		Number of Animals	Walker 256 Ascites Cells		RBC	
-90 min (1 ml/100 g body wt.)	-45 min (0.32 ml)		pHi[b]	FU uptake[c]	pHi	FU uptake
CONTROL None	None	6	6.84[d] (6.78-6.95)[e]	18.62 (14.63-22.51)	7.22 (7.16-7.27)	1.30 (1.16-1.43)
Saline	Saline	4	6.85 (6.81-6.91)	22.54 (22.25-22.99)	7.14[f] (7.06-7.22)	1.47 (1.22-1.85)
Saline	35 mg/ml Oxamate	6	6.84 (6.69-6.91)	21.97 (18.46-28.68)	7.09[f] (6.93-7.17)	1.28 (1.14-1.41)
5 g/kg Glucose	Saline	6	7.02[f] (6.96-7.08)	32.96[f] (28.94-39.76)	7.04[f] (6.87-7.14)	1.29 (1.16-1.36)
5 g/kg Glucose	35 mg/ml Oxamate	6	6.97[f] (6.97-7.04)	30.66[f] (25.42-42.62)	7.08[f] (6.93-7.19)	1.34 (1.17-1.45)

[a] Rats which had been inoculated ip with 10^5 Walker 256 ascites cells seven days previously were given one of four treatments prior to harvesting of the resulting ascites fluid. Each treatment consisted of two ip injections delivered at the times indicated.

[b] The pHi for both cell types was calculated from the distribution of the weak electrolyte, DMO, between intracellular and extracellular fluid following a 35 min DMO exposure.

[c] A separate aliquot of the ascites fluid from each animal was exposed to ^{14}C-FU for 35 min. Results are displayed as dpm of ^{14}C-FU per 10^5 cells.

[d] The numerical means were calculated for display purposes.

[e] Range of values.

[f] A treatment group whose values were significantly different (P < 05) from those of the corresponding control by the Wilcoxon Rank Sum Test.

increase in pHi of the ascites cells which would have facilitated the partitioning of the weakly acidic FU into the ascites cells.

Glucose-oxamate pretreatment resulted in a pHi which was still significantly different from the saline-saline and saline-oxamate pretreated cells, although this pHi was slightly reduced from the level obtained with the glucose-saline pretreatment. This result did not agree with the pHi results reported by Hult and Larson (1976) for Walker 256 im tumor slices exposed to 5 mM glucose and 40 mM oxamate. In that in vitro study, the pHi of the tumor slices following glucose-oxamate pretreatment was similar to control pHi.

Fluorouracil uptake into Walker 256 ascites cells after glucose-oxamate pretreatment reflected the change in pHi, with uptake decreasing slightly, but insignificantly (Table 5-I). This result also supported a simple partitioning hypothesis for control of FU uptake. There was no dissociation of intracellular FU levels from pHi, since the pHi and the FU uptake for those cells pretreated with glucose-oxamate remained at the level obtained for those cells pretreated with glucose-saline.

An examination of these FU uptake results with the FU activity results obtained in Chapter III of this thesis showed no dissociation of FU uptake from FU activity. Indeed, the increase in FU levels which was observed in this study following glucose administration to the ascites cells agreed with the increase in FU activity when glucose

was added to a FU treatment regimen (Figure 3-1). These results added credence to the concept that glucose mediated increases in FU uptake might influence the activity of FU upon neoplastic tissues.

The pH of the ascites fluid showed a significant decrease ($P < .05$) in those groups of animals which received any one of the four pretreatment regimens when compared to the non-injected controls (Table 5-II). The glucose-oxamate pretreatment tended to make the ascites fluid more acidic than the fluid from the saline-saline and saline-oxamate pretreated animals. Significantly ($P < .05$) more acidic than the ascites fluid from any of these groups of animals was the ascites fluid obtained from those animals pretreated with glucose-saline. The pH for this group was approximately 0.1 pH unit below that of the ascites fluid from those animals given the other pretreatments. Stevens and Lehman (1973) have reported an increase in the effect of hexamethylenetetramine (methenamine) upon Ehrlich ascites tumor when it was injected in an acidic solution. The increased effect was due to the greater concentration of the drug which remained in the more acidic ascites fluid. These authors suggested that glucose-induced reductions in pHe of the ascites fluid would have the same effect, though the variability of the pH response to the glucose might lead to more variability in the drug response. It should be noted from the results reported by these investigators, however, that a decrease in pHi relative to pHe following glucose exposure (which was not observed with the Walker 256 ascites cells in this study) might not be as important to successful therapy as the reduction in

Table 5-II pH, Po_2, and Pco_2 of Walker 256 Ascites Fluid following _in vivo_ Treatment with Glucose and/or Sodium Oxamate.

Treatment[a]		Number of Animals	Ascites Fluid		
-90 min (1 ml/100 g body wt.)	-45 min (0.32 ml)		pH	Po_2 (mm Hg)	Pco_2 (mm Hg)
CONTROL None	None	6	7.19^x (7.18-7.20)	3.2^x (00.0-08.1)	34.3^x (25.0-58.5)
Saline	Saline	4	7.11^y (7.08-7.16)	0.3^x (00.0-01.5)	31.2^x (22.6-38.1)
Saline	35 mg/ml Oxamate	6	7.11^y (7.00-7.18)	3.1^x (00.0-10.0)	39.8^x (35.0-51.3)
5 g/kg Glucose	Saline	6	6.97^z (6.91-7.06)	1.3^x (00.0-06.2)	29.8^x (23.4-36.5)
5 g/kg Glucose	35 mg/ml Oxamate	6	7.05^{yz} (6.83-7.16)	12.1^y (06.3-15.9)	38.3^x (28.2-50.8)

[a]See Table 5-1.

[b]pH, Po_2, and Pco_2 were determined following the combination of the harvested ascites fluid with a TRIS-HCl buffer (final TRIS concentration of 0.1 M) at an initial pH of 7.2. Numerical means for each parameter were calculated for display purposes. The range of values is indicated by the figures within parentheses.

[xyz]Superscript letters which are NOT alike designate treatment groups whose values were significantly different ($P < .05$) from one another.

pHe following glucose therapy (which was observed with the Walker 256 ascites).

Red blood cell pHi changes were very similar in pattern to the pHe changes of the ascites fluid (Table 5-I). The only value significantly different from the others was that of the non-injected control RBCs. Intracellular levels of FU in the RBC were not significantly affected by any of the pretreatments (Table 5-I), a dissociation of FU uptake from pHi in the RBC, since alterations in pHi did not result in alterations of intracellular FU levels.

The Pco_2 was not significantly different among any of the pretreatment groups, but when analyzed along with the corresponding pHe values, an interesting relationship was observed (Table 5-II). The lowest pHe was demonstrated in the ascites fluid with the lowest Pco_2. This indicated that the decrease in the pH of the ascites fluid following glucose-saline pretreatment was due to an increase in acidic metabolic products (possibly lactic acid) rather than acidic respiratory products (carbonic acid). Another point concerning the blood gas data was that the two groups with the highest Pco_2 values were those pretreatment groups which received sodium oxamate as part of their pretreatment. These high Pco_2 levels might have been due to a reversal of the Crabtree effect as was observed in Ehrlich ascites cells following their exposure to 40 mM oxamate (Papaconstantinou and Colowick, 1961).

The increase in Pco_2 of the ascites fluid was not mirrored by a decrease in Po_2. On the contrary, those animals which received the glucose-oxamate pretreatment regimen had significantly higher ($P < .05$) Po_2 levels in their ascites fluid. Elwood (1968) has reported an inhibition of both glycolysis and respiration in Sarcoma 37 ascites cells with oxamate concentrations higher than 80 mM. The results obtained from this study yielded preliminary indirect evidence that low concentrations of oxamate ($\cong 10$ mM) might be able to depress both glycolysis (no decrease in pHe - equivalent to less lactic acid) and respiration (increase in Po_2 of ascites fluid) following glucose stimulation of glycolysis.

Two points should be made concerning the assay for pHi within the cells of the Walker 256 ascites fluid. First, the "triple isotope" method of pHi determination as was used for the in vitro study (Hult and Larson, 1976) would have been much easier to use. Unfortunately, preliminary work showed that the use of ^{36}Cl as the marker of extra-cellular space in packed Walker 256 ascites cells produced inflated results for extracellular space with consequent high pHi. The high contamination (15% by volume) of RBC in these samples was per-ceived as the cause of the problem since ^{36}Cl is freely permeable to the RBC membrane. Various procedures were attempted in order to eliminate this contamination, including differential centrifugation, saponin, and freezing under dimethylsulfoxide protection. The

inability of any of these measures to eliminate the RBC contamination without severe compromise of ascites cell viability resulted in the development of the method of measuring pHi which was utilized in this Chapter.

The second point to be made concerns the fact that the pHe was taken at the beginning of the incubation and did not change more than 0.02 pH unit during the time required for the incubation and sectioning of the microhematocrit tubes. The ability of the ascites fluid which had been exposed to glucose as part of the pretreatment to shift the pH of the buffer upon initial combination, but thereafter shift it no further, was a cause of some concern. If it was the ascites cells in the fluid that were producing this acid and if no more acid was being produced during incubation and sectioning, there was the possibility that the cells were not surviving for the length of the experiment. A series of experiments showed that the reason for the anomaly was the absence of substrate (glucose) in the ascites fluid by the time the ascites fluid was combined with the buffer. Thus, the ascites cells were not able to produce more acid after addition of the buffer.

VI. ACTIVITY OF CYTOSINE ARABINOSIDE, CCNU, PROCARBAZINE, AND 5-FLUOROURACIL UPON EHRLICH ASCITES TUMOR AFTER GLUCOSE AND/OR SODIUM OXAMATE PRETREATMENT IN VIVO

Introduction

Ross (1961, 1964) was the first to suggest that the decrease in pHi following glucose administration which had been observed in numerous tumor systems might be conducive to more effective anti-neoplastic therapy with basic agents. Since his proposal, various authors have shown an increase in the antineoplastic activity of a number of basic agents after glucose administration (Ross, 1961; Connors et al., 1964; Stevens and Mosteller, 1969). All of these studies assumed that decreases in pHi had occurred following the addition of glucose to the tumor model being used, but none of the investigators measured pHi in these models. The studies which were described in Chapters II through V of this thesis were the first attempt to correlate pH shifts due to glucose administration with resultant drug effects.

A number of important points pertaining to FU uptake and activity emerged from the preceding studies. These points were the following: 1) Acute in vivo toxicity of FU in non-tumor bearing animals was not changed by the prior administration of glucose and oxamate. 2) The administration of glucose and/or oxamate prior to sub-acute

exposure to FU in a therapeutic regimen did not affect FU toxicity.

3) The activity of FU in an in vivo model (Walker 256 ascites tumor)
was significantly increased by prior administration of a dose of glu-
cose which also increased the levels of FU within ascites tumor cells.
4) The increase in FU activity observed with prior glucose adminis-
tration was dose related to the glucose, and 5) Fluorouracil uptake
in the Walker 256 ascites model was not dissociated from pHi.

Despite these interesting findings, the studies designed to
investigate the possible utility of the theory proposed by Ross (1961)
in antineoplastic chemotherapy used another in vivo model. There
were two reasons for the decision to change the tumor model at this
time. Firstly, because of the unique increase in pHi for Walker 256
following glucose administration, any data relating drug uptake and
effect to alterations in pHi would not be generally applicable to the
majority of neoplasms. Secondly, the size of the anticipated study,
viewed together with the limitation in animal resources, argued
against the use of a rat tumor model and for the use of a mouse tumor
model. So another tumor model was sought which might be used for
this investigation.

Poole (1967) had observed that the pHi of Ehrlich ascites cells
was decreased in vitro by the addition of glucose to the incubation
medium. She also reported a decrease in pHi for Ehrlich cells when
the cells were exposed to the LDH inhibitor, oxamic acid (Poole and

Butler, 1969). Oxamic acid had been shown to be effective upon the

LDH in Ehrlich ascites cells in vitro (Papaconstantinou and Colowick,

1961). FU was reported to be effective upon Ehrlich ascites cells

(Heidelberger et al., 1958) and the inhibition of glycolysis observed

by Woods and Burk (1969) occurred in Ehrlich ascites cells. Thus,

after some preliminary work had indicated that a suitable measure-

ment of drug activity was available, the Ehrlich ascites tumor was

chosen as the model for determining whether the pH of neoplastic

cells might have an influence upon the uptake and activity of a number

of antineoplastic drugs.

The measurement of activity was based upon the elapsed time

until abdominal distention could be visually detected after an animal

had been injected ip with a known number of ascites cells. These

cells were harvested from an animal to which one of the pretreatment

regimens outlined in General Methods had been administered. Follow-

ing harvesting the cells were incubated with either saline or one of the

selected drugs before their inoculation into a new host. Drugs used

were FU (Roche Laboratories, Nutley, NJ), cytosine arabinoside

(CA) (Cytosar®, the Upjohn Co., Kalamazoo, MI), 1-(2-chloroethyl)-

3-cyclohexyl 1-nitrosourea (CCNU) (Cancer Chemotherapy National

Service Center, Bethesda, MD), and procarbazine (PZ) (Matulane®,

Roche Laboratories, Nutley, NJ) a selection which covered acidic,

neutral, and basic portions of the pK spectrum, since CA has an

acidic pK of 4. 5 (Chou et al. , 1975), FU has an acidic pK of 8. 1

(Florey, 1973), PZ has a basic pK of 6. 76 (Roche Laboratories,

1976), and CCNU is a neutral compound (Doerge, 1976). The effects

of each of the pretreatment regimens with each of the chosen drugs

were then measured by the viability of the inoculum. Decreases in

viability, due to either death of ascites cells or a reduction in their

proliferative capacity, were manifested as increases in the time until

abdominal distention.

