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INHIBITION OF THYMIDYLATE SYNTHETASE OF WALKER CARCINOMA BY CHLORAMBUCIL. A POSSIBLE MECHANISM OF ACTION*

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Abstract—Effects of chlorambucil (CA) on growth of Walker carcinoma 256 and on enzymes involved in the synthesis of thymidine monophosphate are described. A single intraperitoneal dose (25 mg/kg) or multiple doses (8 mg/kg/day for 3 days) of CA were "curative" for drug-sensitive (WS) tumors. Thymidylate (dTMP) synthetase activity of WS tumors was significantly decreased (approximately 25%) at 3 hr, reached its lowest level (approximately 50% loss) at 12 hr, and remained at this level up to 60 hr after administration of 25 mg/kg of CA. Dihydrofolate (FAH_2) reductase activity did not change significantly up to 36 hr and then slowly decreased (approximately 20% loss) by 60 hr. Thymidine (TdR) kinase activity of WS tumors was not affected. Treatment of WS tumors with multiple doses of CA also resulted in pronounced inhibition of dTMP synthetase activity (approximately 50% loss), some decrease (approximately 15% loss) in FAH_2 reductase activity and no change in TdR kinase activity. A Chlorambucil-resistant strain (WR) of Walker carcinoma was developed. In contrast to WS, after treatment with CA the enzyme activities of WR tumors remained essentially the same as those from untreated animals. *In vitro* incubation of partially purified dTMP synthetase enzymes from either WS or WR tumors with CA inhibited both enzymes to the same extent (approximately 50% loss at 1.25×10^{-6} M). TdR kinase and FAH_2 reductase activities were not inhibited up to 1.25×10^{-4} M CA. The results of these studies support the concept that CA exerts cytotoxic activity by inhibition of dTMP synthetase.

Activities of dTMP synthetase and TdR kinase were found to be significantly altered in WR tumors as compared to WS tumors. The activity of dTMP synthetase was decreased approximately 20% and that of TdR kinase was increased approximately 35% in WR tumors. Resistance to CA may be due, in part, to increased dependence of WR tumors on the salvage pathway for synthesis of dTMP.

Biological alkylating agents, such as nitrogen mustards, ethyleneimines, and esters of alkylsulphonic acids have received considerable attention for their tumor inhibiting activities [1–3]. These compounds are highly reactive and have been shown to interact with a variety of nucleophilic groups on enzymes [4, 5], nucleic acids [6, 7] and reduced folate coenzymes [8]. A common effect of alkylating agents is the inhibition of DNA synthesis at dose levels which do not inhibit synthesis of other macromolecules [3, 9]. It has been suggested that DNA is the primary target site and that bifunctional alkylating agents act primarily by alkylation and crosslinking of guanine moieties in DNA [7]. However, experiments with whole animals have failed to show a good correlation between the amount of alkylating agent bound to cellular DNA and the sensitivity of the tumor to alkylating agent. Therefore, other investigators have argued in favour of molecular sites other than DNA as the most volnerable targets for these drugs [10–12].

Chlorambucil [*p*-*N*,*N*-di-(β-chloroethyl) aminophenylbutyric acid] is a bifunctional alkylating agent, useful for the treatment of chronic lymphocytic leukemia and testicular carcinoma [2]. It has been shown to inhibit synthesis of DNA and RNA in L-1 tumor cells [13]. Recently, it has been shown that chlorambucil (CA) at "curative" doses can cause accumulation of DNA, RNA, protein and glutathione [11], interfere with the biosynthesis of histone proteins [14], and inhibit incorporation of [^{3}H]thymidine into DNA [15] of drug-sensitive Yoshida ascites sarcoma cells. We have conducted studies to investigate the action of CA using a drug-sensitive (WS) and a drug-resistant (WR) form of the Walker carcinoma 256. The effects of CA on regression of the WS and WR forms of the tumor and activities of selected tumor enzymes involved in the synthesis of thymidine monophosphate (dTMP) were examined. Thymidylate synthetase (EC 2.1.1.6), is responsible for *de novo* dTMP synthesis. It requires tetrahydrofolate (FAH_4) which is produced by dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3). The alternate or salvage pathway for dTMP synthesis is through thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.2.21). These enzymes were selected because dTMP is a regulatory metabolite in DNA synthesis [16]. The results of these studies are presented in this paper.

