metabolically activated [22]. This finding discounts the role of altered membrane permeability and lends support to our proposal that CCl_4 (as well as 1,1-DCE) inhibits calcium pump activity via inhibition of microsomal ATPase. Studies to determine whether CCl_4 or 1,1-DCE enhances back diffusion of calcium from microsomal vesicles would provide additional information on the mechanism by which hepatotoxins alter microsomal sequestration.

In summary, we have shown that hepatotoxic doses of CCl_4 and 1,1-DCE promptly cause pronounced inhibition of hepatic microsomal ATPase activity. Microsomal calcium-dependent ATPase activity can be measured satisfactorily *in vitro* in either the presence or absence of added magnesium. Inhibition of ATPase activity appears to be a common mechanism whereby hepatotoxins diminish the ability of microsomes to sequester calcium. As the microsomal calcium pump is believed to play an active role in maintaining calcium homeostasis in hepatocytes, toxin-induced inhibition of calcium- and/or magnesium-dependent ATPase may be an initial biochemical lesion which triggers a sequence of events which may culminate in cell death.

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Role of intracellular dTTP levels in fluorodeoxyuridine toxicity

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A growing concern in cancer chemotherapy is the apparent heterogeneity among primary tumors in the response to chemotherapeutic agents and the appearance of secondary tumors which are no longer sensitive to the chemotherapeutic agent. Resistance to chemotherapeutic agents has often been linked to changes in enzyme activities which either are required for drug activation or represent the ultimate target of the drug [1]. On the other hand, drug activation may be also regulated by intracellular metabolites and thus variations in intracellular levels of these metabolites may largely affect drug toxicity [2].

5-Fluoroauracil (5-FU) and 5-fluorodeoxyuridine (FdUrd) are used in the treatment of various kinds of cancer [3]. The block in DNA synthesis that results from treatment of cells with FdUrd may be accounted for by inhibition of thymidylate synthase by the intracellular phosphorylation product FdUMP (4). In addition, DNA synthesis may be impaired due to the misincorporation of FdUrd into the DNA [5]. The phosphorylation of FdUrd is catalyzed by thymidine kinase, an enzyme which is inhibited by dTTP in extracts from various cells [6–8]. If this feedback inhibition also occurs in intact cells, the intracellular dTTP pools may influence the rate of FdUrd phosphorylation and, as a result, may also affect FdUrd toxicity. To test this hypothesis we exploited S49 mutants with genetically altered intracellular dTTP pools.

Materials and methods

Materials. [6-^{3}H]FdUrd (20 Ci/mmole), [6-^{3}H]FdUMP (20 Ci/mmole) and [^{3}H(G)]nitrobenzyl-6-thioinosine (16 Ci/mmole) were purchased from Moravek Biochemicals (Brea, CA). [8-^{3}H]dATP (17 Ci/mmole), [*methyl*-^{3}H]dTTP (44 Ci/mmole) and [5-^{3}H]dCTP (25 Ci/mmole) were purchased from ICN (Irvine, CA). [*Methyl*-^{3}H]Thymidine (5 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Purine and pyrimidine nucleotides were purchased from the Sigma Chemical Co. (St. Louis, MO). Poly[d(A,T)], poly[d(G,C)] and *Escherichia coli* DNA polymerase 1 were purchased from the Miles Chemical Co. (Elkhart, IN).

Cell culture. Mouse T-lymphoma (S49) cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% horse serum. The purine nucleoside phosphorylase-deficient (NSU-1) cell line and two mutant derivatives, a dCMP deaminase-deficient (araC-6-1) clone and a clone with altered ribonucleotide reductase (dGuo-200-1), are described elsewhere [9]. A thymidine kinase-deficient (FdUrd-9) clone and a nucleoside transport-deficient (FdUrd-42) clone were selected by virtue of their resistance to 1 μM FdUrd. Selections of S49 cell mutants were performed as described previously [9]. For cell growth experiments, 5×10^4 cells were incubated in microtiter plates containing 0.2 ml of Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated (30 min, 56°) horse serum in the presence of the indicated concentrations of FdUrd. After 72 hr of growth, cells were counted in a Coulter Cell Counter (Coulter Electronics, Hialeah, FL).