Methods

Five male mice which were well swollen with ascites due to the

inoculation of 10^7 Ehrlich ascites tumor cells 12 days previously

were needed for this study. Each mouse was used as an in vivo

reservoir of Ehrlich ascites cells during the pretreatment phase of

the experiment. Two hundred adult male mice were available to

receive the treated cells.

Each of the five animals carrying the ascites tumor was ran-

domly assigned to one of the five pretreatment regimens outlined in

General Methods. The first injection for each pretreatment was

administered 120 minutes prior to harvesting the ascites fluid; the

second injection was delivered 90 minutes later, i. e. , 30 minutes

before harvesting of the ascites fluid.

At zero time tha animal was killed by cervical separation.

Five milliliters of ascites fluid was harvested and transferred to a 25 ml Erlenmeyer flask which already contained 1. 0 ml of solution. This solution consisted of 10 I. U. of heparin, 320 mg of 2-amino-2-(hydroxymethyl)-1, 3-propanediol (TRIS - 2. 8 mmole), and 150 mg disodium ethylenediaminetetraacetate (EDTA) (Mallinckrodt Chemical Works, Saint Louis, MO). The flask and all other glassware used had been previously treated with a 1:100 dilution of Siliclad®. The Siliclad®, EDTA, and Heparin were measures taken in order to minimize the adherance of ascites cells to the glassware and to each other. This solution had been adjusted to pH 7. 2.

Following a five minute equilibration period, 0. 9 ml of the ascites mixture was transferred at five minute intervals to each of five, 5. 0 ml culture tubes. The first tube already held 0. 1 ml of normal saline, while the other four tubes contained a solution which consisted of 50 µg of drug dissolved in 0. 1 ml of saline. The drugs used were FU, CA, CCNU, and PZ (CCNU was dissolved in 95% ethanol). These drug solutions had been freshly prepared just prior to the harvesting of the cells.

After a 30 minute incubation at $37^{o}C$ on a Dubnoff metabolic shaker, 0. 1 ml of the ascites fluid from the initial culture tube (no drug) was injected into each of eight adult male mice. At five minute intervals the ascites-drug mixture which had incubated for 30 minutes was injected in the same manner to eight mice. When eight animals

had been injected with fluid from each of the five culture tubes, 0.1 ml from the saline incubation tube was diluted to 25 ml for counting by a Coulter counter as described in Chapter III of this thesis. This cell count served as a normalization control for the number of cells provided by the animal used for that particular pretreatment regimen.

The newly injected animals were checked at the same time daily (0900) for onset of abdominal distention. When obvious distention became evident, the number of days since inoculation was recorded. Animals which had no abdominal distention after 28 days were considered "cures" and the experiment was terminated. Cumulative data for each drug with each pretreatment regimen were maintained.

The Wilcoxon Rank Sum Test (Wilcoxon and Wilcox, 1964) was used to analyze the differences in effect between each one of the drugs and the saline control for each of the pretreatment regimens.

Differences in the effect of each drug due to the different pretreatment regimens (compared to the non-injected control for that drug) were evaluated using the Fischer Exact Probability Test (Siegel, 1956). For this test any animal which became distended later than 125% of the median time until abdominal distention for the corresponding non-injected controls was considered significantly affected.

Results and Discussion

Except for CA, 50 µg/ml concentrations of all of the drugs were effective in delaying the time until abdominal distention following every pretreatment (Table 6-I). This acidic drug was ineffective upon those ascites cells which had previously been exposed to either glucose-saline or glucose-oxamate pretreatments. All of the drugs utilized had been reported effective either clinically or experimentally in concentrations equal to or less than 50 µg/ml (Oliverio et al., 1964; Oliverio et al., 1970; Chou et al., 1975; Clarkson et al., 1964).

When the ascites cells were incubated with 50 µg/ml of CCNU, this drug was highly effective ($P < .005$) in delaying the time until abdominal distention after all of the pretreatment regimens (Table 6-I). Procarbazine and FU were also effective after all of the pretreatment regimens when compared to saline, though they were not as effective as CCNU in increasing the time until abdominal distention.

There was no significant change in the length of time until distention for either CA or CCNU treated animals after any of the pretreatments (Table 6-II). The glucose-oxamate pretreatment, however, was effective in increasing the time until distention in a significant number of animals ($P < .05$) which had received cells treated with either FU or PZ (Table 6-II). This indication of increased FU and PZ activity upon the Ehrlich ascites cells was not

Table 6-I Number of Days Required for the Production of Grossly Visible Ascites Tumors in Mice after Intraperitoneal Inoculation with Ehrlich Ascites Cells Which Had Been Pretreated in vivo with Glucose and/or Sodium Oxamate and Treated with Selected Drugs.

Pretreatment[a]		Drug Treatment[b]				
-120 min (1 ml/100 g body wt.)	-30 min (0.32 ml)	Saline CONTROL	FU	(50 ug/ml for 30 min) CA	CCNU	PZ
none	none	9.8^c $(8)^d$	10.5^e (8)	10.8^e (8)	20.8^e (8)	11.4^e (8)
saline	saline	10.4 (8)	11.0^e (8)	11.3^e (8)	14.2^e (8)	12.1^e (8)
saline	35 mg/ml Oxamate	9.9 (8)	11.5^e (8)	13.4^e (8)	16.0^e (8)	11.8^e (8)
5 g/kg Glucose	saline	9.9 (8)	11.9^e (8)	10.4 (7)	16.8^e (8)	15.4^e (7)
5 g/kg Glucose	35 mg/ml Oxamate	11.0 (7)	12.4^e (8)	11.4 (8)	18.3^e (8)	19.9^e (8)

[a] ip injections were administered at the times indicated and in the volumes shown to animals bearing Ehrlich ascites tumor.

[b] Ascites fluid from each pretreatment animal was combined with a TRIS-HCl buffer fortified with Heparin and EDTA (initial pH 7.2) and the resultant suspension was incubated with 5-Fluorouracil (FU), cytosine arabinoside (CA), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), procarbazine (PZ), or saline.

[c] Mean time in days until grossly visible abdominal distention for animals injected with 0.1 ml of the pretreated ascites-drug suspension. This figure was calculated for display purposes.

[d] Number of animals used in the determination.

[e] A group of animals inoculated with drug-treated ascites cells which had a significantly ($P < .05$) greater length of time until abdominal distention when compared with the corresponding saline-treated cells from the same pretreatment animal using the Wilcoxon Rank Sum Test.

Table 6-II   Variations in the Activity of Selected Drugs upon Ehrlich Ascites Tumor Cells Following in vivo Pretreatment with Glucose and/or Sodium Oxamate.

Pretreatment[a]		Saline	Drug Treatment[b]			
-120 min (1 ml/100 g body wt.)	-30 min (0.32 ml)		(50 ug/ml for 30 min)			
			FU	CA	CCNU	PZ
CONTROL none	none	9.28[c] 0/8[d]	9.55 0/8	9.87 1/8	13.80 4/8	9.83 0/8
saline	saline	1/8	1/8	3/8	1/8	1/8
saline	35 mg/ml Oxamate	0/8	2/8	3/8	2/8	1/8
5 g/kg Glucose	saline	2/8	1/8	0/7	2/8	3/7
5 g/kg Glucose	35 mg/ml Oxamate	1/7	4/8[e]	2/8	2/8	4/8[e]

[a]See Table 6-I.

[b]See Table 6-I.

[c]Median time in days until grossly visible abdominal distention for eight animals injected with 0.1 ml of the ascites-drug mixture after no in vivo pretreatment of the ascites cells. Drug-treated ascites cell inocula were considered significantly affected by the pretreatment when the animals injected had not displayed signs of abdominal distention at 125% of the median time for the corresponding non-injected controls.

[d]Number in group significantly affected/total number in group.

[e]A group where significantly more animals were affected (P <.05) than in the corresponding control group by the Fischer Exact Probability Test.

observed when the cells had received the other pretreatments, including glucose-saline. Therefore, the addition of sodium oxamate to the glucose pretreatment regimen was effective in significantly increasing the activity of both FU and PZ upon Ehrlich ascites tumor cells.

Previous studies had reported that the concurrent administration of glucose enhanced the antineoplastic effect of FU (Kung et al., 1963; Woods and Burk, 1969; Chapter III). This, however, is the first study to demonstrate a significant increase in FU activity following the prior administration of glucose and another agent under conditions where the administration of glucose alone had shown no enhancement of FU activity. Moreover, a previous study in this thesis (Chapter III), indicated that the other agent, ~10 mM sodium oxamate, was not effective in increasing the glucose mediated enhancement of FU effect upon Walker 256 ascites. Whether the enhancement of FU activity upon Ehrlich ascites cells following glucose-oxamate pretreatment which was observed in the present study was due to an enhancement of the mechanism by which glucose usually simulates FU activity or whether there was an entirely new mechanism involved should be explored.

VII. INTRACELLULAR pH AND DRUG UPTAKE INTO EHRLICH ASCITES TUMOR CELLS FOLLOWING GLUCOSE AND/OR SODIUM OXAMATE PRETREATMENT

Introduction

The Ehrlich ascites tumor had been chosen for the previous study which dealt with the relationship between glucose and/or sodium oxamate pretreatment and drug effect partially because of the pHi work done by Poole (Poole, 1967; Poole and Butler, 1969) and the FU uptake studies done by Woods and Burk (1969). However, in order to complete the investigation of the relationship among glucose and/or oxamate pretreatment, pHi, drug uptake, and drug effect, it was necessary to determine both the pHi of Ehrlich ascites cells and uptake of the various drugs utilized in the activity study. These determinations had to be done under conditions as close as possible to those used in determining the _in vivo_ activity of these drugs. This study was designed to determine drug uptake for FU, CA, CCNU, and PZ along with pHi values in Ehrlich ascites tumor cells for comparison with the results obtained in Chapter VI.

Methods

Thirty adult male mice which had been inoculated with Ehrlich ascites carcinoma cells 15-18 days previously were used for this study. Due to the accumulation of ascites fluid, these animals

weighed 40-48 g.

As each animal was needed, it was assigned to the next pre-treatment regimen from a previously randomized list. Six animals were given each of the five pretreatments described in General Methods; ten experiments were run each week for three weeks. The first injection in the pretreatment regimen was made 120 min prior to harvesting of the cells. The second injection was made 30 min prior to harvesting of the cells. In addition to the pretreatment regimen, each animal (including the non-injected controls) received a 0.2 ml injection which contained 10 mg EDTA and 2 I.U. of heparin two minutes before the ascites cells were harvested. This was done to minimize the clustering of the ascites cells with each other.

The animals were killed by cervical separation and the ascites fluid harvested. This fluid was washed in three times its volume of a TRIS-phosphate buffer at pH 7.2 (the composition of the buffer is presented as Appendix IV). The buffer contained EDTA and hyaluronidase (Nutritional Biochemicals Corp., Cleveland, OH) to further minimize cell stickiness and the resultant adherance of cells to each other. For those animals which had been pretreated with glucose-saline or glucose-oxamate, glucose was added to the buffer solution to a concentration of 1.0 mg/ml.

The cell suspension was centrifuged at 800 x g for two minutes in an IEC Model UV centrifuge, the supernatant was removed, and the

pellet was resuspended in ten volumes of the TRIS-phosphate buffer.
This final cell suspension (20-30 ml) was placed in a 50 ml beaker
upon a Dubnoff metabolic shaker set at a temperature of $37^{\circ}C$. As
in the previous study, this beaker and all of the other glassware had
been previously coated with a 1:100 dilution of Siliclad®according to
the instructions of the manufacturer.

Following a five minute equilibration (15 min after harvesting)
2.9 ml of the ascites suspension was transferred to each of six, 5.0
ml culture tubes. Each tube contained 0.1 ml of one of the following:

1) ^{14}C-Inulin (0.1 μCi) dissolved in saline (New England

Nuclear, Boston, MA)

2) ^{14}C-DMO (0.1 μCi) (New England Nuclear) and

$^{3}H_2O$ (0.5 μCi) (New England Nuclear) dissolved in saline

3) ^{14}C-FU (0.1 μCi) dissolved in saline (Schwarz/Mann,

Orangeburg, NY)

4) ^{3}H-CA (0.2 μCi) dissolved in saline (New England Nuclear)

5) ^{14}C-CCNU (35.83 μCi/mg) dissolved in 0.05 ml 95%

ethanol and 0.05 ml saline

6) ^{14}C-PZ (27.65 μCi/mg) dissolved in saline

The CCNU and PZ were supplied by Dr. Donald Reed, Depart-
ment of Biochemistry, Oregon State University. Solutions of PZ and
CCNU were made just prior to the addition of the ascites suspension
by placing a minute amount of the radioactive drug in the culture tube

and then dissolving it in 0.1 ml of the solvent indicated above. This procedure was followed in order to minimize the decomposition of these two compounds.

The contents of the tubes were mixed with a Vortex mixer and placed on the Dubnoff metabolic shaker for a 15 min incubation at $37^\circ C$. Every five minutes the tubes were thoroughly remixed with the Vortex mixer. Just before termination of the incubation, the pH of the cell suspension remaining in the beaker was determined using the Radiometer Copenhagen blood micro system.

Following the 15 minute incubation, the contents of each of the six culture tubes were poured into a separate 15 ml polycarbonate centrifuge tube. The six centrifuge tubes were centrifuged for one minute at 5600 x g in a Lourdes Model A-2 Betafuge (Lourdes Instrument Co., Brooklyn, NY).

