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Title: A COMPARISON OF THE EFFECT OF pH CHANGE UPON
THE UPTAKE OF 5-FLUOROURACIL INTO RAT LIVER
AND WALKER 256 CARCINOSARCOMA SLICES

Abstract approved: / Dr. Robert E. Larson

An enhancement of the therapeutic effect and/or a reduction in
the toxicity have been shown by a variety of antineoplastic compounds
when glucose is added to the treatment schedule. A possible explana-
tion for this involves a selective change in intracellular pH of the
tumor tissue initiating a greater uptake of the antineoplastic agent.

The present study was carried out to show the effect of altera-
tions in intracellular pH upon the uptake of one of the compounds
studied above, 5-Fluorouracil (5-FU), into both Walker 256 carcino-
sarcoma and normal liver slices. Two methods, the addition of glu-
cose and the addition of glucose plus sodium oxamate to the incubation
medium, were utilized in an attempt to lower intracellular pH selec-
tively in the tumor slices. Intracellular pH was determined by the
distribution of $^{14}$C-labeled 5,5-dimethyl-2,4-oxazolidinedione
between extracellular and intracellular fluid, and uptake of 5-FU was determined by using $^{14}$C-labeled 5-FU.

In the absence of glucose in the incubation medium the intracellular pH of both the liver and tumor slices was measured with good precision and agreed with values reported in the literature. The addition of 5mM glucose resulted in a decrease of intracellular pH within the liver slices, but a significant increase in pH of the tumor slices. Twenty minute preincubation of the slices with 40mM sodium oxamate before glucose addition further lowered the pH of the liver slices, but maintained intracellular pH of the Walker 256 slices at control levels.

Studies of the uptake of 5-FU suggested that it partitions into normal liver slices as a weak acid. The 5-FU uptake into Walker 256 tumor slices was significantly increased by the addition of 5mM glucose and this increase persisted upon the addition of 40mM oxamate. This indicates that 5-Fluorouracil uptake into Walker 256 carcinosarcoma slices was more dependent upon the presence of glucose than alterations in intracellular pH.
A Comparison of the Effect of pH Change Upon the Uptake of 5-Fluorouracil into Rat Liver and Walker 256 Carcinosarcoma Slices

by

Richard Lee Hult

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APPROVED:

Redacted for privacy

Associate Professor of Pharmacology and Toxicology
in charge of major

Redacted for privacy

Head of Department of Pharmacology and Toxicology

Redacted for privacy

Dean of Graduate School

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Typed by Brenda Fadness for Richard Lee Hult
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A warm thanks is also extended to the other members of my graduate committee, Dr. Donald Reed, Dr. Lavern Weber, Dr. Gregory Fink, Dr. Ronald Winters, and Dr. Frank Dost, for their helpful comments during the conception of the problem and their critical review of the manuscript.

Environment plays an important part in any individual's growth. In this regard I wish to thank the Department of Pharmacology and Toxicology for the excellent facilities made available to me and voice a special note of appreciation to Mr. Hudson White, our animal caretaker, for making available a ready supply of healthy laboratory animals. Sincere gratitude is also extended to my contemporaries for their interest and stimulation throughout the course of this research problem.

Finally, I wish to thank my wife, Bonna, for her interest, encouragement, and support during these last three years.
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A COMPARISON OF THE EFFECT OF pH CHANGE UPON THE UPTAKE OF 5-FLUOROURACIL INTO RAT LIVER AND WALKER 256 CARCINOSARCOMA SLICES

INTRODUCTION

The concept of treating cancer with chemical agents has had a long history (over 1000 years) but for the majority of that period successes have been limited. Within the last 30 years, however, there has been a tremendous upsurge of interest and activity in this area leading to a number of new and relatively effective antineoplastic compounds. During this period many problems have been encountered including drug resistance, toxicity, and others. Most of the chemical agents in clinical or experimental use today are limited in their effectiveness by their inherent cytotoxicity upon the normal tissues of the body, especially those tissues with a high rate of proliferation. As a result much effort has been put into the elucidation of the biochemical and physiological differences between normal and neoplastic tissue with the hope that exploitation of these differences could increase antineoplastic effect and reduce toxicity.

