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# SEQUENTIAL INHIBITORY EFFECTS OF ANTITUMOR AGENTS RELATED TO LEVODOPA AND DOPAMINE UPON DNA SYNTHETIC ENZYMES*

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**Abstract**—Novel antitumor agents related to levodopa and dopamine exhibit a selective and rapid inhibition of DNA synthesis as measured by thymidine incorporation. Our investigations have attempted to determine the biochemical basis of the selective inhibition of tumor cells and in this present study we examined the effects of these agents on thymidylate synthase. The dihydroxybenzene derivatives were found to inhibit thymidylate synthase *in situ* at concentrations ranging between 100 and 800 μM. The quinols did not inhibit partially purified thymidylate synthase, although the oxidized quinones did cause inhibition at concentrations between 10 and 100 μM. Time course experiments suggested that the inhibition of thymidylate synthase *in situ* by the dihydroxybenzene derivatives occurs after the inhibition of thymidine incorporation, indicating that an earlier event was critical to the inhibition of DNA synthesis. With the use of a novel *in situ* assay which measured the release of [$^3$H]water from [5-$^3$H] uridine in intact cells, we were able to show that one of the earliest biochemical events is the inhibition of ribonucleotide reductase and that the inhibition of thymidylate synthase, which is delayed by approximately 30 min, was indirectly mediated possibly through effects on ribonucleotide reductase.

Our laboratory has been investigating the antitumor properties, in man and in a variety of experimental tumor systems, of orthodihydroxybenzene derivatives structurally related to levodopa and dopamine [2–4]. Initial biochemical studies indicated that a prompt inhibition of TdR incorporation by intact cells was a characteristic and reproducible biochemical feature of the mode of action of these agents [5]. Our studies of the mechanism of action of these drugs have been directed toward elucidating the molecular basis of this phenomenon.

Recently, we described the effects that the dihydroxybenzene derivatives exert upon mammalian ribonucleotide reductase *in vitro* [6]. Although the reduced forms of the ortho catechols exhibit no direct inhibitory effect upon DNA polymerase α, we demonstrated that in permeabilized cells there is a delayed inhibitory effect upon this enzyme [6]. To describe further the action of these novel antitumor agents, we examined the action of these agents upon a third enzyme that is crucial to the control of DNA synthesis, thymidylate synthase.

The *in vitro* analysis of the effect of inhibitors of the various enzymes involved in DNA synthesis is made difficult, since the cell membrane is impermeable to their immediate substrates. The use of permeabilized cells is one technique used to circumvent this problem. An altervative *in situ* approach for the study of the enzyme thymidylate synthase has been developed which utilizes charcoal filtration to isolate [$^3$H]water released from [5-$^3$H] deoxyuridylate [7]. Since other important enzymes such as ribonucleotide reductase are closely linked to thymidylate synthase, it might be possible, using this method, to examine effects upon ribonucleotide reductase in intact cells.

Several recent reports have demonstrated that the ribonucleotide reductase inhibitor hydroxyurea, which has no direct effect upon thymidylate synthase, does indirectly inhibit the activity of this enzyme *in situ* [7, 8]. Since the ortho catechols inhibit ribonucleotide reductase *in vitro*, the possibility exists that they may also inhibit thymidylate synthase by a similar indirect mechanism. In this report we investigated the effects of several dihydroxybenzene derivatives upon thymidylate synthase activity *in situ* and have established that inhibition does occur and is mediated indirectly. A novel *in situ* technique is described for examining the inhibitory effects of these drugs on ribonucleotide reductase activity. We also examined the relationship between the inhibition of ribonucleotide reductase and thymidylate synthase *in situ*, as well as the correlation of the inhibition of the activity of these enzymes to the inhibition of DNA synthesis.

## MATERIALS AND METHODS

*Materials.* Radiolabeled [$^3$H]dTTP, [$^3$H] thymidine, and [5 and 6-$^3$H]uridine were purchased

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Table 1. Inhibitory effect of catechols on thymidylate synthase activity and DNA synthesis

| Drug | Thymidylate synthase* $IC_{50}$‡ | DNA synthesis† $IC_{50}$ |
|---|---|---|
| 3,4-DHBA | 176 μM | 167 μM |
| Dopamine | 283 μM | 400 μM |
| L-Dopa methyl ester | 416 μM | 699 μM |
| L-Dopa | 794 μM | 1030 μM |
| 5-FU | 114 nM | |
| Hydroxyurea | 78 μM | |

* Thymidylate synthase activity was measured using the [5-$^3$H]deoxyuridine *in situ* assay. The cells were preincubated with drug for 1 hr.