Starting at 40 minutes after harvesting of the cells, 0.1 ml of the supernatant from each centrifuge tube was placed into a separate, tared scintillation vial containing 1.0 ml Soluene® digestant and 50-100 mg of an Ehrlich ascites pellet which had been produced by centrifugation of fresh, dilute ascites fluid at 5600 x g for one minute. This pellet was included in the digest of the supernatant sample to provide a constant quench for counting purposes. Immediately upon removal of the supernatant sample, the balance of the supernatant was decanted and the tube inverted in a test tube rack for drainage.

This process was done at a one tube per minute rate for six minutes using the same tube order in all 30 experiments.

Following the same tube order and rate, each centrifuge tube was wiped completely dry above the pellet with a Kimwipe® and the pellet then was removed by means of a spatula. This pellet was placed in a tared scintillation vial containing 1.0 ml Soluene®. All 12 vials were now reweighed to obtain the weight of the sample contained in each vial.

Upon complete digestion of the tissue (6-8 hr at 55°C), 10 ml of Dimilume® counting fluor was added to each vial and samples were counted for ten minutes. Two channels of a Packard Tricarb Model 3385 liquid scintillation spectrometer were used, one for counting tritium and one for counting carbon-14. The tritium channel was set for 100% gain with the window at 40-1000 while the carbon-14 channel was set for 5% gain with the window at 85-1000.

Standard curves for various weights of Ehrlich ascites cell pellets (50-120 mg) in 1.0 ml Soluene® and 10 ml Dimilume® had already been determined for both isotopes. For the single labelled samples, dpm were calculated directly from cpm using these standard curves. The channels ratio method (Hendler, 1964) was used to separate the counts due to tritium from the counts due to carbon-14 in the one dual-labelled sample.

The dpm for all samples were converted to dpm per 100 mg

sample using the sample weights calculated above. The resultant

^{3}H-saline, ^{14}C-DMO, and ^{14}C-inulin dpm for both supernatant and

pellet, along with the pHe value, were inserted into the pHi equation

developed by Schloerb et al. (1965 - reproduced in Appendix V). The

ensuing pHi values for all six animals of each pretreatment regimen

were pooled and the mean calculated for display purposes. Significant

differences among the sets of values were determined by using the

Wilcoxon Rank Sum Test (Wilcoxon and Wilcox, 1964).

For presentation of the drug uptake data, the dpm of drug per

100 mg of sample were converted to dpm per 100 mg of packed cells,

using the figure for extracellular space which had been calculated

from the ^{14}C-inulin distribution. Then, in order to normalize for

differing amounts of label (drug) in each experiment, the dpm/100 mg

of ascites cells were divided by the dpm/100 mg of supernatant and

the results expressed as this ratio. The six ratios calculated for

each pretreatment were pooled for each of the four drugs and a mean

calculated for display purposes. The Wilcoxon Rank Sum Test was

again used to test for significant differences among the pretreatment

groups.

Results and Discussion

The mean control (non-injected) pHi in Ehrlich ascites cells as

measured by DMO distribution was 7.46 (Table 7-1). This was

approximately 0.2 pH unit higher than that reported by Poole (1967).

The pHi within the ascites cells was significantly (P <.05) reduced

by approximately 0.15 pH unit when either the saline-saline or saline-

oxamate regimens were administered to the animal. An even greater

reduction in pHi accompanied the administration of glucose-saline as

the pretreatment schedule. The mean pHi observed following this

pretreatment (7.08) was 0.38 pH unit below that of the non-injected

control animals. This decrease in pHi corresponds to an increase in

$[H^+]$ activity of almost 60%. Both glucose and sodium oxamate in the

pretreatment regimen resulted in a pHi which was only slightly lower

than with the saline-oxamate regimen (Table 7-1). These pH values

were significantly (P <.05) higher, however, than the pHi of those

Ehrlich cells exposed to glucose-saline as the pretreatment. Thus,

glucose by itself appeared to be more effective in reducing pHi than

the combination of glucose and the LDH inhibitor, oxamate. This

finding is in contrast to the results reported by Poole and Butler

(1969). They observed similar decreases in pHi with either glucose

alone or glucose plus sodium oxamate in their in vitro incubation.

The FU uptake into the ascites cells was significantly increased

when the cells were exposed to glucose as part of the pretreatment

regimen (Table 7-I). This increase in intracellular FU concentra-

tions of approximately six-fold was enhanced by the addition of sodium

oxamate to the pretreatment regimen. Thus, as was found in the case

Table 7-I   Intracellular pH (pHi) and the Uptake of 5-Fluorouracil (FU), Cytosine Arabinoside (CA), 1-Cyclohexyl-3-(2-chloroethyl)-1-nitrosourea (CCNU), and Procarbazine (PZ) in Ehrlich Ascites Tumor Cells Following _in vivo_ Treatment with Glucose and/or Sodium Oxamate.

Treatment[a]		Number of Animals	pHi[c]	Drug Uptake Ratio[b] (Drug/100 mg tissue / Drug/100 mg supernatant)			
-120 min (1 ml/100 g body wt.)	-30 min			FU	CA	CCNU	PZ
none	none	6	7.46^d 7.29-7.76^e	1.36 0.89-1.94	2.34 1.34-3.35	29.8 23.2-34.7	1.48 1.27-1.81
saline	saline	6	7.33^f 7.16-7.59	1.55 1.09-3.34	2.61 1.48-4.37	25.0 18.0-38.4	1.53 1.17-1.81
saline	35 mg/ml Oxamate	6	7.31^f 7.20-7.38	1.72 1.42-2.13	2.73 1.57-5.06	28.5 21.4-36.6	1.47 0.90-1.83
5 g/kg Glucose	saline	6	7.08^g 6.98-7.20	11.84^f 4.01-19.44	2.70 1.79-3.02	25.8 13.0-38.7	1.61 1.00-2.30
5 g/kg Glucose	35 mg/ml Oxamate	6	7.25^f 7.07-7.44	13.10^f 8.75-19.92	1.99 0.47-3.49	28.8 19.1-47.6	1.94^f 1.70-2.14

[a] Mice bearing grossly visible Ehrlich ascites tumor were given two ip injections at the times indicated and of the volumes shown.

[b] Harvested ascites cells were washed and resuspended in a TRIS-PO$_4$ buffer at pH 7.2.   Uptake during a 15 min exposure to one of the various drugs (radioactively labelled) is displayed.

[c] pHi was calculated from the distribution of the weak electrolyte, DMO, between extracellular and intracellular fluid during a 15 min exposure.

[d] Means for all parameters were calculated for display purposes.

[e] Range of values.

[f] A treatment group significantly (P <.05) different from the control group by the Wilcoxon Rank Sum Test.

[g] A treatment group significantly (P <.05) different from all other groups by the Wilcoxon Rank Sum Test.

of Walker 256 im carcinosarcoma slices (Hult and Larson, 1976),

Ehrlich ascites tumor cells showed a dissociation of FU uptake from

pHi. The addition of glucose to the pretreatment schedule significantly

reduced pHi, but also significantly increased the intracellular FU

concentration. This result was in direct opposition to a partitioning

hypothesis since a lower pHi should have led to lower intracellular

levels of the weakly acidic FU. Glucose-oxamate pretreatment re-

sulted in a slight reduction (insignificant) in pHi when compared to the

pHi values obtained with either the saline-saline or saline-oxamate

pretreatments, yet the FU uptake for glucose-oxamate pretreated

cells remained at least six-fold higher than the corresponding uptake

for the saline-saline or saline-oxamate pretreatments.

The increased FU uptake observed in this study was reflected

in a significant increase in drug effect only in those ascites cells

from the animal administered the glucose-oxamate pretreatment

(Table 6-II). Pretreatment with the glucose-saline regimen did not

result in a significant increase in drug effect even though the present

study showed FU levels to be increased. Thus, the apparent dissocia-

tion of FU uptake from pHi within Ehrlich ascites cells was further

compounded by the dissociation of FU activity from FU uptake.

The uptake of PZ was significantly increased (P <. 05) by the

glucose-oxamate pretreatment (Table 7-I). Here again the shift in

drug uptake did not correlate with the shift in pHi, a dissociation of

drug uptake from pHi. In this case the significant increase in drug uptake occurred after the glucose-oxamate pretreatment, a pretreatment which caused no shift in pHi. Despite the pHi fluctuation, the PZ uptake after glucose-saline pretreatment was essentially unchanged. Here the effects of the pretreatment regimens upon drug activity (time until abdominal distention, Table 6-II) reflected the uptake of the drug, with the only significant increase in PZ effect upon Ehrlich ascites cells occurring following glucose-oxamate pretreatment.

None of the pretreatments altered the uptake of CCNU into the Ehrlich ascites cells (Table 7-I). Intracellular pH changes had no influence upon the uptake of this drug as might have been expected since CCNU is a neutral nonionized compound. Its uptake ratio was consistently greater than any of the other drugs employed, probably due to its high lipid solubility. This large drug uptake ratio was mirrored by drug effect (Table 6-I), which was much greater for CCNU than for any of the other drugs at any of the pretreatments except for PZ activity following glucose-oxamate pretreatment. After this pretreatment a PZ cell/plasma concentration ratio of approximately 2.0 achieved similar effects upon the Ehrlich ascites cells to the effects observed with CCNU cell/plasma concentration ratios of 24-28 (Table 7-I). This indicated that the amount of drug in the cells was not the only factor to consider when evaluating the potential effects upon drug activity. Also to be considered was the activity of

the drug once within the cell.

CA uptake was also unchanged following any of the pretreatment regimens (Table 7-I). This corresponded with the similarity of the CA effects upon the ascites cells shown in the last Chapter. Of interest here was the fact that the alteration in pHi due to glucose-saline pretreatment had no effect upon either the CA uptake or CA activity. Thus, the uptake of CA was more dependent upon other factors than the changes in pHi of the Ehrlich ascites cells.

As mentioned above, in direct contrast to the work of Poole and Butler (1969), the pHi within the ascites cells in this study was not reduced by the administration of glucose-oxamate to the pH levels obtained by the administration of glucose-saline. The advantage of this dual therapy, mentioned in the General Introduction, would be the slower decrease in pHe because of the intracellular formation of acidic phosphorylated glycolytic intermediates instead of the freely diffusable lactic acid. Therefore, if partitioning of the drug into the cell were a factor, the less acidic nature of pHe for at least the first 20-30 minutes following oxamate administration would tend to favor the partitioning of basic drugs into the cell and not just into the region of the tumor. Whether this inability to alter the pHi by glucose and oxamate was due to a dosage differential for the oxamate (Poole and Butler used 40 mM, while the concentration in these studies was about 10 mM) or whether it was due to some type of buffering capacity

for the phosphorylated intermediates by the Ehrlich ascites cells

in vivo deserves further study.

The method used in this study for the determination of pHi and

drug uptake was similar to the method utilized by Poole for her in

vitro incubations (Poole, 1967; Poole and Butler, 1969). The method

developed in Chapter V for pHi and drug uptake into Walker 256 ascites

cells was tried initially, but it was discovered that the stickiness of

the Ehrlich ascites cells as compared to the Walker 256 ascites cells,

precluded their being separated for counting in the Coulter counter as

was required in that method. The incubation medium used in this

method (TRIS-phosphate buffer with EDTA and hyaluronidase) was

designed to minimize the cellular aggregation, but this process still

occurred to a slight extent. The minimal clustering which occurred

did not seem to affect this method of analysis for pHi and drug uptake.

VIII. SUMMARY AND CONCLUSIONS

A number of important points with regard to the use of glucose and/or sodium oxamate as adjuncts in antineoplastic therapy have become apparent from the series of studies reported herein. To briefly summarize, these points were: 1) Acute _in vivo_ toxicity of FU was not changed by the prior administration of glucose and oxamate in non-tumor bearing animals. 2) The administration of glucose and/or oxamate prior to sub-acute exposure to FU in a therapeutic regimen did not affect FU toxicity. 3) The administration of glucose prior to FU therapy led to both higher FU levels within Walker 256 ascites cells and significant increases in FU activity upon Walker 256 ascites tumor. 4) Glucose induced enhancement of FU activity was dose related. 5) Fluorouracil uptake into Walker 256 ascites cells (and resultant FU activity), in contrast to earlier results obtained with Walker 256 im tumor (Hult and Larson, 1976), followed a pattern predictable from the pHi and the pK of the drug. These observations were drawn from the experiments outlined in Chapters II through V and pertained specifically to FU activity upon Walker 256 ascites tumor.

In addition, the studies with Ehrlich ascites tumor suggested the following: 1) A number of antineoplastic drugs were probably more dependent upon factors other than pHi for their intracellular

concentration. 2) Among these factors, the state of the cell metabolism might be of importance (i. e., has the glycolytic metabolism been stimulated or inhibited?. . . has nucleic acid metabolism been stimulated or inhibited?) 3) Glucose enhancement of intracellular FU accumulation did not necessarily lead to increased activity of the drug in vivo. 4) A neutral compound such as CCNU was incorporated into the neoplastic cells similarly at all pH values observed, and similar CCNU activity within the in vivo model mirrored this uniformity of uptake. 5) A concentration of 10 mM sodium oxamate was not effective in lowering the pHi in Ehrlich ascites cells, in contrast to earlier results obtained with 40 mM oxamate (Poole and Butler, 1969).

It should be noted at this point that whenever intracellular drug concentrations were measured, what was actually measured was the radioactive label and not the concentration of the intact drug. Although minimal times were utilized for equilibration of the substance, both anabolic and catabolic processes as well as spontantous degradative processes might have occurred leading to a diffusion of the label among many moieties. Since the same time intervals were used for all samples, the results can be equated as long as the point stated above is kept in mind.