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MATERIALS AND METHODS

Tumor transplantation. Six-week-old male Wistar rats (Canadian Breeding Farm and Laboratories Ltd., Quebec, Canada) weighing 140–160 g were used for all experiments. Walker carcinoma tumor sensitive to alkylating agents was obtained through the courtesy of Prof. A. C. Wallace, Dept. of Pathology, Univ. of Western Ontario, London, Canada and was propagated by regular transplantation into male rats every 10 days. A suspension of tumor cells from a 10–12 day old drug-sensitive and drug-resistant tumor was prepared in sterile saline [17] and 0.2 ml of this suspension containing approximately 2×10^5 cells was injected i.m. into the ventromedial region of each thigh. Tumor resistant to CA was obtained by repeated treatment with progressively increasing doses of the drug. The resistance was maintained by regular treatment with 25 mg/kg of CA every 8 days after implantation of the tumor in animals. Measurements of tumor size were made with calipers and tumor weight was calculated on the assumption that tumors were prolate spheroids with a density of 1.0 [18].

Treatment schedules. CA solution was made fresh daily by dissolving in a minimum vol. of 95% ethanol and diluting with 1% sodium bicarbonate solution (ethanol–bicarbonate solution ratio 1:5). Concentrations were adjusted so that all animals received 0.2–0.3 ml of solution. The drug was administered by the i.p. route. Control animals were given the same vol. of ethanol–bicarbonate solution. Treatment was initiated on day 8 after tumor transplantation.

For enzyme studies, two treatment schedules were employed: (i) a single dose (10 mg/kg or 25 mg/kg) of CA was administered and rats were sacrificed at 3, 6, 9, 12, 18, 24, 36, 48, and 60 hr after treatment, (ii) to study the effects of multiple doses of CA, 5 mg/kg/day or 8 mg/kg/day of drug was administered on 3 consecutive days and rats were sacrificed 24 hr after the last treatment.

Tumor tissue. The rats were killed by cervical dislocation. The tumor tissue was carefully separated from muscle and necrotic tissue and was cooled in a petri dish on ice. Extracts for enzyme activity measurements were prepared either from fresh tumor or from tissue stored at −70° for 1–2 days. Enzyme activities were stable in the frozen tissue.

Extracts were obtained by homogenizing 2 g tissue in 8 ml of Tris–HCl buffer (50 mM, pH 7.4) containing 1 mM EDTA and 10 mM 2-mercaptoethanol. Homogenization was at 4000 rpm for 2 min in a glass–teflon homogenizer. The homogenate was centrifuged at 37,000 *g* for 30 min and the supernatant was retained for the assays of enzyme activities.

Enzyme activities. Reactions were initiated by addition of substrates and corrections were made for substrate blanks. Spectrophotometric assays were conducted at 25° in a Unicam SP800 or SP1700 equipped with program controller and scale expansion accessories. In this report one enzyme unit refers to the synthesis of 1 nmole of product per hr and enzyme activity is expressed per mg protein determined by the method of Lowry *et al.* [19].

Thymidylate synthetase activity was assayed by a spectrophotometric method [20]. The assay mixture contained: Tris HCl buffer, pH 7.4, 110 μmoles; 2-mercaptoethanol, 30 μmoles; formaldehyde, 7 μmoles; *dl*-L-FAH_4, 0.2 μmole; $MgCl_2$, 60 μmoles; EDTA, 0.02 μmole, and enzyme extract in a total vol. of 1.0 ml. All components (except dUMP) were incubated with the enzyme for 10 min at 25° and then the reaction was initiated by adding 0.2 μmole of dUMP.