Enzyme assays. Thymidine kinase activity was assayed in cell extracts using [^{3}H]thymidine according to the procedure described by Cheng and Prusoff [10]. The number of nucleoside transport binding sites was determined by measuring the binding of the radioactive nucleoside transport inhibitor nitrobenzylthioinosine as described by Wiley *et al.* [11].

Uptake and binding of [^{3}H]*FdUrd.* For studies of [^{3}H]-FdUrd uptake, S49 cells 2×10^6 cells/ml were incubated at 37° in Dulbecco's Modified Eagle's Medium supplemented with 10% horse serum in the presence of 1 μCi/ml [^{3}H]-FdUrd (20 Ci/mmole) after various incubation times. Aliquots (1 ml) were layered over 0.3 ml of silicone–oil mix, cells were separated by centrifugation at 8000 *g* for 4 min as described by Wiley *et al.* [11], and the radioactivity associated with the cell pellet was determined. In several experiments, the cell pellet was extracted with 0.4 M perchloric acid (PCA), and the radioactivity in FdUrd nucleotides was determined by thin-layer chromatography on polyethyleneimine cellulose plates [12]. More than 90% of the radioactivity in the cell extracts was found in FdUMP.

Measurements of covalently bound FdUrd. For measurements of FdUMP binding to thymidylate synthase, cells were incubated as described above in the presence of the indicated concentrations of [^{3}H]FdUrd (20 Ci/mmole). Thymidylate synthase-bound [^{3}H]FdUrd was measured after this incubation by trichloroacetic acid precipitation onto glass fiber filters as described by Washtien and Santi [13].

Intracellular dTTP measurements. Nucleotides were extracted with 0.4 M PCA at 0° for 30 min, and dTTP levels were assayed by the DNA polymerase method as described by Hunting and Henderson [14], modified as previously described [15].

Results and discussion

Four mutant S49 lymphoma cell lines were used in this study. NSU-1 cells are the parent purine nucleoside phosphorylase-deficient cells from which two additional mutants were selected as described by Weinberg *et al.* [9]. The NSU-1 cells have similar dNTP pools as wild-type cells and thus for the purpose of this study are similar to the wild-type cells [9]. Two mutants selected from NSU-1 cells have altered intracellular dTTP levels. dGuo-200-1 cells were selected for resistance to deoxyguanosine and contain a mutation in the protein M_1 subunit of ribonucleoside diphosphate reductase, which causes the loss of normal feedback inhibition by dATP and in elevated dNTP levels [9]. The mean intracellular level of dTTP in dGuo cells is 19.8 ± 3.1 pmoles/10^6 cells as compared to 6.1 ± 2.3 pmoles/10^6 cells in NSU-1 cells. AraC-1-6 cells are deficient in dCMP deaminase activity, an enzyme participating in dTTP synthesis. As a consequence of this mutation, these cells have decreased dTTP levels (3.6 ± 0.9 pmoles/10^6 cells). Two additional mutants selected for resistance to 1 μM FdUrd with impaired thymidine salvage abilities were also used: FdUrd-9 is a thymidine kinase-deficient mutant (<1% of wild-type activity), and FdUrd-42 is a nucleoside transport-deficient S49 lymphoma mutant (<2% of wild-type number of transport sites). Both dGuo-200-1 cells and araC-1-6 cells have normal levels of nucleoside transport and thymidine kinase activities (within ±15% of wild-type levels).