The addition of glucose to the treatment schedule of various antineoplastic compounds was shown to be of beneficial therapeutic effect in a number of animal tumor systems (Ross, 1961; Kung et al. , 1963; Connors et al. , 1964; Stevens and Mosteller, 1969). Presumably the increased drug activity, as measured by inhibition of tumor growth, was attributable to a decrease in tumor pH which had been caused by the glucose. Since most of the drugs utilized were basic compounds, a decrease in tumor pH would have led to a greater uptake of the drug in question with a subsequent increase in drug activity. All of the investigators suggested that this mechanism might be the reason for the increase in drug activity which they had seen, and at least one (Ross, 1961) recommended that new antineoplastic drugs should be designed which were more effective in acidic media. Indeed, some compounds were subsequently designed with the recommendation of Ross in mind (Papanastassiou et al. , 1966; Papanastassiou et al. , 1967; Stevens and Lehman, 1973). No study has appeared, however, which was designed to determine whether a cause and effect relationship among glucose administration, tumor pH, drug uptake, and drug activity in fact existed.

One of the drugs which showed greater activity upon experimental tumors when glucose was added to its treatment schedule was the weakly acidic FU (Kung et al. , 1963). This report became the basis for a clinical discussion concerning the merits of adjunct

glucose in FU therapy. Various investigators found that the addition of glucose to FU therapy was either beneficial (Lemon, 1960; Lemon et al., 1963; Lahiri, 1968) or inconsequential (Cressy and Schell, 1965; Hall et al., 1966; Cressy and Schell, 1966). This discussion seemed to end when Kessel and Hall (1969) reported that while glucose did increase the amount of intracellular FU in numerous experimental tumors, there was no corresponding increase in antineoplastic effect. The increase in tumor intracellular FU levels following glucose exposure was ascribable to glucose stimulation of FU incorporation into ribonucleotides and RNA. The rate of FU uptake into the cell was not changed, but was still dependent upon simple diffusion as had been previously shown by Jacquez (1958). Because the stimulation of FU anabolism into RNA lowered free FU levels more FU was able to diffuse into the cell. These results overshadowed earlier work from the same laboratory which had indicated that the incorporation of FU into RNA was directly correlated to drug activity in certain murine leukemias (Kessel et al., 1966).

The implication was clear. Various investigators had demonstrated that the major determinant of FU cytotoxicity was the inhibition of DNA synthesis by 5-fluoro-2'-deoxyuridine-5'-monophosphate, a competitive inhibitor of thymidylate synthetase (Danneberg et al., 1958; Lindner, 1959). Although glucose had increased the intracellular concentration of FU, the drug was being channeled into an anabolic

pathway which was of minor consequence to its antineoplastic activity;
i.e., the inhibition of RNA synthesis.

The data reported herein support the hypothesis that glucose
can increase the intracellular concentration of FU in neoplastic
tissues. This effect upon FU concentration was not attributable to
an alteration in pHi by the glucose. In Walker 256 ascites cells the
administration of the glucose-saline pretreatment increased the pHi
of the cells, supporting data obtained previously (Schloerb et al.,
1965; Hult and Larson, 1976). In contrast, the pHi for Ehrlich ascites
cells decreased upon the administration of glucose-saline as had also
been previously noted (Poole, 1967). Yet the intracellular concentra-
tion of FU following this pretreatment was increased in both tumor
systems.

Pretreatment of either tumor model with glucose-oxamate also
resulted in increased intracellular FU levels. In the Ehrlich ascites
tumor cells this increase occurred despite a pHi which was compar-
able to control values, a further indication that pH changes were not
influencing intracellular FU levels.

The FU activity upon these two tumor models did not always
correlate with the uptake data. For the Walker 256 ascites tumor
cells the increase in FU concentration after glucose-saline adminis-
tration was accompanied by an increase in drug activity upon the
tumor. This increase in FU activity following glucose-saline

pretreatment was not demonstrated by the Ehrlich ascites tumor even though the FU uptake in this tumor system was increased six-fold over control levels. In contrast, the Walker 256 ascites cells showed only a two-fold increase in FU uptake. It is of interest to note that the significant increase in activity occurred in that tumor system (Walker 256) where the pHi of the cells increased, thus arguing against the enzyme activation hypothesis of Kung et al. (1963) which was based upon the ability of glucose to reduce pHi.

The prior administration of glucose and oxamate to Walker 256 ascites cells resulted in FU activity similar to that associated with glucose-saline pretreatment. Thus, the higher levels of FU within the cell following both the glucose-saline and the glucose-oxamate pretreatments were mirrored by increases in the FU activity upon the tumor. These increases in FU activity were not accompanied by increases in FU toxicity to the host. Adjunct glucose therapy might thus be expected to increase the therapeutic index of FU in those instances where it is effective in enhancing FU antineoplastic activity.

Ehrlich ascites cells from donor animals pretreated with glucose and oxamate were more susceptible to FU than those cells exposed to any of the other pretreatments. This increase in FU activity happened despite the fact that FU levels in Ehrlich ascites cells following glucose-oxamate pretreatment were similar to those found following glucose-saline pretreatment. These results suggested

that not only was FU uptake independent of pHi as was mentioned

above, but FU activity was independent from intracellular FU levels.

The addition of sodium oxamate to the treatment schedule appeared to

stimulate factors which enhanced the cellular response to the high

levels of FU associated with glucose exposure: i. e. , there was a

difference in the susceptibility of the cell to the cytotoxicity of a

particular amount of the drug.

The concept of differing susceptibility was shown by a study

which indicated that HeLa cells were more susceptible to a combina-

tion of glucose and oxamate than Walker 256 tumor (Nagarajan and

Colowick, 1969). The glucose depleted available ATP through stimu-

lation of glycolysis and the resynthesis of ATP was blocked by oxamate

in HeLa cells, but Walker 256 had other methods of producing ATP

and thus was not as affected. The fact that oxamate-glucose pre-

treatment did not have any significant effect upon Walker 256 in this

study supports that data, and the relative susceptibility of Ehrlich

ascites cells to glucose-oxamate as opposed to glucose-saline may

be an indication a similar mechanism was at work in the Ehrlich

tumor system.

Differing susceptibility of a neoplasm to a particular toxic

mechanism might also be used to explain the contradictory results

which have been demonstrated when glucose was added to FU therapy.

Those investigations which reported the inhibition of DNA synthesis

as the toxic mechanism for FU (Danneberg et al. , 1958; Lindner,
1959) indicated that incorporation of FU into fraudulent RNA was
also toxic to neoplastic cells, but only at FU concentrations substanti-
ally higher than those required for inhibition of DNA synthesis. Ad-
junct glucose therapy might be increasing the amount of fraudulent
RNA to the levels needed for cytotoxicity.

It should be noted that FU incorporation into RNA was not
measured in these studies. Until this work is done, it might be
argued that in susceptible cells glucose was enhancing the progress
of FU along the anabolic pathway leading to the inhibition of DNA
synthesis by the deoxyribonucleotide. The literature at this time
(Kessel et al. , 1966; Kessel and Hall, 1969; Itzhaki, 1972) argues
against this view as glucose administration to tumor systems under-
going FU therapy has led to a stimulation only of the incorporation of
FU into RNA.

The incorporation of FU into ribonucleotides might be only an
intermediate step in the toxic mechanism. Woods and Burk (1969),
in contrast to an earlier report by Bosch et al. (1958), demonstrated
that FU was an effective inhibitor of glycolysis. Glucose was a
necessary requirement for this inhibition. The inhibition appeared
to be an increase in the Pasteur effect as there was no discernible
change in the levels of activated phosphate. Using a series of pyrim-
idines, both halogenated and non-substituted, Woods and Burk found

that the amount of inhibition correlated well with earlier reports

of the in vivo cytotoxic activity of these compounds.

It was suggested by these authors that the inhibition of glycolysis

by pyrimidines in the presence of glucose might be due to either a

glucose mediated reduction in pHi which would effect the enzymes

of glycolytic metabolism, stimulation of glycolytic metabolism with

resultant trapping of inorganic phosphate, or an unknown mechanism.

The first of these hypotheses was similar to Kung's hypothesis con-

cerning the possible effects of glucose upon the lethal synthesis of FU

to the deoxyribonucleotide. As mentioned above in connection with

this latter hypothesis, the increase in FU activity upon Walker 256

ascites cells which was shown in a system where the pHi was also

increasing does not support this type of hypothesis.

To test the second hypothesis, Woods and Burk added inorganic

phosphate to their in vitro system and discovered that the inhibition

of glycolysis was reversed. A logical hypothesis based upon this

observation and others enumerated earlier is that glucose does indeed

increase intracellular FU, probably by acting as a ribose donor as

suggested by Kessel and Hall (1969) and thus increases the rate of FU

transformation into ribonucleotides with subsequent incorporation into

RNA. This process would use available supplies of inorganic phos-

phate through the formation of the ATP needed for the phosphorylation

of the pyrimidine nucleoside. An inhibition of glycolysis would occur

due to a lack of phosphate substrate and in spite of the relatively low levels of cellular ATP. Sodium oxamate might possibly add to this inhibition either directly (inhibition of LDH) or indirectly (further depression of ATP levels).

The antineoplastic activity of FU which is attributable to the inhibition of DNA synthesis might thus be enhanced by glucose in one of two ways. One of these would be an increase in the amount of fraudulent RNA to the extent that it also becomes part of the toxic picture at comparable FU concentrations. The other would be an inhibition of glycolysis. Relative susceptibility of individual neoplasms to either of these mechanisms as compared to inhibition of DNA synthesis by FU would determine the relative success of adjunct glucose therapy. Further work is needed to determine whether either or both of these mechanisms are responsible for the increases in FU activity which have been seen in vivo.

Negative and positive therapeutic effects have now been described for the addition of glucose to FU in neoplastic therapy. This dichotomy has occurred in both animal models (Kung et al., 1963; Kessel et al., 1966; Kessel and Hall, 1969; Sections III and VI of this thesis) and in the clinic (Lemon et al., 1963; Cressy and Schell, 1965; Crdssy and Schell, 1966; Hall et al., 1966; Lahiri, 1968). In commenting upon the reasons for these contradictory results the following points might be made.

Initially it can be stated that the addition of glucose to a FU treatment schedule probably does lead to an increased intracellular concentration of FU. This increase in intracellular FU levels is not dependent upon pHi within the cells. Moreover, the high intracellular concentrations of FU produced by the administration of glucose need not result in an increase in effect of the FU. Whether the cytotoxic effect of FU in such situations is due to increased amounts of fraudulent RNA, inhibition of glycolysis, or some other mechanism, it is the susceptability of the particular tumor under study to this toxic mechanism that will determine whether an enhancement of FU activity is seen. This concept becomes significant when applied to the clinical studies noted above since each one utilized a variety of tumor systems.

The dose of glucose appears to be an important determinant of the results obtained. The fact that the activity of FU upon Walker 256 ascites cells was dependent upon the dose of glucose (Section IV) agreed with the clinical experience of Lahiri (1968) and argued against the results obtained from those clinical studies which showed negative effects. As noted previously these studies used smaller amounts of glucose than those which reported positive effects.

The addition of other substances to the therapeutic protocol might or might not lead to the desired effect. The concurrent administration of sodium oxamate along with glucose should have resulted in a reduction of the pHi of the Ehrlich ascites cells as was reported

by Poole (1967). This reduction in pHi did not occur, probably because of the concentration differential (10 mM in this study, 40 mM in the study of Poole). Nevertheless, the change in pHi was not essential to the ability of sodium oxamate to influence the activity of the glucose-stimulated, increased levels of FU upon the Ehrlich ascites cells. It thus becomes important to analyze all of the possible effects of a compound upon a tumor system in order to determine its relative utility. Compounds such as sodium oxamate not only can affect the intracellular pH, but they also can affect other metabolic parameters which may influence the total picture.

Because of these points, the clinical data utilizing glucose with FU becomes suspect. Each neoplastic disease state should be considered separately when trying to determine whether the addition of glucose to FU treatment regimens will result in increased activity or reduced toxicity. In addition, any studies should be conducted with a variety of doses of glucose as was done by Lahiri (1968) for it appears that different amounts of glucose might be indicated for the treatment of different neoplasms.

A number of comments based upon the results reported herein might also be made concerning the feasibility of using adjunct glucose with basic antineoplastic compounds. This hypothesis depended upon the ability of glucose to lower the extracellular pH in a tumor system. Since neoplasms have a lethargic circulation, a reduction of the

extracellular pH might facilitate the concentrating of basic drugs in the area of the tumor as was shown by Stevens and Lehman (1973). In this regard it should be noted that the pH of the Walker 256 ascites fluid decreased when those cells were exposed to glucose-saline even though their pHi increased.

The ratio of the antilogs of the pH values for intracellular and extracellular water might also be an important factor. Although a decrease in extracellular pH might allow more of the basic drug into the area of the tumor, it is the relationship of the pHi within the neoplastic cell to the extracellular pH which determines the amount of drug which will enter the cell under a straight partitioning theory. In the case of Walker 256 tumor for instance, even though a decrease in extracellular pH took place, the increase in pHi within the tumor cells would have a tendency to negate this effect.

The extracellular pH of the Ehrlich ascites fluid was held at 7.2 by the TRIS-phosphate buffering system. Following various pretreatments the pHi of the Ehrlich ascites cells varied over a range of almost 0.5 pH unit. The prior administration of glucose-saline produced the lowest pHi, a situation which should have been conducive to a greater uptake of basic drugs and a smaller uptake of acidic drugs if partitioning were the main factor which determined drug levels.