One such attempt was the addition of glucose to the treatment schedule of several chemotherapeutic agents, including 5-Fluorouracil (5-FU) (Ross, 1961; Kung, et al., 1963; Connors, et al., 1964; Ross, 1964). Utilizing inhibition of tumor growth as the measurement of drug effectiveness, these studies reported enhanced antineoplastic
activity and/or reduced toxicity in experimental animals. This con-
cept was lent clinical support by a study (Lemon, et al., 1963) which
reported a decrease in the toxic effects to cancer patients of intra-
venously infused 5-FU upon the addition of glucose.

Most investigators have suggested that the observed effects
might be due to a selective lowering of pH within the neoplastic tissue
after the administration of glucose. This conclusion was based upon
the historic observation by Warburg et al. (1924) that many tumors
utilize aerobic glycolysis as a primary energy source with the sub-
sequent production of lactic acid and consequent lowering of pH. In-
deed, pH values for various experimental tumors as measured by the
microelectrode technique have shown a significant decrease in pH
following glucose administration (Voegtlin, et al., 1935; Kahler and
Robertson, 1943).

Two clinical studies (Hall, et al., 1966; Cressy and Schell,
1966) have shown no difference in terms of antineoplastic activity
and/or toxicity between saline and 5% dextrose in water as the infusion
medium in 5-FU therapy. These authors, however, used only 50%
and 25% of the total glucose load utilized in the clinical study which
showed decreased toxicity. This in turn was only about 15% of that
used in the animal studies. Thus, the possibility exists that the
amount of glucose utilized is critical.
Oxamic acid, a specific inhibitor of lactic dehydrogenase (LDH) was effective in inhibiting the growth of Walker 256 carcinosarcoma when administered with glucose by systemic infusion in the region of the tumor (Reynolds, et al., 1963). Oxamate has been reported as an inhibitor of LDH in HeLa cells (Goldberg and Colowick, 1965) and Ehrlich ascites carcinoma cells (Papaconstantinou and Colowick, 1961). In addition, oxamate lowered intracellular pH of Ehrlich ascites cells through the build-up of acidic glycolytic intermediates (Poole and Butler, 1969).

The purpose of the present investigation was twofold: first, to determine whether the extent of 5-FU partitioning into neoplastic tissue as well as into normal liver tissue was dependent upon pH; secondly, to investigate the relative efficiency of a combination of glucose plus oxamate as opposed to glucose alone in reducing intracellular pH.
EXPERIMENTAL

Male Sprague-Dawley rats weighing 150-200 g from our rat colony were used. They were housed, five per cage, at a temperature of 21-22°C with a 12-hour light and dark cycle. Food and water were supplied ad libitum throughout the study.

The Walker 256 carcinosarcoma tumor line was obtained from the National Cancer Institute (NCI), Bethesda, Maryland. It was propagated weekly as an intraperitoneal ascites tumor using the protocol of NCI (Geran, et al., 1972). Solid, 7-10 day old, intramuscular tumors were obtained from the thigh, again using the NCI protocol (Geran, et al., 1972).

The distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO) between extracellular and intracellular fluid was utilized for the measurement of intracellular pH. The original method as set forth by Waddell and Butler (1959) was modified by the incorporation of a mixture of three radionuclides similar to that described by Schloerb and Grantham (1965), but refined to allow simultaneous counting of all three radionuclides in the same sample by employing the Automatic External Standardization (AES) feature of a Packard Tricarb Model 3375 liquid scintillation spectrometer (Kuhl and Winters, 1974).

The mixture of radionuclides $^{3}H_{2}O$-saline, $^{14}C$-DMO, and $^{36}$Cl-saline was prepared each day from stock concentrates as
follows: 0.2 ml \( ^{14} \text{C-DMO} \) (0.5 uCi - New England Nuclear, Boston, Mass.) in ethyl acetate was allowed to evaporate to dryness, redisolved in 0.4 ml of normal saline, and combined with 0.4 ml \( ^3 \text{H}_2 \text{O-saline} \) (4.0 uCi - New England Nuclear) and 0.2 ml \( ^{36} \text{Cl-saline} \) (0.5 uCi - Amersham Searle Corp., Arlington Heights, Ill.).

Liquid scintillation spectrometry was also used for the determination of 5-FU uptake, the working solution prepared each day by diluting 0.1 ml of the stock concentrate of \( ^{14} \text{C-5-FU} \) (0.25 uCi - Schwarz/Mann, Orangeburg, New York) in water to one milliliter with saline.