The sensitivities of the various S49 mutants to growth inhibition by FdUrd are compared in Fig. 1. As expected, the two mutants deficient in thymidine kinase (FdUrd-9) and nucleoside transport (FdUrd-42) were resistant to growth inhibition by FdUrd concentration of >100 nM. In addition, the mutant cells with defective ribonucleoside diphosphate reductase activity (dGuo-200-1) were also less sensitive to growth inhibition by FdUrd as compared to wild-type cells (50% inhibition at 10 and 1 nM respectively). In contrast, the dCMP deaminase-deficient mutant cells (araC-6-1) were sensitive to significantly lower concentrations of FdUrd (50% inhibition at 0.3 nM). Thus, in cells with normal thymidine transport and thymidine kinase activities the degree of resistance to FdUrd seems to correlate with the intracellular dTTP levels, e.g. dGuo-200-1 cells had the largest dTTP pools and were more resistant to growth inhibition by FdUrd while araC-6-1 had the lowest dTTP pools and were the most sensitive to exogenous FdUrd.

To elucidate the mechanism by which dTTP pool size may affect cell sensitivity to FdUrd, we measured [^{3}H]-FdUrd uptake by the various S49 lymphoma mutants (Fig. 2). Again, as expected, both the nucleoside transport-deficient line (FdUrd-42) and the thymidine kinase-deficient line (FdUrd-9) did not take up significant amounts of FdUrd. On the other hand, in the mutants with both normal thymidine kinase and nucleoside transport activities, there was an inverse relationship between dTTP levels and the rate of FdUrd uptake. Of these latter cell lines, the dGuo 200-1 cell line contained the largest dTTP pools and in parallel had a slower rate of FdUrd uptake, while the araC-6-1 cell line had the smallest dTTP pool and took up FdUrd at a faster rate. Since nucleoside phosphorylation is the rate-limiting step in nucleoside uptake [16], these results

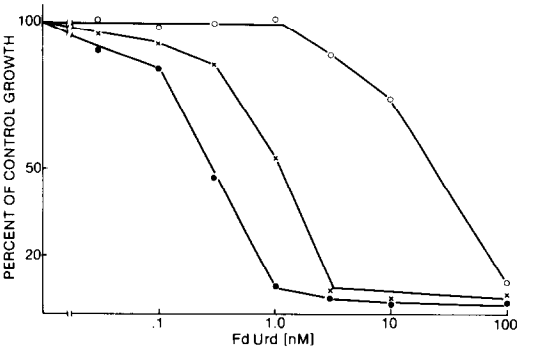

Fig. 1. Growth inhibition of various S49 mutants by FdUrd. Cells (2.5×10^5/ml) were incubated in medium containing the indicated FdUrd concentrations. After growth for 72 hr the number of viable cells was counted in the Coulter Counter, as described in Materials and Methods. Key: the parent line (NSU-1, ×——×); dCMP deaminase-deficient cells (araC-6-1, ●——●); cells with feedback-resistant ribonucleotide reductase (dGuo-200-1, ○——○); thymidine kinase-deficient cells (FdUrd-9, □); and transport-deficient cells (FdUrd-42, ■). The results represent the mean of a single experiment done in triplicate cultures. The range of the triplicate samples did not exceed ±10% of the mean. Three additional experiments gave similar results.

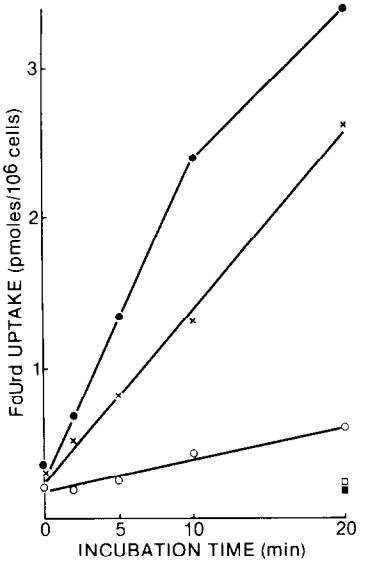

Fig. 2. [^{3}H]FdUrd uptake by S49 lymphoma mutants. Cells (2×10^6/ml) were incubated in Dulbecco's Modified Eagle's Medium in the presence of 1 μCi [^{3}H]FdUrd (20 Ci/mmole). At the indicated times, 1.0-ml aliquots were removed, and [^{3}H]FdUrd uptake was determined as described in Materials and Methods. Key: parent cell line (NSU-1, ×——×); dCMP deaminase-deficient cells (araC-6-1, ●——●); cells with feedback-resistant ribonucleotide reductase activity (dGuo-200-1, ○——○); thymidine kinase-deficient cells (FdUrd-9, □); and nucleoside transport-deficient cells (FdUrd-42, ■).