† DNA synthesis was determined using the TdR incorporation method. The drug and labeled thymidine were added at the same time.

‡ $IC_{50}$ = the concentration resulting in 50% inhibition of activity.

from New England Nuclear, Boston, MA. Radiolabeled [5-$^3$H]deoxyuridine and [5-$^3$H]dUMP were obtained from Schwarz/Mann, Cambridge, MA, and Amersham, Arlington Heights, IL, respectively. Unlabeled nucleotides and nucleosides were obtained from P-L Biochemicals, Milwaukee, WI. 3,4-Dihydroxybenzylamine (3,4-DHBA*) was prepared from the parent nitrile by catalytic hydrogenation as described previously [9]. All other drugs were prepared synthetically and fully characterized [3].

The origin and maintenance of L1210 lymphocytic leukemia cells have been described [2]. Briefly, the cells were maintained in a suspension of Eagle's minimum essential medium containing 10% fetal bovine serum, 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a 5% $CO_2$ humidified air incubator at 37°. The medium was also supplemented with 50 μM 2-mercaptoethanol [10]. The technique for radiolabeled precursor incorporation has been described previously [2]. The cells were checked for the presence of mycoplasm using the radiolabeled technique described by Schneider *et al.* [11].

*Thymidylate synthase enzyme assay.* A crude cell-free extract was prepared from L1210 leukemia cells carried in $BDF_1$ male mice for 7 days. The cells (*ca.* $1 \times 10^{10}$) were harvested from the ascitic fluid by centrifugation and washed twice. The cells were resuspended in 1.5 vol. of 20 mM Tris, 1 mM DTE (pH 7.4). The cells were homogenized with a Potter–Elvehjem homogenizer, and the homogenate was centrifuged successively at 3,000 rpm for 10 min and at 100,000 *g* for 90 min. The enzyme activity was detected in the supernatant fluid. Thymidylate synthase activity was assayed essentially as described by Navalgund *et al.* [12] except that 8 mM DTE was used instead of mercaptoethanol, 50 μM dUMP with 1 μCi of [5-$^3$H]dUMP instead of 67 μM dUMP with 1.3 μCi of [5-$^3$H]dUMP, and no bovine serum albumin was used. Thymidylate synthase activity was determined using the charcoal filtration method described by Roberts [13].

* Abbreviations: 3,4-DHBA, 3,4-dihydroxybenzylamine; DTE, dithioerythritol; $IC_{50}$, concentration resulting in a 50% inhibitory response; and 5-FU, 5-fluorouracil.

*Permeabilization.* To analyze thymidylate synthase activity using [5-$^3$H]dUMP, L1210 cells were first permeabilized by treatment with lysolecithin [14]. Exponentially growing L1210 cells were washed twice and resuspended at $1 \times 10^8$ cells/ml in a buffered solution containing 250 μg/ml of lysolecithin. The cells were placed on ice for 1 min. Permeabilized cells ($1 \times 10^7$) were added to the thymidylate synthase assay mixture containing: 50 μM dUMP; 1 μCi [$^3$H]dUMP; 0.1 mM tetrahydrofolate; 0.05% formalin; 8 mM DTE; and 60 mM NaF. Thymidylate synthase activity was determined using the charcoal filtration method described by Roberts [13].

In situ *thymidylate synthase assays.* Two methods were used to examine thymidylate synthase activity *in situ.* The first method is the same procedure described by Rode *et al.* [7] which measures the tritiated $H_2O$ released from [5-$^3$H]dUMP which is formed in cells from [5-$^3$H]deoxyuridine added to the medium by the action of thymidylate synthase. Cells were collected by centrifugation and resuspended in fresh medium at $2 \times 10^6$ cells/ml. [5-$^3$H] UdR (0.33 μCi, 5 μM) was added to 100-μl samples of cell suspension to start the reaction. The second method is a variation of this technique in which [5-$^3$H]uridine is added to the medium. In this method uridine must first be converted to deoxyuridylate by the action of the enzyme ribonucleotide reductase prior to its availability as a substrate for thymidylate synthase. This method is similar to the previous procedure except that [5-$^3$H]uridine (2 μCi) is used instead of deoxyuridine.