Dihydrofolate reductase was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm [21]. The reaction mixture contained: Tris–HCl buffer, pH 7.0, 100 μmoles; KCl, 150 μmoles; NADPH, 0.1 μmole; FAH_2, 0.05 μmole; mercaptoethanol, 10 μmoles; enzyme extract; and water to make a final vol. of 1.0 ml. A molar extinction coefficient of 12,000 [22] at 340 nm was used for calculation of activity.

Thymidine kinase activity was determined by the rate of ^{14}C incorporation from [2-^{14}C]thymidine into dTMP, dTDP, and dTTP [23]. The assay mixture contained: ATP, 5 μmoles; $MgCl_2$, 5 μmoles; NaF, 0.5 μmole; enzyme extract; mercaptoethanol 5 μmoles; [2-^{14}C]thymidine (S.A. 59 mCi/m-mole), 2.5 mμmoles; and Tris HCl buffer pH 7.5, 10 μmoles in a total vol. of 0.25 ml. After incubating the reaction mixture at 37° for 15 min, the reaction was terminated by immersing the tubes in boiling water for 3 min. The pyrimidine deoxyribonucleotides were separated by descending chromatography (16–18 hr) with isopropanol-1% $(NH_4)_2SO_4$ in water (2:1) as solvent. Positions of the deoxyribonucleotides were located under ultraviolet light with the aid of reference standards. The spots were cut out and placed into 10 ml of scintillation fluid for counts of radioactivity. The scintillation fluid contained: PPO, 4 g; POPOP, 0.1 g; Triton X-100, 500 ml and toluene, 1000 ml. R_f values were: thymidine, 0.72; dTMP, 0.33; dTDP, 0.20 and dTTP, 0.08. Radioactivity was determined in a Nuclear Chicago Unilex II counter.

In vitro inactivation experiments were carried out by incubation of the cell free extract or dialyzed ammonium sulfate fraction (40–60%) from WS and WR tumors with different concentrations of CA at 37° for 60 min. The samples from reaction mixture were removed at 15 min intervals and assayed for dTMP synthetase, TdR kinase and FAH_2 reductase activities using the standard assay procedures. Enzyme controls were run simultaneously to verify that the alterations in enzymatic activity were not due to inactivation at 37°.

Statistical analysis. The *t*-test was used for comparison of the treatment values.

Chemicals. dl-L-Tetrahydrofolic acid was prepared by the method of Gupta *et al.* [8]. Dihydrofolate was synthesized by the procedure of Blakley [24] and stored as a fine suspension in 0.001 N HCl in vials at −100°. Concentrations of FAH_2 [24], FAH_4 [20] and NADPH [25] were determined spectrophotometrically using published molar extinction coefficients.

Chemicals were obtained from the following commercial sources: Folic acid, 2-mercaptoethanol, Tris (Sigma 7-9) and unlabelled nucleosides and nucleotides, Sigma Chemical Co. St. Louis, MO; EDTA (disodium salt), toluene (scintillation grade) and trichloroacetic acid, Fisher Scientific Co.; chlorambucil, Cyclochemical Corp. CA; [2-^{14}C]thymidine, Schwarz

Mann, Toronto, Ontario; and PPO, POPOP and Triton X-100, Amersham Searle, Oakville, Ontario.

RESULTS

Effect of CA upon the growth of drug-sensitive and drug-resistant tumors

Tumors were palpable 6 days after transplantation and were 16–18 mm in diameter when treatments were initiated on day 8. Untreated rats died between 16 and 20 days after transplantation. Figure 1 shows the growth and regression of WS tumors in control and CA treated animals. Single doses of CA were administered at 5, 10 and 25 mg/kg. A significant decrease in tumor size compared to the control was observed 36–48 hr after treatment. The decrease in growth rate of the tumor was related to the dose of CA administered. At the maximum tolerated dose (25 mg/kg), complete eradication of the tumor mass was achieved in 80–85 per cent of the animals. No tumors could be detected by palpation up to 40 days and no mortality was recorded up to 60 days after treatment. This dose is considered a "curative" dose in these experiments. Similarly, treatment with multiple doses of CA (5 mg/kg/day or 8 mg/kg/day) for 3 days was also found to inhibit proliferation of WS tumors and eventually eradicate the tumor mass in all animals (Fig. 1).