The incubation medium was Krebs Solution (Hukovic's modification [Laurence and Bacharach, 1964]) omitting the glucose. Oxamate experiments had 40 mM oxamate included in the incubation medium by the addition of an oxamic acid concentrate which had been prepared by dissolving oxamic acid in saline and adjusting to pH 7.4 with sodium hydroxide. A series of experiments was also conducted using standard Krebs as the incubation medium. Fresh glucose concentrates along with the radioactive solutions were available for addition to the incubation medium.

The animals were sacrificed by decapitation and the liver or tumor was rapidly excised and placed in iced incubation medium at a pH of 7.2. Within 30 minutes of excision six slices approximately 0.5mm in thickness and weighing 80-120 mg were cut from a section
of tissue 2.5 cm in diameter using a Stadie-Riggs tissue slicer modified to keep the tissue chilled during slicing. Each slice was placed in a tared, 25 ml Erlenmeyer flask containing five milliliters of incubation medium. After weighing, the slices were placed on a Dubnoff metabolic shaker at 37°C and bubbled continuously with a 95%-O₂/5%-CO₂ mixture in order to maintain pH at 7.4.

Following a 20 minute equilibration period the slices were exposed to both glucose (5, 10, or 25 mM) for 5, 10, or 20 minutes and 0.1 ml of either the radionuclide mixture or the 5-FU working solution for 16 minutes (preliminary work had shown all four radionuclides to be equilibrated within 16 minutes). The minimal exposure time was especially important to permit the assumption that disintegrations per minute (dpm) in the 5-FU uptake experiments were predominantly ¹⁴C-5-FU and not its metabolites. Investigators have shown 5-FU to be partially metabolized 60-90 minutes after exposure both in vivo and in vitro (Chaudhuri, Mukherjee, and Heidelberger, 1958; Mukherjee and Heidelberger, 1960). In all cases the times of addition were adjusted so that the times of removal coincided.

Incubations were terminated by removing each slice from its flask and reweighing it in a tared scintillation vial containing one milliliter of digestant. For the pH determinations, tissue was digested in Soluene® with the counting fluor Dimilum® (Packard Inc., Downers Grove, Ill.). The tissue for the 5-FU uptake study was
digested in Protosol® (New England Nuclear) and counted in a toluene based fluor consisting of 5.0 g PPO (2, 5-diphenyloxazole) and 0.5 g Dimethyl-POPOP (1, 4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene) in one liter of toluene.

The remaining incubation medium was transferred to a five milliliter centrifuge tube and the pH immediately determined with a Sargent pH meter - model DR and microelectrode #S-30070-10 calibrated carefully over the pH range 5.0 through 9.0 to give pH readings with a precision of ±0.02 pH units. After a 20 minute centrifugation at 250 x g, 0.1 ml of the supernatant was placed in a scintillation vial. This vial already contained a non-radioactive, 80-120 mg piece of the tissue under study along with one milliliter of digestant in order to control for quench. From the dpm in this vial and the difference in weight of the slice before and after incubation, the amount of radioactivity in the experimental samples due to adhering incubation medium could be determined.

Fifty-minute counts were obtained for both the sample and its corresponding incubation medium using the spectrometer settings suggested by Kuhl and Winters (1974) for these three radionuclides. The resultant data were analyzed for dpm (Kuhl and Winters, 1974). These data, together with the external pH value, were utilized to calculate internal pH according to the equation set forth by Schloerb
and Grantham (1965) using a Monroe model 1785 programmable calculator.

The 5-FU uptake was estimated in slices not exposed to the other radionuclides but otherwise identically treated. As above, dpm due to adhering incubation fluid was subtracted from tissue sample dpm and the results for each slice converted to $10^{-2}$ pMole 5-FU per milligram of tissue.