## RESULTS

*Inhibition of thymidylate synthase* in situ. The initial studies were designed to determine the effects of the antitumor agents bearing the dihydroxybenzene moiety upon thymidylate synthase activity *in situ.* Using [5-$^3$H]deoxyuridine as the labeled precursor, this assay measures the release of [$^3$H]water formed as the result of the action of thymidylate synthase on [5-$^3$H]dUMP. Table 1 describes the results of these experiments for several structurally related catechols and compares their relative order of potencies as inhibitors of thymidylate synthase with their abilities to inhibit thymidine incorporation

Table 2. Effect of DTE on the inhibition of thymidylate synthase by oxidized 3,4-DHBA

| DTE (mM) | $IC_{50}$* ($\mu$M) |
|---|---|
| 0.2 | <10 |
| 0.6 | 45 |
| 1.1 | 105 |
| 5.1 | 135 |

Thymidylate synthase was prepared and assayed as described, except that the concentration of DTE was varied as indicated. The quinone form of 3,4-DHBA was prepared as described previously [5].

* $IC_{50}$ = concentration of oxidized 3,4-DHBA resulting in 50% inhibition of enzyme activity.

in intact cells. We have shown previously that these catechols selectively inhibit DNA synthesis with little or no effect on RNA or protein synthesis [5].

*Partially purified thymidylate synthase.* We examined the inhibitory effect of 3,4-DHBA on the activity of the enzymes using a partially purified cell-free extract to determine whether the observed inhibitory effect upon thymidylate synthase was mediated directly or indirectly. Similar to the findings of Rode *et al.* [7] and Reddy and Pardee [8] who examined the effects of the ribonucleotide reductase inhibitor, hydroxyurea, on thymidylate synthase, we found that while the catechols had an inhibitory effect on thymidylate synthase *in situ* they had no inhibitory effect against the isolated enzyme. This result suggests that the *in situ* inhibitory effect involves an indirect mechanism.

One possible indirect mechanism is that the drug must be activated, perhaps by oxidation to the reactive quinone form, in order to inhibit thymidylate synthase. To investigate this possibility we examined the effect of the oxidized form of 3,4-DHBA upon thymidylate synthase activity. The results (Table 2) revealed that the oxidized form was a very potent inhibitor of this enzyme, although the inhibition was dependent on the concentration of the reducing agent DTE which incompletely counteracted the effect of the quinone. To determine whether the inhibitory effect was reversible, the partially purified enzyme was incubated in the presence of 5 mM 3,4-DHBA for 45 min at 22°. The drug was removed by passing the sample (0.1 ml) through a 1-ml column of G-25 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 10 mM Tris, pH 7.6. The enzyme was eluted with the same buffer, and the first 0.3-ml fraction following the void volume was collected. The inhibition was irreversible, since enzyme activity was not restored following passage of the drug-treated enzyme through the Sephadex G-25 column. While this result indicates that the oxidized 3,4-DHBA can inhibit thymidylate synthase, the following experiment suggested that this is not the mechanism of action *in situ*. Exponentially growing L1210 cells were exposed to 1 mM 3,4-DHBA for 2 hr. Thymidylate synthase activity was inhibited by more than 80% as determined by the [5-$^3$H]UdR *in situ* assay. Cell-free extracts were prepared from drug-treated and control cells ($1.5 \times 10^8$ cells). Our results revealed that the thymidylate synthase activity was not inhibited in the extract prepared from the drug-treated cells. The inhibition was reversed by disruption of the cells which argues against inhibition by an irreversible quinone oxidation mechanism.