The growth rates of untreated WR tumors and of tumors treated with a single dose of 25 mg/kg are shown in Fig. 2. The growth of the treated tumors was only slightly slower than WR tumors growth in untreated animals. Administration of multiple doses of CA 8 mg/kg/day for 3 days also failed to retard growth of WR tumors.

Effect of CA on the activities of enzymes in drug-sensitive and drug-resistant tumors

A. *Single dose treatment.* Figure 3 shows activities of the enzymes in WS and WR tumors from control animals and from animals given 25 mg/kg of CA. A pronounced inhibition of dTMP synthetase activity was observed in WS tumors after CA treatment (Fig. 3A). The synthetase activity was inhibited (approximately 25 per cent) at 3 hr (first sample analyzed post medication), reached its lowest level (approximately 50 per cent of zero time) at 12 hr and the activity remained at this level up to 60 hr (last sample analyzed) after administration of the alkylating agent. In contrast, treatment with CA of rats bearing drug-resistant tumors resulted only in a slight decrease (approximately 10%, Fig. 3A) at 3 hr but thereafter activity rapidly recovered from inhibition and reached the same level as in the control group by 9 hr post treatment. The activities of dTMP synthetase in untreated WS and WR tumors remained essentially unchanged during this period (Fig. 3A). Thymidine kinase activity was not affected ($P < 0.05$) after a single dose of CA treatment in WS or WR tumors (Fig. 3B). There appeared to be no consistent difference in FAH_2 reductase activity between control and treatment groups in WS tumors up to 24 hr after treatment but activity in the treated group was less than in the control group ($P < 0.05$) at 36, 48 and 60 hr after administration of CA (Fig. 3C). The FAH_2 reductase activity of WR tumors remained unchanged after treatment with the drug.

Administration of a lower dose (10 mg kg) of CA also resulted in a rapid decrease of dTMP synthetase activity in WS tumors (maximum reduction 40 per

