Studies have been carried out to elucidate the mechanism of the selective antitumor action of 5-aziridinyl-2,4-dinitrobenzamide (CB 1954) against the British line of Walker 256 tumor. This drug, at a concentration of 1 \( \mu \text{g/ml} \), produced biochemical effects in ascites tumor cells, which appear to be identical to those of the difunctional alkylating agent, melphalan. The incorporation of thymidine-\(^{3}H\) into DNA was inhibited progressively and, after 4 to 12 hr of incubation of tumor cells with the drug, proceeded at about one-half the normal rate. The utilization of radioactivity from uridine-6-\(^{3}H\) for DNA synthesis was also diminished by drug treatment, whereas no corresponding effect on \( \text{de novo} \) purine, RNA, or protein synthesis was noted. Increased concentrations of CB 1954 produced only a slightly greater effect on thymidine incorporation into DNA. In contrast, thymidine incorporation into the DNA of six other rodent tumors, including a U. S. line of the Walker tumor, which were resistant to the carcinostatic effects of CB 1954, was unaffected by drug treatment at the usual concentrations. However, in a line of the British Walker tumor that had acquired resistance to CB 1954, thymidine incorporation was depressed by CB 1954, suggesting that the initial damage produced by drug treatment was readily reversible. In a survey comparing incorporation of isotopically labeled precursors into the 3 lines of the Walker tumor, the rate of thymidine incorporation into the DNA's of tumors resistant to CB 1954 was increased.

INTRODUCTION

CB 1954 has been demonstrated to be a potent and selective inhibitor of the British Walker carcinoma 256 (3). CB 1954 had the highest therapeutic index ever reported for this tumor, and its structural specificity permitted few alterations in functional groups without great loss of biological activity (7). CB 1954 was quite atypical as an antitumor alkylating agent in that it was monofunctional and had a very narrow spectrum of antitumor activity. However, a line of the Walker tumor with acquired resistance to CB 1954 was cross-resistant to melphalan (6). The carcinostatic properties of CB 1954 could be reversed by coadministration of AIC or purine ribonucleosides, suggesting that the drug also had properties of a purine antagonist (5).

The importance of understanding the mechanism of action of this drug was suggested particularly by its narrow antitumor specificity, its simple but novel chemical structure for a tumor-inhibitory drug, and its sparing toxicity against the hematopoietic system (2). A preliminary report on this investigation has appeared (8).

MATERIALS AND METHODS

Drugs and Radioactive Compounds. CB 1954 was dissolved in dimethyl sulfoxide and stored at 0\(^\circ\). Aliquots of this solution were added to water for addition to the cell suspensions. Corresponding quantities of dimethyl sulfoxide were added to control incubates. Melphalan (20 mg) was dissolved in 0.2 ml ethanol:HCl (98:2) and diluted to at least 20 ml with buffer solution consisting of dipotassium hydrogen phosphate, 2%, and propylene glycol, 45% in water. Propylene glycol buffer was added to control flasks.

The following quantities of labeled precursors were added per ml of cell suspension: adenine-8-\(^{14}C\), 59 mCi/mmmole, 0.2 \( \mu \text{Ci/ml} \) for Procedure 2 (see below) and 1.5 \( \mu \text{Ci/ml} \) for Procedure 4; formate-\(^{14}C\), 41.7 mCi/mmmole, 2.5 \( \mu \text{Ci/ml} \); glycine-2-\(^{3}H\), 2 \( \mu \text{Ci/ml} \); leucine-4,5-\(^{3}H\), 19 Ci/mmmole, 5 \( \mu \text{Ci/ml} \); uridine-5-\(^{3}H\), 2 \( \mu \text{Ci/ml} \); uridine-6-\(^{3}H\), 3 \( \mu \text{Ci/ml} \); thymidine-methyl-\(^{3}H\), 5 \( \mu \text{Ci/ml} \); and AIC-2-\(^{14}C\), 24.2 mCi/mmmole, 0.5 \( \mu \text{Ci/ml} \).

These radioactive compounds came from the Radiochemical Centre, Amersham, England, except for AIC, which was purchased from Calatomic, Los Angeles, Calif.

Tumors. Three lines of the Walker 256 tumor were used. The WS was the British line and was sensitive to CB 1954, while the WR was a line derived from it with acquired resistance to CB 1954 (6). The WZ was the U. S. line of the Walker that was naturally resistant to CB 1954. No...
differences could be shown with respect to DNA content of these tumor lines. The WS, WR, and WZ tumors contained 59.4, 61.5, and 55.2 μg DNA per mg dry weight, respectively. All 3 lines were passaged weekly in Wistar rats by i.p. injection of 2 × 10⁶ cells. The Yoshida tumor was passaged similarly.