Drug levels within the Ehrlich ascites cells however were not influenced by the changes in pH. FU uptake following the various

pretreatments has already been discussed and was found to be independent of pHi.

The change in pHi produced by the glucose-saline pretreatment regimen produced no change in the uptake of either CCNU or CA. For CCNU this was understandable since its neutrality at physiological pH should provide protection against slight shifts in that range. The uptake of CA, a fairly strong acid, should be affected by pH shifts if simple partitioning is the major determinant of its uptake. This did not occur (another dissociation of uptake from pHi) which leads to speculation relative to the factors which may be involved in the uptake of CA.

Procarbazine uptake into Ehrlich ascites cells was also dissociated from pHi. In this instance, however, the drug concentration changed while pHi did not. This occurred following the glucose-oxamate pretreatment which resulted in an increase in the intracellular PZ concentration. This basic compound was not influenced by the decrease in pH of the Ehrlich cells following glucose-saline pretreatment which again is in opposition to a simple partitioning hypothesis.

Drug activity for CA, CCNU, and PZ reflected the pattern for drug uptake. Neither CCNU or CA showed any shifts in activity after the various pretreatments. The increased cellular accumulation of PZ was reflected by an increase in drug activity. The ability of the

combination of sodium oxamate and glucose to affect the uptake and activity of PZ within this tumor system is a phenomenon which deserves further study.

Thus for all four of the drugs employed factors other than pHi determined their uptake and resultant activity. The ability of glucose to increase the activity of a drug depends upon both the individual tumor and the individual drug. Support for this conclusion is provided by these results which indicate that the tumor may not respond to glucose exposure with a decrease in pHi, the drug uptake into any particular tumor may be more dependent upon factors other than simple pK related partitioning of the drug into the tumor (this is especially true for drugs involved with tumor metabolism), and the uptake of the drug into any tumor and resultant activity of the drug may or may not be correlated. Thus, the general hypothesis dealing with the administration of glucose as an adjunct in antineoplastic therapy either in respect to a type of drugs (based upon pK) or a single drug (FU) needs reevaluation in light of the individuality of the drug-tumor response.

Oxamic acid as an adjunct to antineoplastic therapy based upon lowered pHi is suspect as well due to this tumor and drug individuality. A drug which does partition into the cell based solely upon the pHi and pHe within the tumor might have its activity enhanced with concurrent oxamate therapy, but at oxamate concentrations higher than those used

in these studies. The ability of oxamate, when administered along with glucose, to stimulate the activity of the glucose-induced high levels of FU within Ehrlich ascites tumor cells and to significantly increase both the intracellular concentration and the activity of PZ within Ehrlich ascites tumor cells indicates a need for further study of adjunct oxamate therapy. These studies would concern themselves with the ability of oxamate to alter biochemical parameters rather than the physiochemical parameter of pH.

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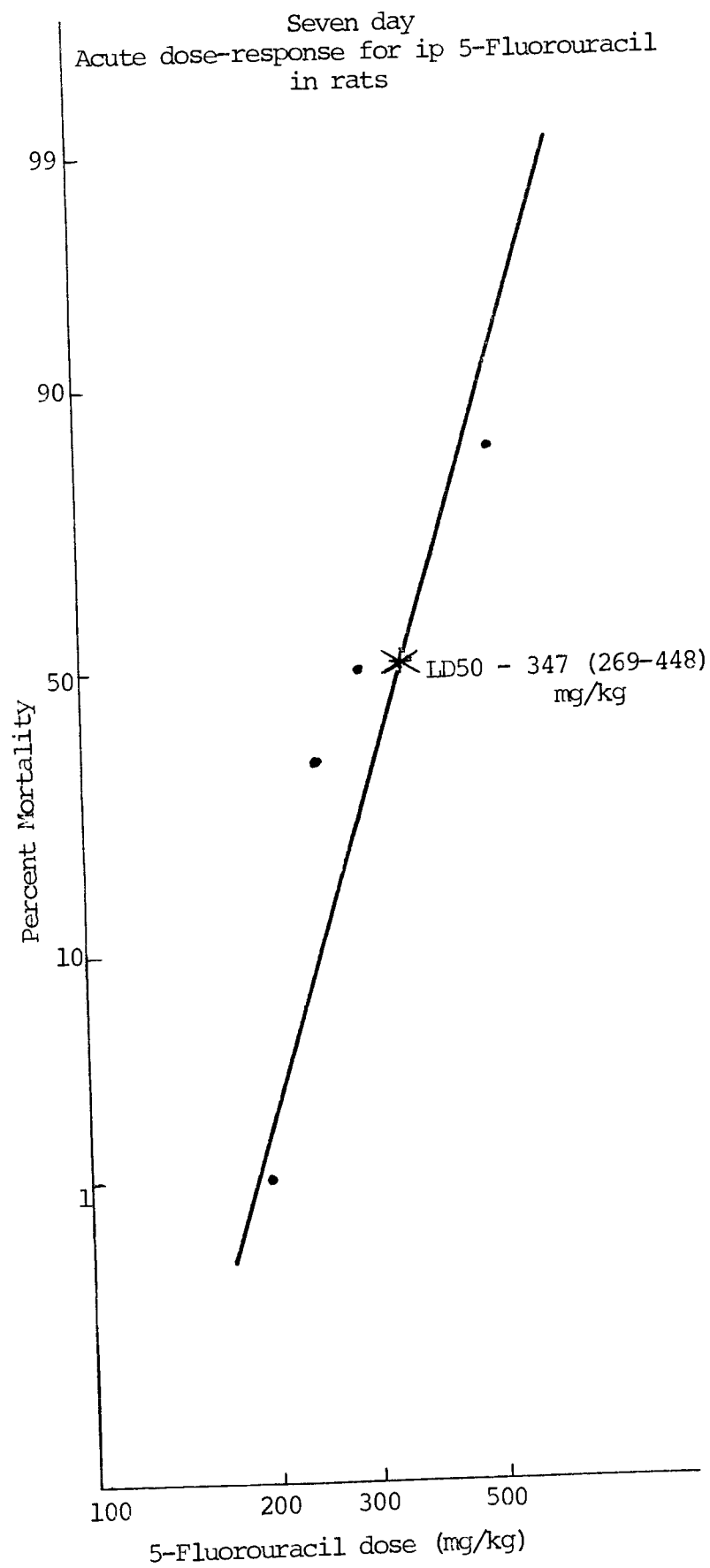
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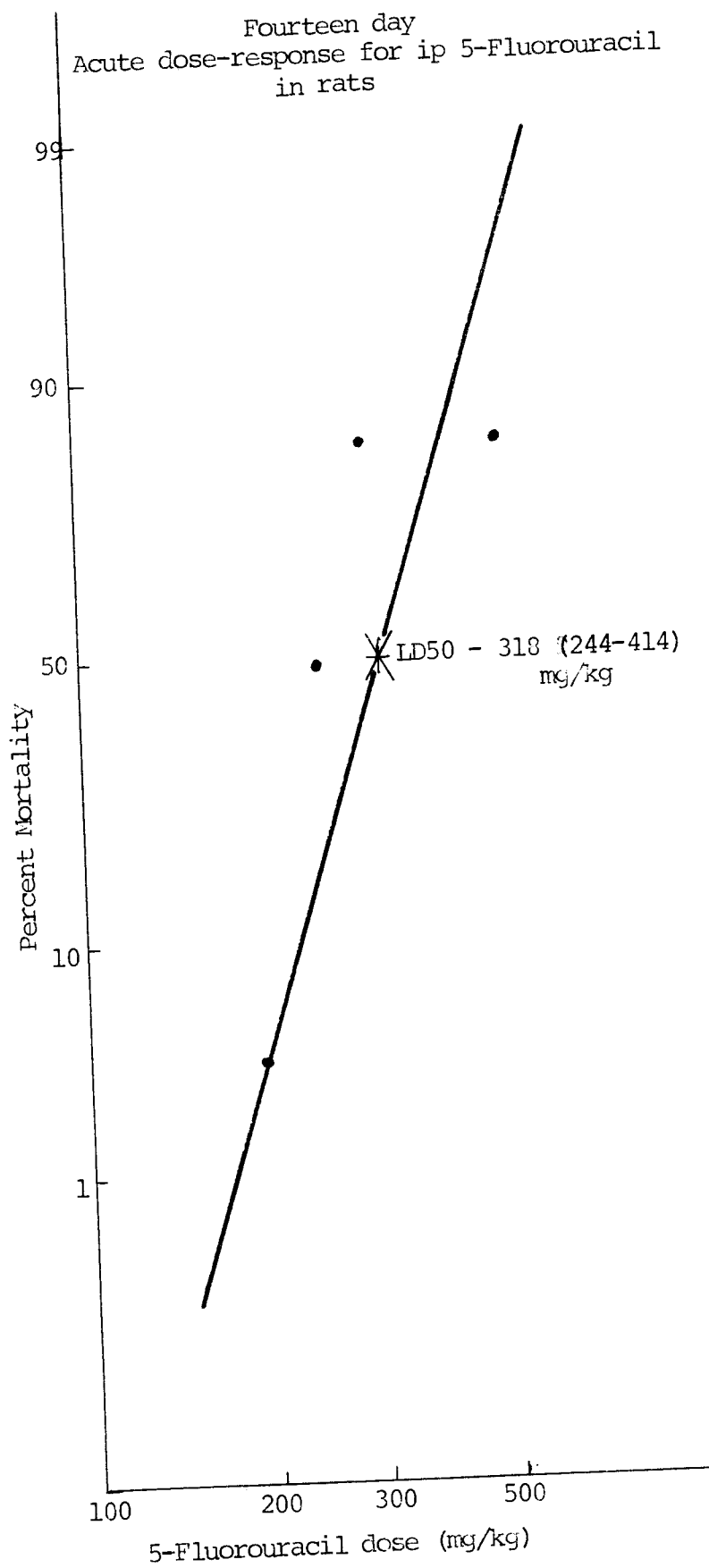
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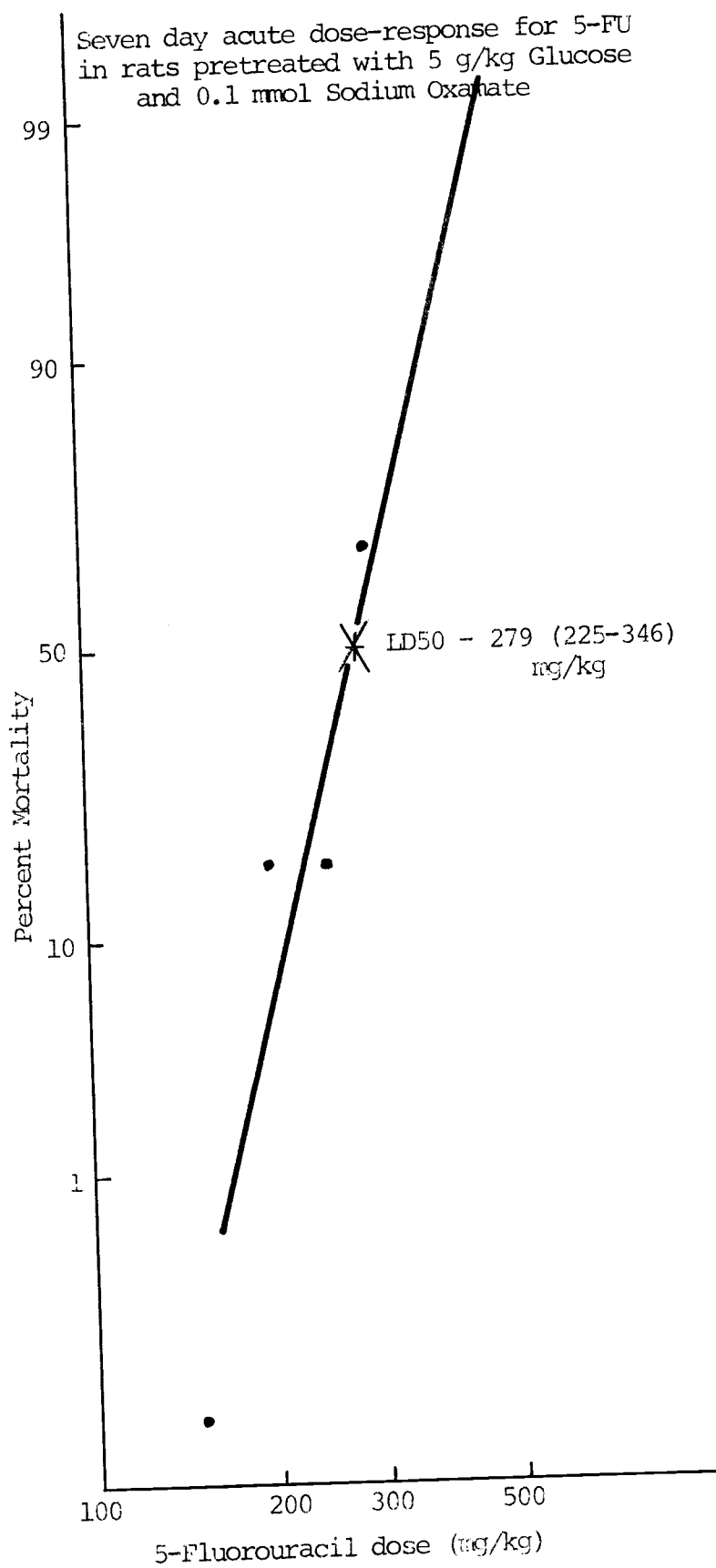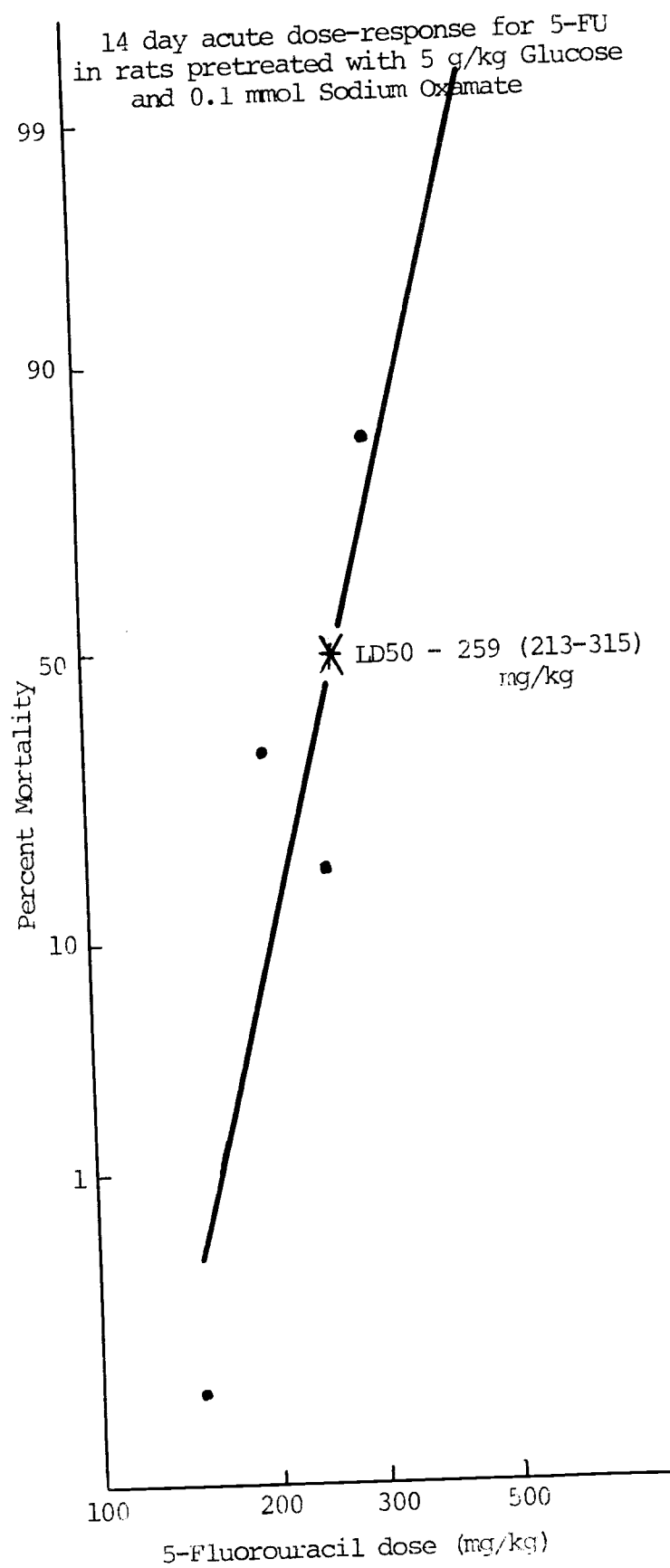
APPENDICES