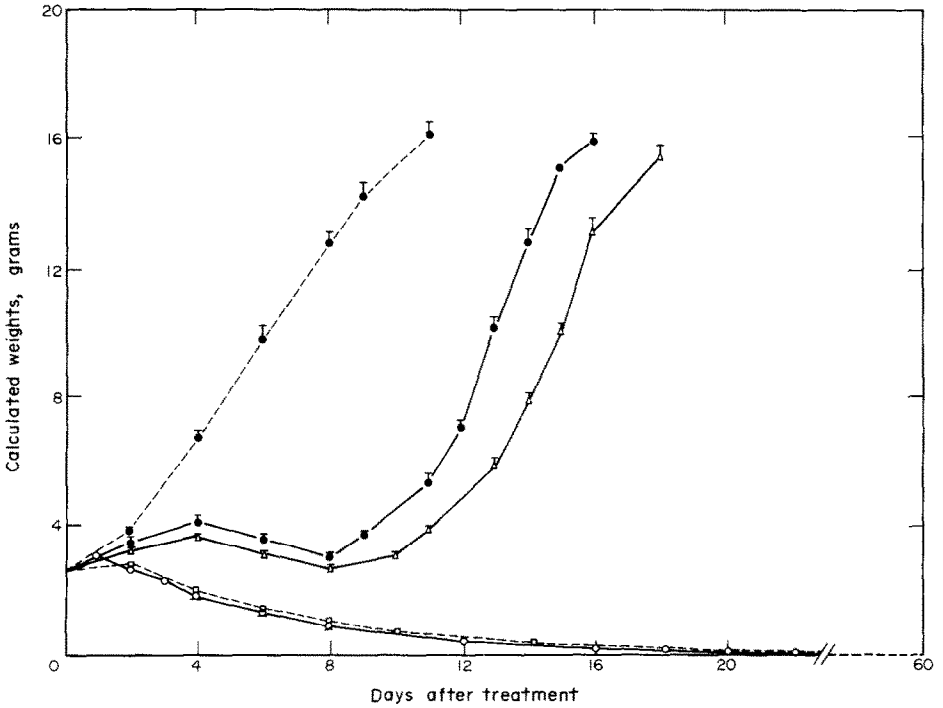

Fig. 1. Effects of i.p. doses of chlorambucil on the growth of sensitive Walker carcinoma. ●---● control; —●— single dose (5 mg/kg); —△— single dose (10 mg/kg); —○— single dose (25 mg/kg); □---□ multiple doses (8 mg/kg/day for 3 days). Each point is the mean value for 16–20 tumors. Vertical bars indicate standard error.

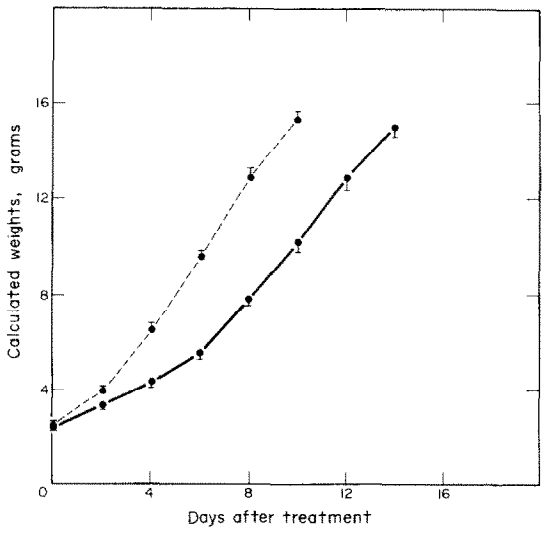

Fig. 2. Effects of a single i.p. dose of chlorambucil (25 mg/kg) on the growth of chlorambucil-resistant Walker carcinoma. ● ● control; treated animals --●--. Each point is the mean value for 16–20 tumors. Vertical bars indicate standard error.

cent compared to control animals at 9 hr). After 9 hr the enzyme activity steadily increased and by 48 hr reached the same levels as in the control group (data not shown). At this dose CA afforded only temporary regression of tumor.

It is interesting to note that dTMP synthetase activity of WR tumors was significantly lower (approximately 20 per cent) than control WS tumors. On the other hand, TdR kinase activity was significantly higher (approximately 30–40 per cent) in control WR tumors than in control WS tumors.

B. *Multiple doses treatment.* The effects of multiple doses of CA (8 mg/kg/day) for 3 days on dTMP synthetase, TdR kinase and FAH_2 reductase activities of WS and WR tumors are shown in Fig. 4. After CA treatment of WS tumors there was pronounced inhibition of dTMP synthetase activity (approximately 50 per cent), some decrease in FAH_2 reductase activity (approximately 15 per cent) and no significant change in TdR kinase activity. The dTMP synthetase, FAH_2 reductase and TdR kinase activities in WR tumors remained essentially unchanged after administration of CA in multiple doses. These results are in agreement with data on enzymes obtained after administration of a single "curative" dose of CA (*vide supra*).