Four mouse tumors were used and were maintained by routine passaging in the ascites form. They were the Gardner lymphosarcoma and TLX 5 in CBA/Lac mice and the NK lymphoma and ADJ/PC6A (PC6) in C- mice. They were all resistant to CB 1954 (6).

Incorporation Procedures. Ascites tumor cells were removed from the rat or mouse into 0.3% NaCl solution to hemolyze red cells. The suspension was centrifuged at 700 × g for 5 min, and the tumor cells were resuspended in 2 ml 0.9% NaCl solution for counting on a Coulter Model F × particle counter. The cells were then suspended in Tissue Culture Medium 199:horse serum (60:40) at a concentration of 1.5 × 10⁶ cells/ml. They were all resistant to CB 1954 (6).

Procedure 1 allowed for rapid assay of total uptake of radioactivity in the cells. In Procedure 2 the acid-soluble fraction has been extracted and the remaining radioactivity was incorporated into the acid-insoluble cell residue. The difference in radioactivity between Filters 1 and 2 represents the pool fraction, which was not measurable in thymidine-³H or leucine-²H experiments but which was extensive when labeled uridine, adenine, or AIC were used. Procedure 3 results in hydrolysis of nucleic acids, and the residue on the filter is largely protein. The difference in radioactivity between Filters 2 and 3 represents largely incorporation into nucleic acids. In Procedure 4, RNA has been hydrolyzed to soluble mononucleotides which are washed into the filtrate. Thus, if a precursor such as labeled thymidine is used which is incorporated solely into DNA, radioactivity on Filters 2 and 4 should be identical. The close agreement of the 2 methods in Chart 1 validates procedure 4 as a quantitative measure for incorporation into DNA exclusively, providing that labeled precursors solely for nucleic acids are used.

RESULTS

Effect on Major Biosynthetic Reactions of the Sensitive Walker Tumor. Walker WS ascites cells were incubated in Tissue Culture Medium 199:horse serum in the presence and absence of CB 1954, and isotopically labeled compounds were added to detect drug-induced alterations in biosynthetic reactions. The drug (at 1 μg/ml or 4 × 10⁻⁶ M) did not affect the incorporation of leucine-³H into cellular protein, that of uridine-5-³H into total nucleic acids, or that of thymidine-³H into DNA immediately upon addition. At about 2 hr of incubation in the presence of CB 1954, an effect on thymidine incorporation became measurable and, after 4 hr of exposure to the drug, thymidine incorporation into DNA was markedly depressed, whereas the formation of protein from leucine-³H and the incorporation of adenine-¹⁴C or uridine-5-³H into nucleic acids (mainly RNA) remained unaffected. The conversion of uridine-6-³H into the pyrimidines of DNA exclusively was depressed by CB 1954 qualitatively like that of thymidine-³H incorporation, although to a lesser extent (Chart 2). When adenine-¹⁴C incorporation into DNA alone was tested, CB 1954 produced a depression similar to that seen with uridine-6-³H into DNA. When incubation of cells with CB 1954 exceeded 4 hr, the selective effects on incorporation of the labeled compounds became less pronounced. The drug effect on thymidine incorporation was concentration dependent. The dose-response curve for this drug effect, measured after 4 hr of exposure to the drugs (Chart 3), was rather flat in comparison to that of mephalan. Because of the effect of CB 1954 at 1 μg/ml, both in the present in vitro metabolic system and its tumoricidal action in vitro during a 2-hr incubation (6), this concentration was selected for most of the biochemical studies.

Lack of Effect on de Novo Purine Formation in the Sensitive Walker Tumor (WS). The antagonism of the action of CB 1954 by AIC and purine ribosylnucleosides (5) suggested that the drug blocked at an early stage of de novo purine biosynthesis and that the availability of exog-
Chart 1. Validation of alkali digestion-filtration technique for quantitating thymidine-\( ^3 \text{H} \) incorporation into DNA of Walker WS ascites cells. Tumor cells were removed from rats, incubated for 4 hr in presence (\( \bullet, \Delta \)) or absence (\( O, \Delta \)) of 1 \( \mu \text{g} \) CB 1954 per ml of suspension, and added to flasks containing thymidine-\( ^3 \text{H} \) for further incubation (see "Materials and Methods"). Samples were removed periodically and either filtered and treated with 0.2 \( \% \) perchloric acid (\( O, \Delta \) Filter Procedure 2 (see "Materials and Methods")), or mixed with 1 \( \% \) NaOH, stored overnight, and then filtered (\( \bullet, \Delta \) Filter 4).