APPENDIX I

The four graphs in this appendix are plots of the raw data obtained from the experiments described in Chapter II. Dose-response lines were fitted to this raw data by linear regression, and the equation for the "best fit" regression line for each set of data is indicated. These lines were then used for a median lethal dose (LD50) analysis according to the method of Litchfield and Wilcoxon (1949).
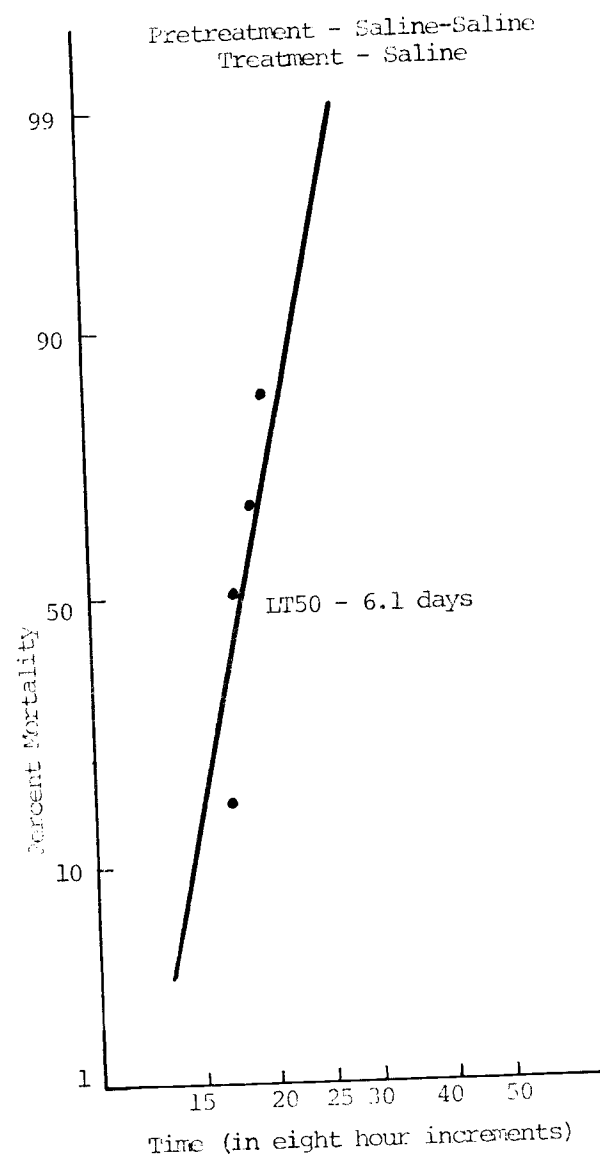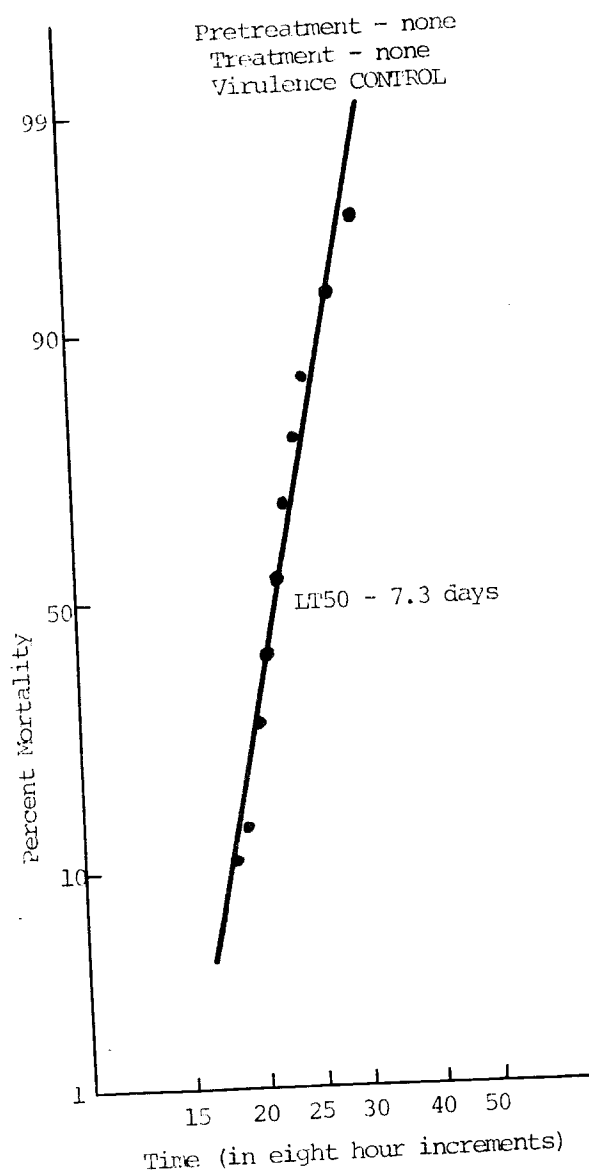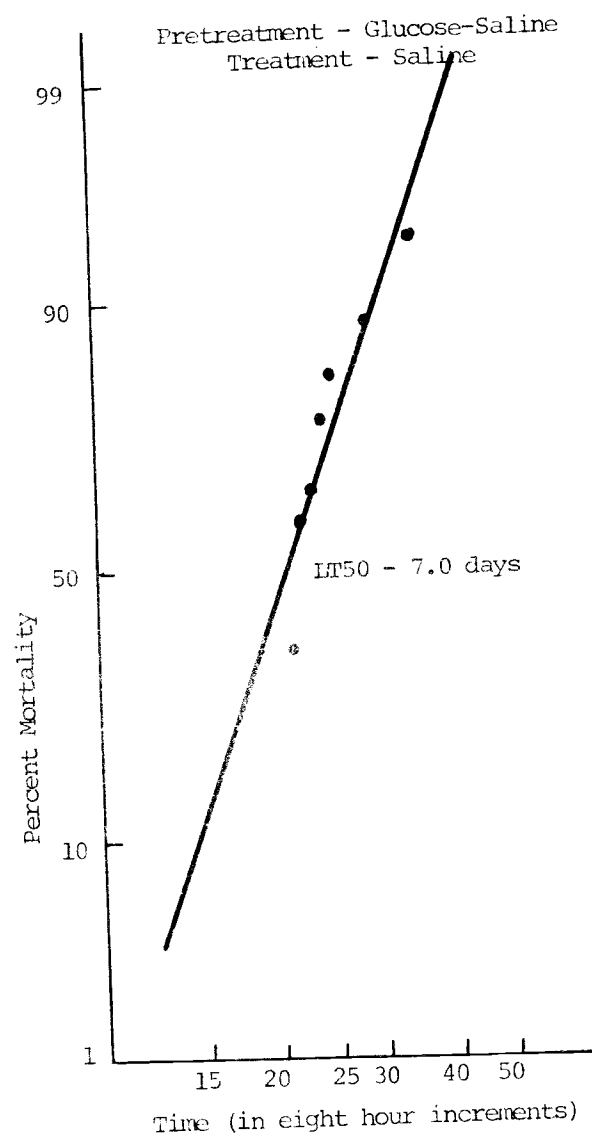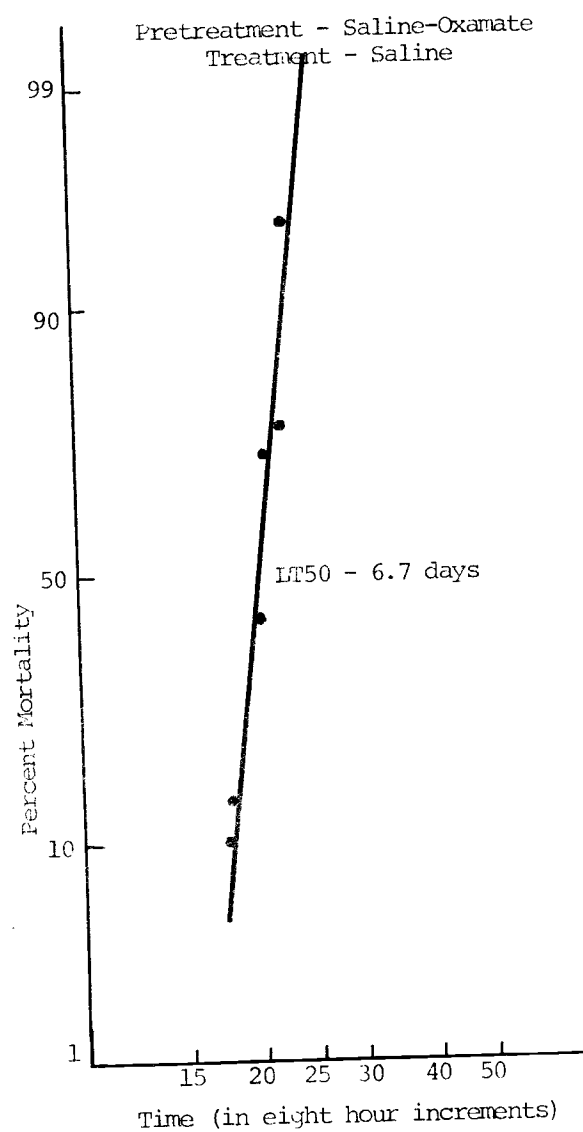
Seven day
Acute dose-response for ip 5-Fluorouracil
in rats

Percent Mortality

5-Fluorouracil dose (mg/kg)

LD50 - 347 (269-448)
mg/kg

Fourteen day
Acute dose-response for ip 5-Fluorouracil
in rats

Percent Mortality

99

90

50

10

1

LD50 - 318 (244-414)
mg/kg

100   200   300   500

5-Fluorouracil dose (mg/kg)

Seven day acute dose-response for 5-FU in rats pretreated with 5 g/kg Glucose and 0.1 mmol Sodium Oxanate

LD50 – 279 (225-346) mg/kg

14 day acute dose-response for 5-FU
in rats pretreated with 5 g/kg Glucose
and 0.1 mmol Sodium Oxamate