In vitro *experiments*

These studies were undertaken to determine if CA has the ability to inactivate the same enzymes in cell free extract. The effect of different concentrations of CA on dTMP synthetase activity of WS and WR tumors are shown in Fig. 5. The dTMP synthetase activities of both WS and WR tumors were inhibited to almost the same extent in the presence of CA. The loss of dTMP synthetase activity was found to be related to the concentration of CA in the reaction mixture and the duration of incubation. At 1.25×10^{-6} M concentration of CA, approximately 50 per cent loss of dTMP synthetase activity was observed after 60 min of incubation. There was a further decrease in activity when the concentration of alkylating agent was increased to 1.25×10^{-5} M; only 25 per cent of the initial activity remained after 30 min. Dialysis of CA-inactivated enzyme against Tris–HCl, pH 7.5 or Tris buffer containing mercaptoethanol 0.05 M failed to restore enzyme activity. The substrate, dUMP (up to 500 μM) also failed to stabilize dTMP synthetase from CA inactivation. There was little or no inhibition of TdR kinase and FAH_2 reductase activities up to 1.25 ± 10^{-4} M concentration of CA.

DISCUSSION

The response of WS tumors after treatment with CA was essentially similar to that reported for the solid form of plasmacytoma in hamsters with other alkylating agents in earlier stages of tumor regression [26]. Our results, however, differ from the effect of CA on sensitive Yoshida ascites sarcoma [11], where a single dose (8 mg/kg) of the drug was reported to be "curative". One obvious reason for the difference in sensitivity of drug-sensitive Walker carcinoma to CA as compared to that of drug-sensitive Yoshida ascites sarcoma is that in these studies the solid form of the tumor was used. Other investigators have also reported that sensitivity of a tumor to the same drug can vary according to its site of implantation [27, 28]. Of particular interest was the observation that multiple treatments with CA were more effective in eradication of tumor mass than a maximal tolerated single dose (25 mg/kg) even though the total dose (15 mg kg) for multiple treatments was lower than for the single treatment.

The most striking aspect of the enzymatic data was the marked and relatively early reduction in dTMP synthetase activity after CA treatment in drug-sensitive tumors. The regression of tumor growth and eventually complete "cure" was found to be related to the decrease in dTMP synthetase activity. In contrast, dTMP synthetase activity of drug-resistant tumors remained essentially the same as the control animals after treatment with CA. The *in vitro* inactivation experiments showed that of the three enzymes studied, only dTMP synthetase activity was inhibited by CA, in the drug-sensitive tumors. Since dTMP synthetase is essential for cell division, these results suggest that tumor regression was due in part to the loss of activity of this key enzyme.

Recent results indicate that incorporation of [^{3}H]deoxyuridine into DNA was selectively inhibited by CA in drug-sensitive Walker carcinoma ascites cells*. In contrast, uptake of [^{3}H]thymidine was not affected. These results also provide support for the key role of dTMP synthetase inhibition in relation to cytotoxicity of CA.

The slight decrease in FAH_2 reductase activity was likely a secondary response because it was not observed until 36 hr after administration of CA. One possible reason for decreased FAH_2 reductase activity may be the decreased requirement for reduced folate coenzymes.

* S. K. Srinivasan and V. S. Gupta, unpublished results.