Chart 2. Effect of 4-hr preincubation of Walker WS cells with 1 \( \mu \text{g} \) CB 1954 or melphalan per ml of suspension on subsequent incorporation of isotopic precursors for cell components. Leucine-\( ^3 \text{H} \) (Leu) was incorporated into protein (Procedure 2); adenine-\( ^4 \text{C} \) (Ad) labeled nucleic acids, principally RNA (Procedure 2); thymidine-\( ^3 \text{H} \) and uridine-6-\( ^3 \text{H} \) were converted into DNA (Procedure 4). O, control cultures; \( \bullet \), CB 1954-treated cells; \( \Delta \), melphalan-treated cells.

Chart 3. Effect of drug concentration during preincubation on subsequent rate of incorporation of thymidine-\( ^3 \text{H} \) into DNA of Walker WS cells. Procedure as in Chart 1. Drugs: \( \bullet \), CB 1954; \( \Delta \), melphalan.

Actions of CB 1954

measured by calculating the radioactivity in the hot perchloric acid extracts of the cell samples (Procedures 2 and 3), again no drug-produced effect was noted. The unaffected incorporation of formate-\( ^1 \text{C} \), more than 80% of which is converted into hot perchloric acid-extractable nucleotide products, further demonstrated that de novo synthesis was not specifically inhibited by CB 1954. Moreover, CB 1954 did not affect the incorporation rates of adenine-\( ^4 \text{C} \) and AIC-\( ^4 \text{C} \) into RNA (Filters 1 and 2). An increased rate of utilization of these compounds might have been expected if CB 1954 had blocked de novo purine biosynthesis.

Comparative Studies with Melphalan in the Sensitive Walker Tumor (WS). For purpose of comparison, experiments corresponding to those described for CB 1954 were performed with melphalan, a difunctional alkylating agent. A concentration of 1 \( \mu \text{g} / \text{ml} \) was selected because this drug concentration had been shown to be in the same inhibitory range on Walker cells as 1 \( \mu \text{g} / \text{ml} \) for CB 1954 (6). No immediate drug effect could be observed after incubation of cells with that concentration of melphalan; but 4 hr after drug exposure, thymidine-\( ^3 \text{H} \) incorporation into DNA was selectively depressed, whereas adenine-\( ^4 \text{C} \) or uridine-\( ^3 \text{H} \) incorporation into total nucleic acids and leucine-\( ^3 \text{H} \) incorporation into proteins continued unaltered (Chart 2). DNA synthesis as measured by the conversion of uridine-6-\( ^3 \text{H} \) into DNA was also depressed by treatment with melphalan, although this drug action consistently was less pronounced than that on thymidine incorporation into DNA. Thus, the biochemical effects of CB 1954 and melphalan bore close qualitative and quantitative similarity.

Effects of CB 1954 on Thymidine Incorporation by Other Tumors. At concentrations up to 10 \( \mu \text{g} / \text{ml} \), preincubation with CB 1954 for 4 hr did not inhibit thymidine incorporation by any of the tumors other than the WS line (Chart 4). This correlates well with the antitumor activity of CB 1954 since only the WS tumor responds to it (6).
Biochemical Comparison of Walker Tumor Lines. In addition to the standard line of the Walker tumor sensitive to CB 1954 (WS), 2 lines of the Walker tumor were available which were insensitive to CB 1954. The WZ line behaved like other tumors naturally resistant to CB 1954 in that its pattern of thymidine incorporation was unaltered by incubation with CB 1954 in the usual concentration range (Chart 4). Although concentrations of CB 1954 greater than 10 μg/ml depressed thymidine incorporation into that line of tumor, this effect appeared to be nonspecific and was observed for all tumors studied. AIC, which had completely prevented the effect of CB 1954 on thymidine incorporation in the WS tumor (5), still was able partially to antagonize the effect of high concentrations of CB 1954 on the WZ tumor. Thus, while CB 1954 at 300 μg/ml depressed thymidine incorporation to 12% of control values of the WZ tumor, in the presence of AIC at 1 mg/ml the maximum depression reached only 24% of control.

Thymidine incorporation was also inhibited by CB 1954 in the WR tumor which had acquired resistance to CB 1954. At a concentration of 1 μg/ml of CB 1954, thymidine incorporation was depressed almost as much as that of the WS tumor (Chart 5). The incorporation of adenine-14C or uridine-6-3H into DNA was also diminished, indicating that the drug was affecting DNA synthesis. The incorporation of leucine-3H or adenine-14C into protein and nucleic acids, respectively, was unaltered.

The relative incorporation rates of various precursors into the Walker tumors are shown in Table 1. No biochemical differences were apparent that might account for their differing sensitivities to CB 1954. The only difference seen was that the resistant lines WR and WZ incorporated thymidine at a greater rate than the sensitive WS line.