LD50 - 259 (213-315) mg/kg

Percent Mortality

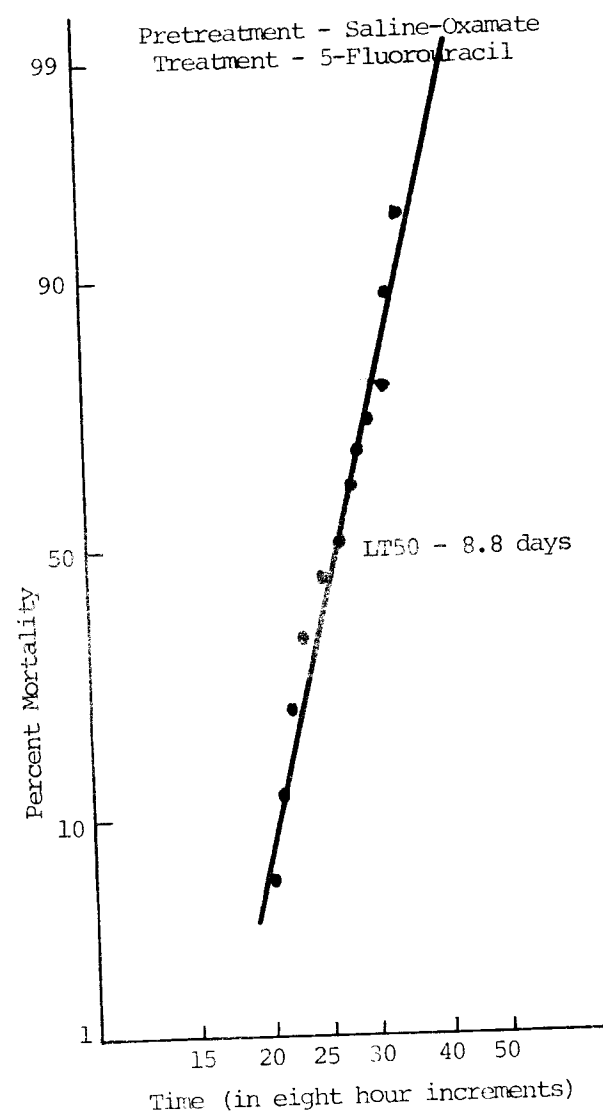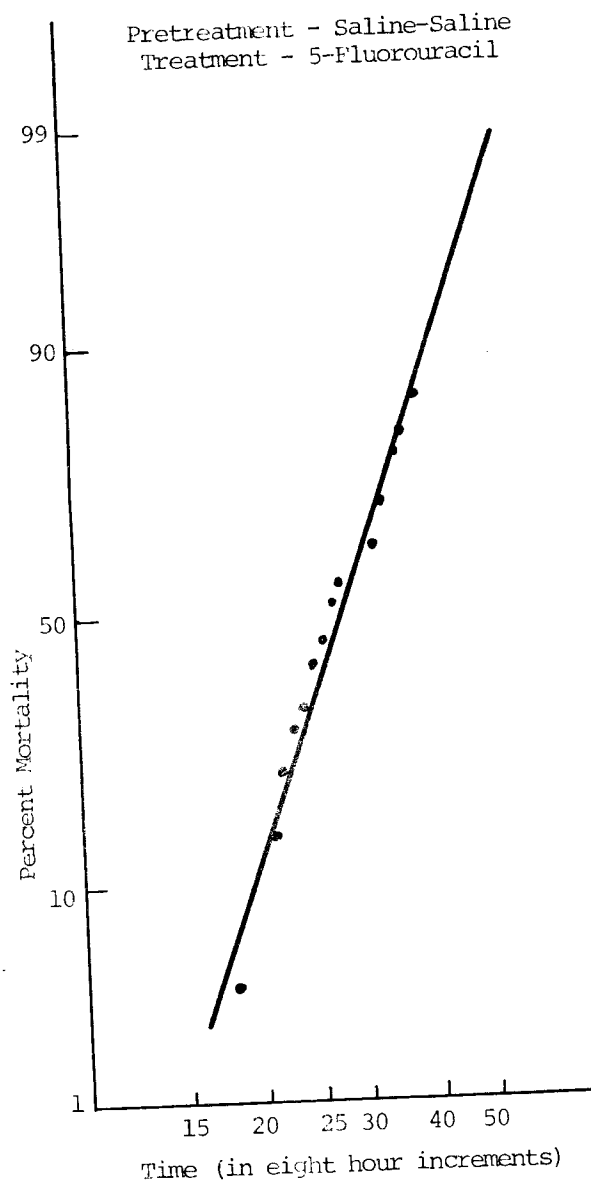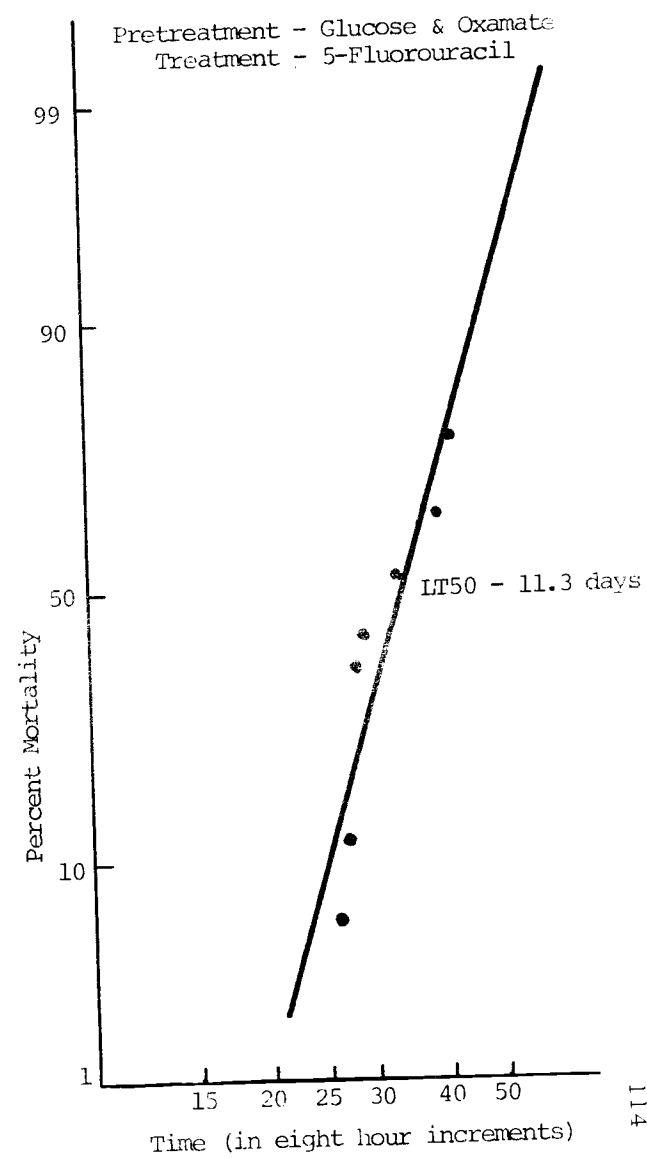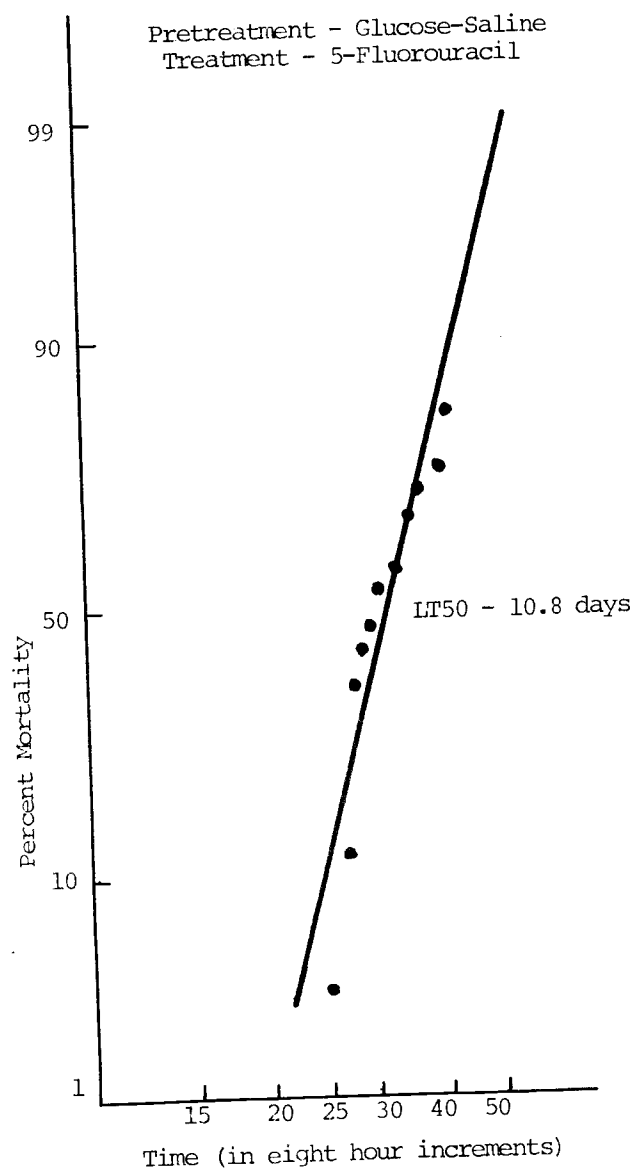5-Fluorouracil dose (mg/kg)

APPENDIX II

The eight graphs in this appendix are plots of the raw data obtained from the experiments described in Chapter III. Time-percent effect lines were fitted to the raw data by linear regression. These lines were then used for a median lethal time (LT50) analysis according to the method of Litchfield (1949).
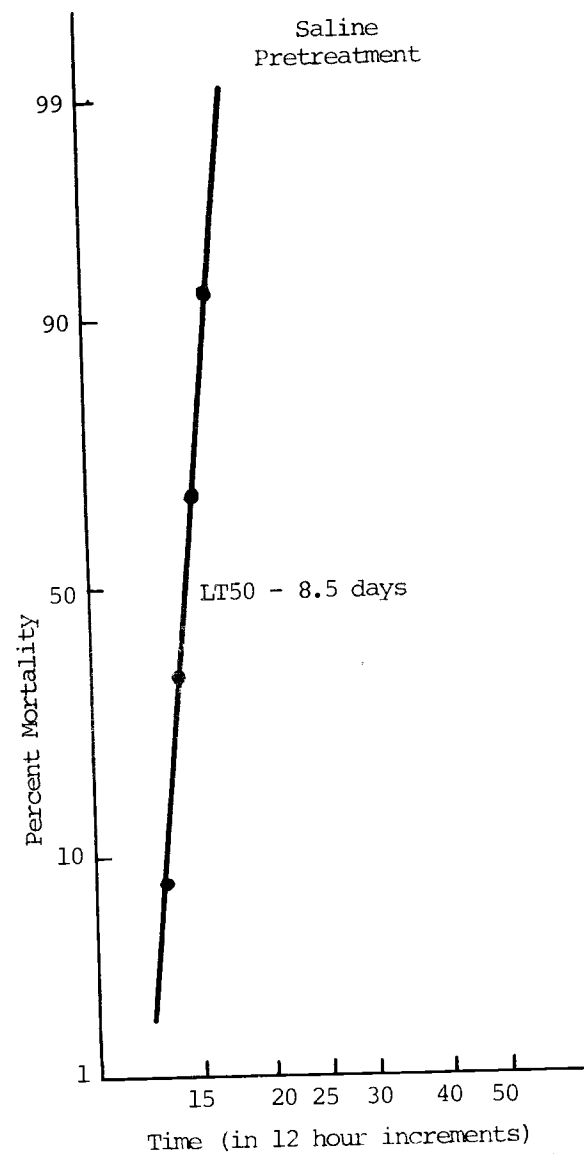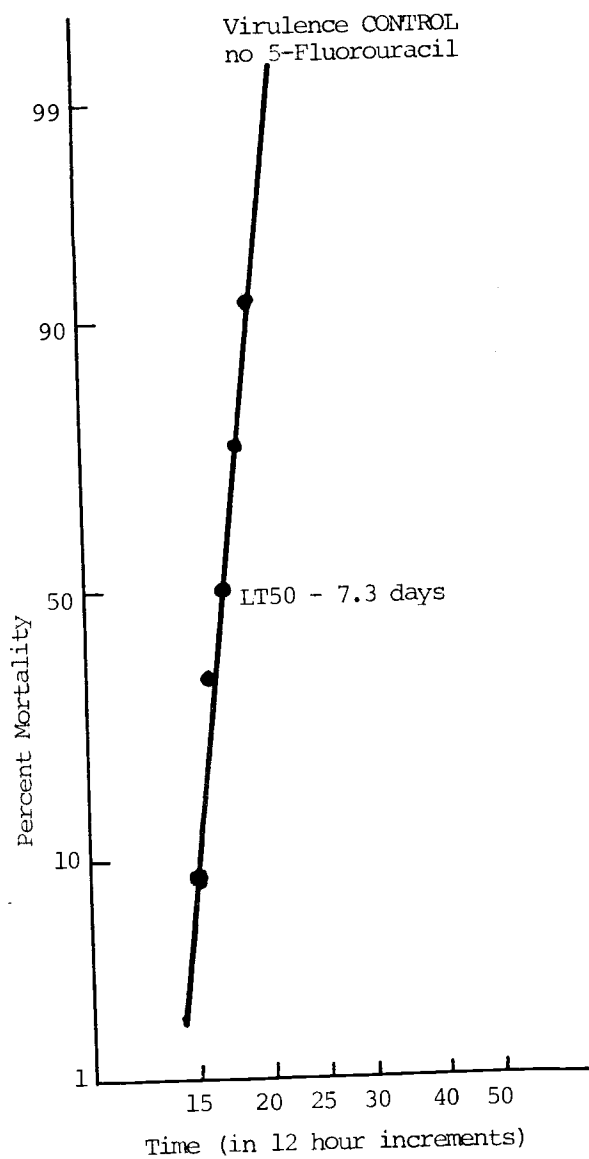
Pretreatment – none
Treatment – none
Virulence CONTROL