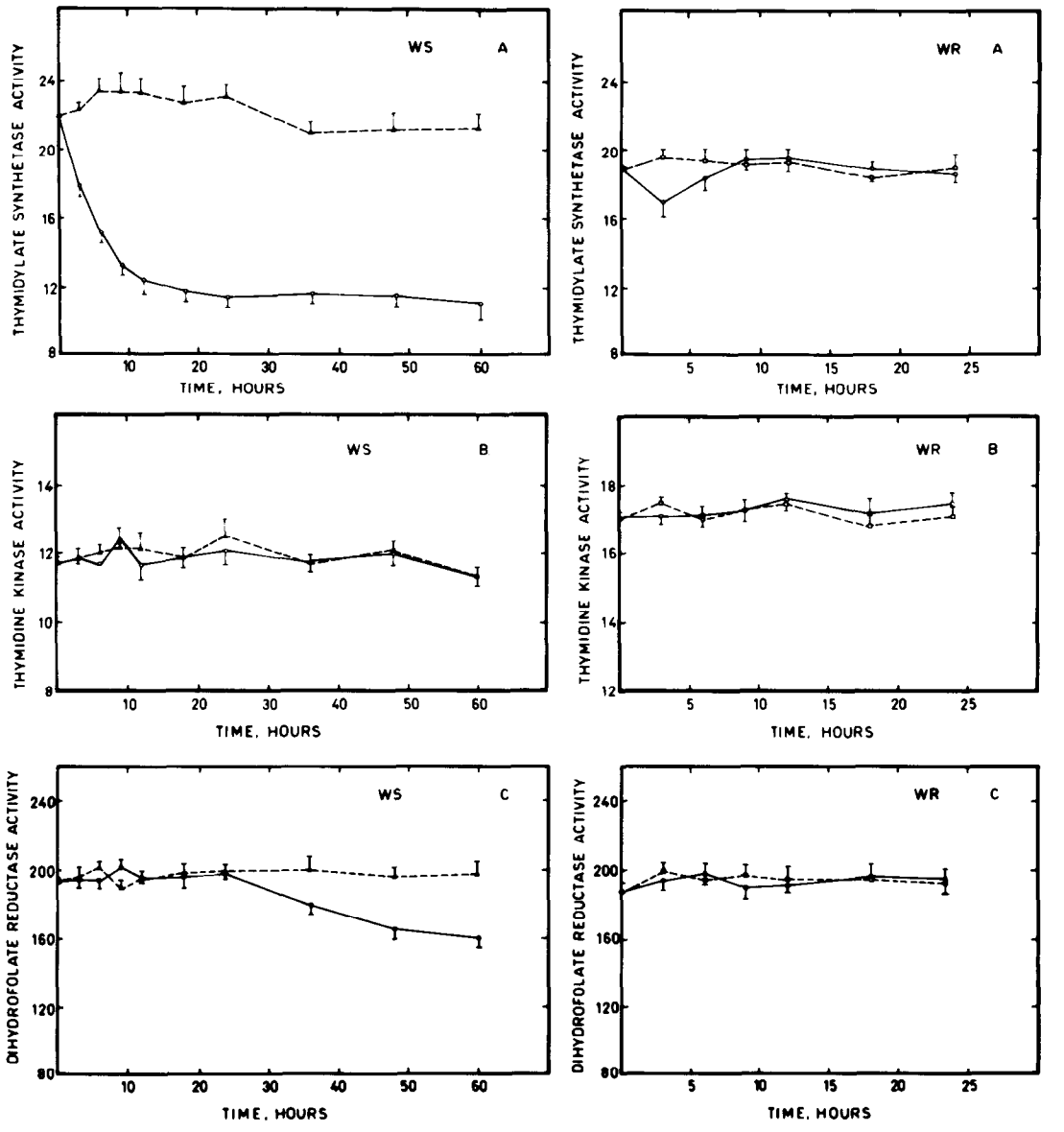

Fig. 3. Effects of a single i.p. dose of chlorambucil (25 mg/kg) on thymidylate synthetase (A); thymidine kinase (B) and dihydrofolate reductase (C) activities of sensitive (WS) and chlorambucil-resistant (WR) Walker carcinoma. The results obtained with tumors from treated animals are shown by the solid line —— ; the dotted line ----- show activities of tumors from control animals. Each point represents a mean value for 16–20 tumors. Vertical bars indicate standard error.