For the internal pH study the six values from each experiment were pooled and the mean and standard deviation determined. Any slice which deviated two standard deviations or more from this mean was considered an aberrant value and was omitted. It was never necessary to exclude more than one slice from any experiment. In those cases where more than one experiment was used to determine the data point, only slices remaining were pooled. For all data means and 95% confidence limits were determined. This same procedure was followed for the 5-FU uptake study. Differences among means of the treatments were evaluated for significance by Student's "t" test (Goldstein, 1964).
RESULTS

As measured by $^{14}$C-DMO distribution, the intracellular pH of normal rat liver slices and Walker 256 tumor slices was measured with excellent precision (Table I). The control value for Walker 256 tumor tissue (7.12 ± 0.07) agreed closely with the value of 7.19 found by Schloerb, et al. (1965) in vivo for Walker 256 tumors. In addition the control value for liver found here (7.32 ± 0.10) agreed well with the in vivo value of 7.39 reported by Kahler and Robertson (1943) who used microelectrode techniques.

The internal pH of liver slices was significantly lowered (P < .05) by the addition of 5 mM glucose to the incubation medium (Table I). This drop of approximately 0.16 pH units occurred both when glucose was present in the initial incubation medium and when glucose was added to an incubating slice. Five millimolar glucose is the amount suggested for Krebs Solution and corresponds to a glucose concentration of 90 mg% (or a load of approximately 0.5 g/kg). The decrease in pH in liver slices coupled with the close agreement of our control (Krebs minus glucose) incubation data to in vivo measurements suggested that these tissues in vitro were responding to the addition of glucose in normal concentrations much as tissues in vivo (glucose already available - 90 mg%) would respond to a loading dose of glucose. The glucose load of 0.5 g/kg is 67% of the
<table>
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<td>A Time of (min.)</td>
<td>C Time (min.) with Additions (B - A)</td>
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<td>1) CONTROL no glucose</td>
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<td>2) 5 mM glucose</td>
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<td>7.42 ± .17*</td>
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<td>36</td>
<td></td>
</tr>
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<td>3) 5 mM glucose</td>
<td>26</td>
<td>7.36 ± .03*</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>4) 5 mM glucose</td>
<td>20</td>
<td>7.42 ± .04*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>5) 5 mM glucose</td>
<td>0</td>
<td>7.44 ± .11*</td>
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<td></td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>6) 25 mM glucose</td>
<td>20</td>
<td>7.51 ± .10*</td>
</tr>
<tr>
<td></td>
<td>40</td>
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<tr>
<td>7) 5 mM glucose 40 mM oxamate</td>
<td>20</td>
<td>7.07 ± .20*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>(17)</td>
</tr>
<tr>
<td>8) 10 mM glucose 40 mM oxamate</td>
<td>40</td>
<td>7.14 ± .04</td>
</tr>
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Values for intracellular pH are given as mean ± standard deviation. The figures in parentheses indicate the number of slices used for each determination. The symbol * indicates a value significantly (P < .05) different from that of the corresponding control. Except in experiment #5, equilibration in glucose-free Krebs Solution or glucose-free Krebs plus 40 mM oxamate was for 20 minutes prior to the addition of glucose. 

$^3$H$_2$O, $^{14}$C-DMO, and $^{36}$Cl in saline were added 16 minutes prior to termination of the experiment, but no sooner than 20 minutes after the start of equilibration.
load used in the clinical study which showed decreased toxicity (Lemon, et al., 1963) and 10% of the value utilized in the whole animal studies (Ross, 1961; Kung, et al., 1963; Connors, et al., 1964; Ross, 1964). Liver slice incubation with higher concentrations of glucose (10 or 25 mM) resulted in extremely variable data possibly due to osmotic or toxic factors.

The addition of 40 mM oxamate to the incubation medium lowered internal pH of the liver slices an additional amount to a level approximately 0.25 pH units below the control (Table I). This corresponds to an increase of $H^+$ activity of about 32% above control levels.

The 5-FU uptake into liver slices was not affected by the addition of glucose (Figure 1); however, the combination of oxamate and glucose lowered the uptake from $43.61 \times 10^{-2}$ to $41.11 \times 10^{-2}$ pMole/mg tissue, a decrease just significant at the $P < .05$ level. This decrease in uptake with increasing $H^+$ activity suggests that 5-FU was partitioning into liver tissue as a weak acid.