LT50 – 7.3 days

Percent Mortality

Time (in eight hour increments)

Pretreatment – Saline-Saline
Treatment – Saline

LT50 – 6.1 days

Percent Mortality

Time (in eight hour increments)

111

Pretreatment – Saline-Oxamate
Treatment – Saline

LT50 – 6.7 days

Percent Mortality

Time (in eight hour increments)

Pretreatment – Glucose-Saline
Treatment – Saline

LT50 – 7.0 days

Percent Mortality

Time (in eight hour increments)

Pretreatment - Saline-Saline
Treatment - 5-Fluorouracil

Percent Mortality

Time (in eight hour increments)

Pretreatment - Saline-Oxamate
Treatment - 5-Fluorouracil

Percent Mortality

LT50 - 8.8 days

Time (in eight hour increments)

Pretreatment – Glucose-Saline
Treatment – 5-Fluorouracil

LT50 – 10.8 days

Percent Mortality

Time (in eight hour increments)

Pretreatment – Glucose & Oxamate
Treatment – 5-Fluorouracil

LT50 – 11.3 days

Percent Mortality

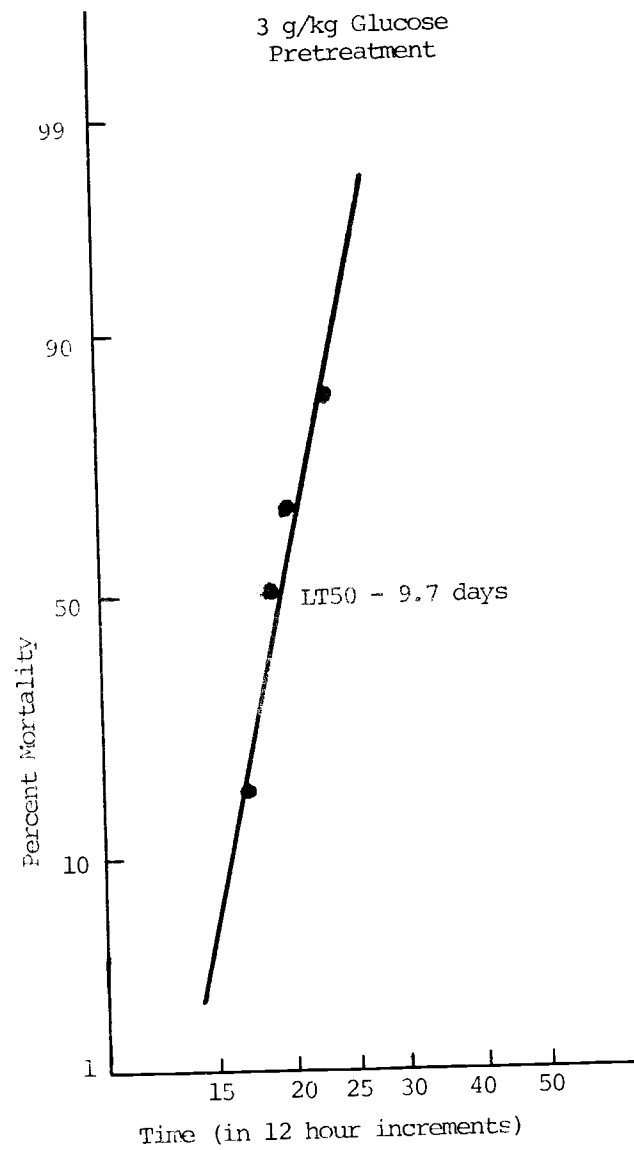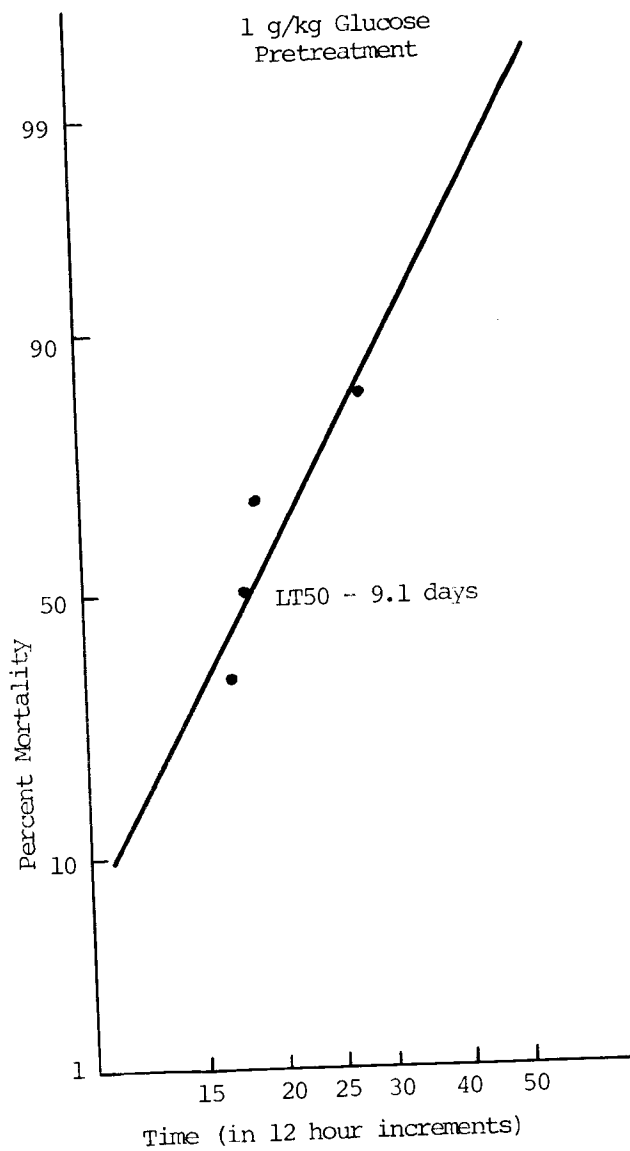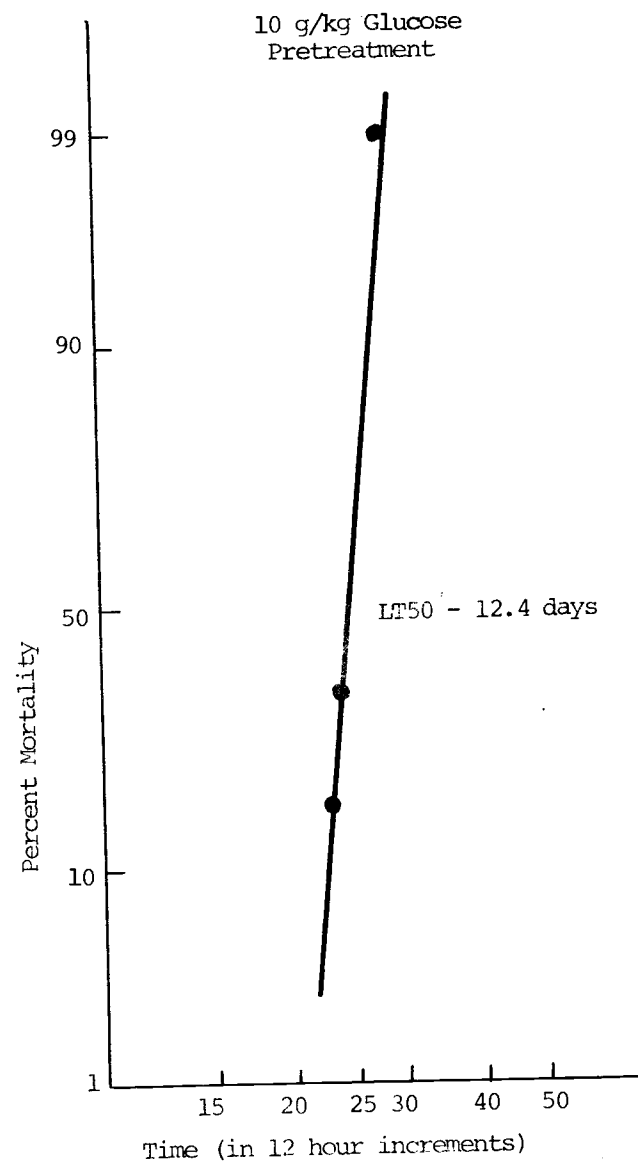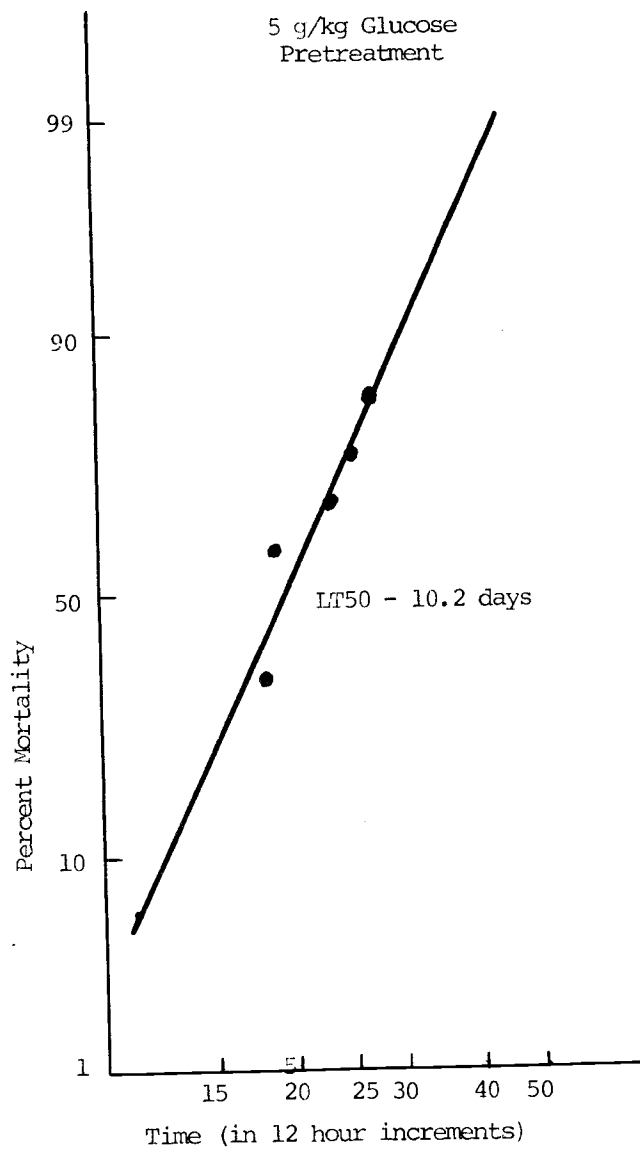Time (in eight hour increments)

114

APPENDIX III

The eight graphs in this appendix are plots of the raw data obtained from the experiments described in Chapter IV. Time-percent effect lines were fitted to the raw data by linear regression. These lines were then used for a median lethal time (LT50) analysis according to the method of Litchfield (1949).

Virulence CONTROL
no 5-Fluorouracil

LT50 - 7.3 days

Percent Mortality

Time (in 12 hour increments)

Saline
Pretreatment

LT50 - 8.5 days

Percent Mortality

Time (in 12 hour increments)

0.25 g/kg Glucose
Pretreatment

LT50 — 8.6 days

Percent Mortality

Time (in 12 hour increments)

0.5 g/kg Glucose
Pretreatment

LT50 — 10.8 days

Percent Mortality

Time (in 12 hour increments)

1 g/kg Glucose
Pretreatment

LT50 – 9.1 days

Percent Mortality

Time (in 12 hour increments)

3 g/kg Glucose
Pretreatment

LT50 – 9.7 days

Percent Mortality

Time (in 12 hour increments)

5 g/kg Glucose
Pretreatment

LT50 - 10.2 days

Percent Mortality

Time (in 12 hour increments)

10 g/kg Glucose
Pretreatment

LT50 - 12.4 days

Percent Mortality

Time (in 12 hour increments)

APPENDIX IV

The composition of the TRIS-PO$_4$ buffer used in Chapter VII

was the following:

Compound	Amount (gm/100 ml)
2-amino-2-(hydroxymethyl)-1,3,propanediol [TRIS]	2.4230 (0.2 M)
disodium ethylenediamine-tetraacetate [EDTA]	.1000
NaCl	.4725
KCl	.0243
KH$_2$PO$_4$	.2685
Na$_2$HPO$_4$	.6665
Hyaluronidase	.0150

pH was adjusted to 7.2 by using concentrated HCl. The

hyaluronidase was added just prior to use.

APPENDIX V

The equation for the calculation of pHi which was used in Chapter VII was a modified form of the original equation as described by Waddell and Butler (1959). This modification, which was first proposed by Schloerb et al. (1965), can be employed when isotopes are being used to measure the parameters needed to solve the equation. In this case the three isotopes used were:

1) ^3H-as ^3H_2O measures total sample water

2) ^{14}C-Inulin measures sample extracellular water

3) ^{14}C-DMO measures pHi by its distribution within the sample.

With these three substances as the markers, the equation becomes:

$$pHi = \frac{6.13}{(pKa-DMO)} + \log \{[\frac{DMO_t\ ^3H_2O_p}{DMO_p\ ^3H_2O_t}(1+\frac{In_t\ ^3H_2O_p}{1.05\cdot In_p\ ^3H_2O_t - In_t\ ^3H_2O_p})$$

$$-(\frac{In_t\ ^3H_2O_p}{1.05\ In_p\ ^3H_2O_t - In_t\ ^3H_2O_p})]\times[1\times10^{(pHe-6.13)}]-1\}$$

in which the subscript "t" indicates the dpm of that isotope in the tissue sample and the subscript "p" indicates the dpm of that isotope in a sample of extracellular fluid.