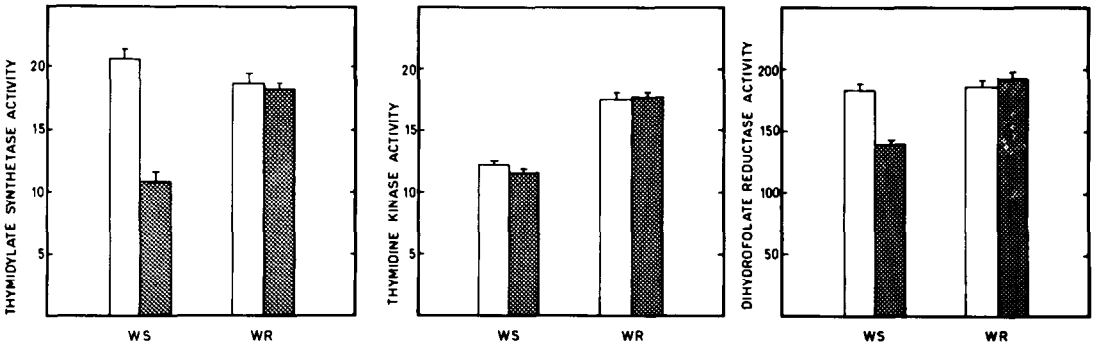

Fig. 4. Effects of multiple doses (8 mg/kg/day for 3 days) of alkylating agent on thymidylate synthetase, thymidine kinase and dihydrofolate reductase activities of sensitive (WS) and resistant (WR) Walker carcinoma. The results obtained from control animals are shown by open bars; the stippled bars indicate enzyme activities of tumors from treated animals. Each bar represents the mean value (± the standard error) for 16–20 tumors.

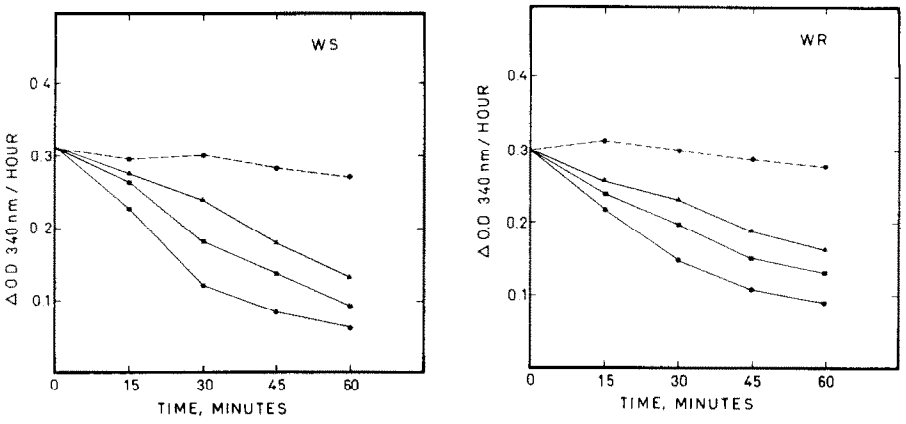

Fig. 5. *In vitro* inhibition of thymidylate synthetase from sensitive (WS) and resistant (WR) Walker carcinoma on treatment with chlorambucil. Control ●—●; chlorambucil 1×10^{-6} M, ▲—▲: 1×10^{-5} M ■—■ and 1×10^{-4} M ●—●.

Although, acquired resistance to alkylating agents is a common phenomenon observed in both experimental animals and man, the biochemical mechanisms responsible for the development of resistance to this class of drugs are poorly understood [29]. Yoshida ascites sarcoma cells resistant to CA were shown to have decreased permeability and lower protein binding capacity as compared to drug-sensitive cells [30]. In the present investigation, the activity of dTMP synthetase was decreased and that of TdR kinase was increased in drug-resistant tumors. Since TdR kinase activity was not inhibited on treatment with CA *in vivo* or *in vitro*, it is possible that increased levels of TdR kinase activity in WR tumors may also be a contributory factor in the resistance to this alkylating agent.

In summary, of the three enzymes studied, dTMP synthetase was the only enzyme whose activity was markedly decreased upon treatment with CA *in vivo* and *in vitro*. These results suggest that anti-tumor activity of CA may occur by inhibition of the reactions leading to DNA synthesis rather than by alkylation of DNA. Experiments are now in progress to determine further the relevance of the effect on dTMP synthetase and its relation to cytotoxic activity by use of other bifunctional alkylating agents. Studies are also underway to investigate the interaction between CA and dTMP synthetase by the use of [^{14}C]chlorambucil and purified enzyme from Walker carcinoma.

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