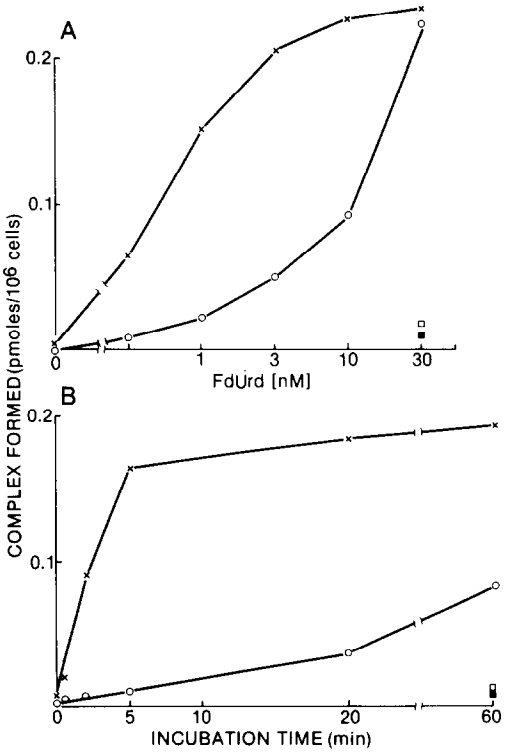

Fig. 3. Kinetics of [^{3}H]FdUrd–thymidylate synthase complex formation in various S49 lymphoma mutants. S49 cells (2×10^6) were incubated in 1.0 ml of growth medium with various concentrations of [^{3}H]FdUrd (20 Ci/mmole) for 2 hr (A), or in the presence of 50 nM [^{3}H]FdUrd for various periods of time (B). At the end of the incubation periods, cells were harvested, and the amount of [^{3}H]FdUrd–thymidylate synthase complex was assayed as described in Materials and Methods. Key: the parent cell line (NSU-1, ×——×); cells with feedback-resistant ribonucleotide reductase activity (dGuo-200-1, ○——○); thymidine kinase-deficient cells (FdUrd-9, □); and nucleoside transport-deficient cells (FdUrd-42, ■).

suggest an important role for dTTP in the regulation of thymidine kinase activity in intact cells.

FdUMP, the phosphorylation product of FdUrd, blocks dTTP synthesis by inhibiting thymidylate synthase to which it is virtually irreversibly bound [4]. It is thus possible to measure the degree of inhibition of thymidylate synthase by measuring the binding of [^{3}H]FdUMP to thymidylate synthase [13]. To find out whether the variations observed in the rates of FdUrd uptake can affect the degree of inhibition of thymidylate synthase, we measured the formation of [^{3}H]FdUMP–thymidylate synthase complex in mutant S49 cells incubated in the presence of FdUrd (Fig. 3). A higher extracellular concentration of FdUrd was required for maximal FdUMP binding in dGuo-200-1 cells than in the wild-type cells (Fig. 3A). In addition, the rate of FdUMP binding to thymidylate synthase was slower in the dGuo-200-1 cells than in the wild-type cells (Fig. 3B). However, the maximal amounts of FdUMP bound to thymidylate synthase after prolonged incubation time (2 hr), and at high extracellular FdUrd concentrations (30 nM), were comparable in both cell populations (Fig. 3A). These results support the hypothesis that the decreased rate of FdUMP formation in dGuo-200-1 cells is responsible for the decreased thymidylate synthase inhibition in these cells compared to wild-type cells. The nucleoside transport-deficient cells (FdUrd-42) and the thymidine kinase-deficient cells (FdUrd-9) did not bind any FdUMP even after lengthy incubations (Fig. 3B). In addition, dGuo-200-1 cells reached the maximum amount of FdUMP binding at higher extracellular FdUrd concentration than the wild-type cells (Fig. 3A); this correlates with the slower rate of FdUrd uptake and relative growth resistance of these cells to FdUrd.