In the Walker 256 tumor slices the addition of 5 mM glucose raised the internal pH from $7.12 \pm .07$ to $7.44 \pm .11$ (Table I). This increase corresponds quite closely to that found in vivo by Schloerb et al. (1965) when a glucose load of 6.0 g/kg was administered to Walker 256 tumor bearing rats (7.19 to 7.36). These investigators suggested that this increase in intracellular pH of Walker 256 upon glucose administration (as opposed to the decrease seen in other
Figure 1. 5-Fluorouracil uptake into liver slices. a) mean ± standard deviation  
   b) number of slices used in making the determination  
   c) significantly different from the control value (P < .05). 
   Brackets indicate 95% confidence limits.
tumors [Voegtlin et al., 1935; Kahler and Robertson, 1943]) was due to a greater buffering capacity of the tumor. The 0.3 pH unit increase indicated by the data corresponds to a decrease of 50% in H⁺ activity. Neither the period of exposure to glucose nor increased glucose concentrations altered the response (Table I). Uptake of 5-FU was significantly increased upon the addition of glucose (Figure 2), an indication that 5-FU was also partitioning in this tissue as a weak acid as was found in liver slices.

When oxamate and glucose were both present in the incubation medium there was no net change in pH from that of the control (Table I). Moreover, higher concentrations of glucose and/or longer incubations with oxamate did not affect this value.

Under these conditions 5-FU uptake was found to have increased to similar levels found with glucose alone (Figure 2). This increase had occurred despite the fact that pH was at control levels. Thus, while the addition of oxamate apparently negated the effects of glucose upon tumor intracellular pH, it did not influence the enhancement of 5-FU uptake.
5-Fluorouracil Uptake into Walker 256 Carcinosarcoma Slices

Figure 2. 5-Fluorouracil uptake into Walker 256 carcinosarcoma slices.

a) mean ± standard deviation  b) number of slices used in making the determination  c) significantly different from the control value (P < .05). Brackets indicate 95% confidence limits.
DISCUSSION

Kung et al. (1963) suggested that the increase in effect of 5-FU at a lower pH might be due to an increase in the undissociated form of the compound, the form that he had shown to probably combine with enzymes. Since 5-FU is a weak acid (pKa = 8.0 ± 0.1 [Florey, 1973]), a lowering of pH will increase the amount of substrate available to react. The authors noted that other explanations were possible including: (1) pH alterations might be affecting the enzymes involved in the lethal synthesis of 5-fluoro-2'-deoxyuridine-5'-monophosphate from 5-FU; (2) pH lowering might enhance the transport of 5-FU into the cell.

Ross (1961) advanced the concept that basic drugs partition selectively into tumor tissue because of its inherent lower intracellular pH. Any treatment such as glucose administration which increases the pH difference between normal and neoplastic tissue was thought to cause more of the basic drug to partition into the tumor tissue. Lemon (1963) noted that this might be what was happening in the case of 5-FU, thinking 5-FU weakly basic.

The data presented here tend to argue against this theory that 5-FU partitioning into tissues depends upon pH. In this study uptake of 5-FU into Walker 256 slices is more dependent upon the presence of glucose than internal pH changes. In contrast, liver slices show
no correlation between 5-FU uptake and the presence of glucose. The data do suggest however, that 5-FU is partitioning as a weak acid in liver tissues.

Both glucose and pyrimidines have been shown to cross certain cellular membranes by facilitated diffusion (Fingl and Woodbury, 1970). Perhaps the addition of glucose stimulates this carrier process in tumor tissue leading to the incorporation of more 5-FU into the cell. One way of testing this possibility would be the addition of insulin to the system. This hormone stimulates the transport of glucose across cellular membranes and might thus be expected to enhance cellular uptake of 5-FU. This hypothesis leads to speculation as to the number of tumors in which carrier activation by glucose could take place as well as the question of the concentration of glucose needed for both activation and maximum intensity of effect.

In addition, although these in vitro data do not support the theory that lowering tissue pH leads to an increase in 5-FU uptake, the question of whether lowering intracellular pH increases the effectiveness of 5-FU by increasing the proportion of undissociated species within the cells is not answered. In this regard it should be noted that while glucose did not significantly lower intracellular pH, inhibition of LDH by oxamate was effective in lowering pH both in liver and tumor cells. If lower tumor pH does lead to increased 5-FU
activity, the combination therapy of 5-FU with local injections of oxamater might be of some utility.


APPENDIX
**KREBS SOLUTION**
(Modified by Hukovic)

Composition (g/l)

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<tr>
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<td>MgSO₄·7H₂O</td>
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