DISCUSSION

Studies of the effects of CB 1954 on the utilization of labeled precursors for the major constituents of ascites cells reveal the selective drug action on the incorporation of thymidine-3H or of uridine-6-3H into DNA, whereas RNA and protein formation were not altered. This action, which required at least 2 hr of contact of the cells with the drug, would be expected for an alkylating agent, which normally inhibits the synthesis of DNA to a greater extent than that of RNA (12). In the same tumor system, corresponding experiments with melphalan, a difunctional alkylating agent, provided similar results. Since CB 1954 is monofunctional, polyfunctionality is evidently not essential for producing these effects. Although investigations of this type do not exclude other mechanisms of action, Venitt (11) has also provided evidence that, at least in bacteria, CB 1954 exerts its cytotoxic effect by alkylation of DNA and subsequent hindrance of DNA replication and cell division. Interference with the S phase of the cell cycle would permit the continuation of RNA and protein synthesis while DNA synthesis was inhibited, thus blocking progression of the cells through the cell cycle.

It was observed consistently that the inhibitory effect of CB 1954 on the conversion of uridine to DNA was slightly less pronounced than that on the conversion of thymidine to DNA (Chart 2). This would suggest that CB 1954, in addition to its effects on DNA synthesis, may also produce a specific action on thymidine uptake or the conversions preceding polymerization. Alternatively, the multistep con-

![Image of Chart 4](https://example.com/chart4.png)

Chart 4. Effect of preincubation of tumor cells with varying concentrations of CB 1954 on subsequent rate of incorporation of thymidine-3H into DNA. Walker WS tumor (●) was the only one in which drug produced growth-inhibitory effect. Naturally resistant tumor lines: △, American Walker line, WZ; ■, Yoshida rat sarcoma; ○, Gardner lymphosarcoma; ▲, NK lymphoma; O, TLX 5 lymphoma; x, mouse plasma cell tumor ADJ/PC6A.

![Image of Chart 5](https://example.com/chart5.png)

Chart 5. Effect of preincubation with CB 1954 on thymidine incorporation into tumor cells, as in Chart 4. Walker WS tumor (●) was susceptible to CB 1954 growth inhibition; WR (O) had acquired resistance to CB 1954 through continuous passage in presence of that drug.
demonstrated with respect to thymidine incorporation of glycine, formate, AIC, or adenine was unaltered by drug (11). To alkylating agents (9) or for bacteria resistant to CB 1954 ability to repair the DNA damage more rapidly, as has been this tumor line differs from the sensitive one only in its thymidine incorporation system (Chart 5). It is possible that line of the British Walker tumor which had acquired sensitivity of the WS tumor to therapy by CB 1954 was also and its thymidine incorporation was unaffected. The unique strain (WZ). The latter tumor line was resistant to CB 1954, line of the Walker tumor (WS) did not extend to the U. S. but overcome the actions of CB 1954 implied that the latter treatment of the tumor cells. The ability of AIC to could not be demonstrated since the utilization of labeled agent, CB 1954 might also inhibit at a discrete stage of de novo purine synthesis. Since AIC is readily incorporated into purines, it appeared likely that CB 1954 specifically blocked or alkylated an enzyme involved in the reaction sequence prior to the formation of AIC ribotide. The administration of AIC or other purines would then have provided the products of the blocked reaction, thereby overcoming the drug effects. However, such a drug action could not be demonstrated since the utilization of labeled glycine, formate, AIC, or adenine was unaltered by drug treatment of the tumor cells. The ability of AIC to overcome the actions of CB 1954 implied that the latter might be an antagonist of AIC in some as yet unidentified biochemical system.

The remarkable specificity of CB 1954 against the British line of the Walker tumor (WS) did not extend to the U. S. strain (WZ). The latter tumor line was resistant to CB 1954, and its thymidine incorporation was unaffected. The unique sensitivity of the WS tumor to therapy by CB 1954 was also demonstrated with respect to thymidine incorporation (Chart 4), suggesting that this biochemical test might be suitable to predict the inhibitory response of tissues to CB 1954.

Of the resistant tumors examined, only the WR tumor, a line of the British Walker tumor which had acquired resistance to CB 1954 (6), was found to respond in the thymidine incorporation system (Chart 5). It is possible that this tumor line differs from the sensitive one only in its ability to repair the DNA damage more rapidly, as has been proposed for other tumor systems with acquired resistance to alkylating agents (9) or for bacteria resistant to CB 1954 (11). No single biochemical explanation can be provided for the difference in the response to CB 1954 of the 3 lines of Walker tumor (Table 1). The incorporation patterns of the 3 tumors incubating under similar conditions resembled each other closely, except that tumors insensitive to CB 1954 were more active in their rate of incorporation of thymidine. A similar finding has been noted for sensitive and resistant lines of the Yoshida tumor when incubated in vitro (4). However, this greater incorporation of thymidine by the resistant tumor lines remains unexplained.

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Studies on the Mechanism of Action of 5-Aziridinyl-2,4-dinitrobenzamide in Tumor Cells

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