In conclusion, our results demonstrate a role for intracellular dTTP levels in the regulation of thymidine kinase activity in intact S49 cells. *In situ*, inhibition of thymidine kinase activity by high dTTP levels caused a slower rate of FdUrd phosphorylation and decreased inactivation of thymidylate synthase, resulting in resistance to growth inhibition by exogenous FdUrd concentrations. These observations suggest a possible role for intracellular dTTP levels of various tumor cells in determining their degree of sensitivity to FdUrd. Moreover, these results also suggest that manipulations of intracellular dTTP levels by drug combination chemotherapy might be a possible approach in potentiating FdUrd cytotoxicity.

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Glutathione *S*-transferase activity during pregnancy in the mouse: effects of *trans*-stilbene oxide pretreatment

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Thioether formation catalyzed by glutathione *S*-transferases (GSHTr, EC 2.5.1.18) represents one of the major mechanisms of *in vivo* detoxication of xenobiotics [1–3]. Recent studies have shown that metabolism of vicinal dihalogens by GSHTr may lead to formation of potent mutagens [4–6]. In mammals, essentially all organs are known to possess this enzyme system [1, 7]. In contrast to the liver, which contains several forms of GSHTr [1], human placenta is reported to possess a single form of this enzyme [8, 9], and its activity is not altered following exposure of pregnant women to the constituents of cigarette smoke [10, 11]. GSHTr activity has also been detected in the placentas of rabbits, guinea pigs [12, 13], rats [14], and monkeys [15]. Data are also available on the changes in the titers of placental GSHTr with gestational age in rabbits and guinea pigs [12, 13]. However, similar information is not available at present for the mouse placental enzyme.

Because of obvious pharmaco-toxicological implications, alterations in the drug-metabolizing enzymes following exposure of pregnant animals to different chemicals have received considerable attention [16–23]. Most of these investigations deal with cytochrome P-450-dependent monooxygenase activity, and little is known about the effects these chemicals have on GSHTr. Earlier, Bell *et al.* [22] reported noninducibility of hepatic GSHTr after treatment of pregnant rats with the classical inducer phenobarbital. However, in subsequent studies by Rouet *et al.* [23], induction of liver GSHTr was observed in pregnant rats and mice exposed to 5,6-benzoflavone. In these reports, possible alteration in the placental GSHTr was not considered. Thus, the question of inducibility of GSHTr during pregnancy warrants further examination. Although in males of both mice and rats the liver GSHTr are inducible enzymes and a number of chemicals including pesticides and carcinogens have been shown to be inducers, the mouse liver enzymes seem to be more responsive [1, 24, 25]. Therefore, the objectives of the present study were to examine (i) the quantitative changes in the mouse hepatic and placental GSHTr activity due to pregnancy, and (ii) the effects of exposure to a potent inducer of this enzyme system, namely *trans*-stilbene oxide (TSO) [26–28].

Materials and methods

Young virgin mice of the CD_1 strain were purchased from the Charles River Breeding Laboratories and housed for at least 1 week prior to use. Purina 5001 mouse chow and water were given *ad lib.* Animal rooms were on a 12-hr light/dark schedule. The date on which a mating plug was observed was designated as day zero of pregnancy. TSO (Aldrich Chemical Co.) in corn oil was administered intraperitoneally once daily for 3 days on either days 9, 10, and 11 or on days 15, 16 and 17 of gestation at doses of 0 (control), 100, 300, or 500 mg/kg. Animals were killed 24 hr after the last dose (on day 12 or 18 of gestation) by cervical dislocation. Placentas and livers were examined *in situ* for signs of toxicity, removed quickly, weighed, and placed in cold 50 mM Tris buffer, pH 7.4, containing 0.25 M sucrose and 1.0 mM EDTA. Placentas were pooled by litter while maternal livers were processed individually to isolate cytosol by differential centrifugation [25].

Cytosolic GSHTr activity was assayed spectrophotometrically at 37° using 105,000 *g* supernatant fluid as the