*Permeabilized cells.* An alternative mechanism to explain the inability of the catechols to inhibit isolated thymidylate synthase is that the inhibition observed *in situ* resulted from an indirect allosteric effect [8]. If this were the case, it might be possible to detect inhibition of thymidylate synthase by 3,4-DHBA through the use of permeabilized cells that maintain nearly normal cellular conditions yet can utilize [5-$^3$H]dUMP as the labeled precursor [14]. Our results, however, showed considerable variation in the extent of the inhibition of thymidylate synthase using permeabilized cells. The inhibition at 1 mM 3,4-DHBA ranged from 18 to 41% in different experiments. This variation may have resulted from measuring, in permeabilized cells, enzymatic activity that was contributed by both nuclear and cytoplasmic thymidylate synthase. There is considerable latent enzyme present in the cytoplasm [15], which would be activated by the permeabilization procedure. However, since the cytoplasmic enzyme is not involved in nuclear DNA synthesis, this portion of the thymidylate synthase activity would not be susceptible to inhibition by the mechanism proposed by Reddy and Pardee [8].

Another possibility was that deoxyuridine kinase was inhibited *in situ*. To investigate this possibility we used the permeabilized cell technique to measure thymidylate synthase activity using [5-$^3$H]deoxy uridine as the precursor. This precursor has to be phosphorylated first to dUMP by deoxyuridine kinase before it can be a substrate for thymidylate synthase. Thymidylate synthase activity was not inhibited by 3,4-HBA, which would indicate that the supply of dUMP was unaffected and, hence, deoxyuridine kinase was active.

[5-$^3$H]*Uridine* in situ *studies.* In a recent study we demonstrated that catechols related to levodopa and dopamine directly inhibit ribonucleotide reductase in permeabilized cells. In the present study we wanted to determine if it would be possible to assess the inhibitory effect of 3,4-DHBA on ribonucleotide reductase using a variation of the thymidylate synthase *in situ* assay. Using [5-$^3$H]uridine as the labeled precursor, this assay measures the release of [$^3$H]-water formed as the result of the action of thymidylate synthase on [5-$^3$H]dUMP which in turn is formed from [5-$^3$H]uridylate by ribonucleotide reductase. The inhibition of the release of [$^3$H]water using this assay should depend on the additive effect of the drug on ribonucleotide reductase and thymidylate synthase. The effect on the latter enzyme was independently assessed using the same assay but with [5-$^3$H]deoxyuridine as the labeled precursor. An indication of the effect of 3,4-DHBA on ribonucleotide reductase could be deduced from an analysis of these two assays.

The results (Fig. 1) indicated that 3,4-DHBA did inhibit the formation of [$^3$H]water from [5-$^3$H]uridine *in situ*, but this process was less sensitive to inhibition by the drug than was found using the [5-$^3$H]deoxy-

uridine *in situ* assay. To investigate this unexpected result, we performed a time course experiment to determine the time of onset of inhibition. The results shown in Fig. 2 indicate that with the uridine assay the inhibition was found to occur immediately, while the time of onset of inhibition for the deoxyuridine assay was delayed for approximately 20–30 min after exposure to the drug. These results suggested that the assays are measuring two different inhibitory effects of 3,4-DHBA. Specifically, the initial inhibitory effect of 3,4-DHBA observed with the [$^3$H] uridine assay is due to the inhibition of ribonucleotide reductase alone and only later does 3,4-DHBA have an indirect inhibitory effect on thymidylate synthase, as detected using the [5-$^3$H]deoxyuridine *in situ* assay.

*Effect of time on inhibition.* The delay in the time of onset of inhibition of thymidylate synthase would suggest that the inhibition of this enzyme by 3,4-DHBA will be reduced by limiting the exposure time to the drug. On the other hand, since the onset of inhibition was very rapid with the [$^3$H]uridine *in situ* assay, limiting the exposure time to the drug should have had little or no effect on the inhibitory response. Figure 3 shows the results of an experiment designed to determine the effect of exposure time upon the inhibition using the two *in situ* assays. The results indicate that the inhibition detected with the [$^3$H] deoxyuridine assay was time dependent whereas the inhibition found with the [$^3$H]uridine was unaffected by length of exposure to the 3,4-DHBA. This result provides additional support for the notion that the two *in situ* assays measured different inhibitory effects caused by 3,4-DHAB.

As shown in Table 1, the relative order of potencies for the inhibition of thymidylate synthase using the [$^3$H]deoxyuridine *in situ* assay and for the inhibition of TdR incorporation agree closely. We therefore wanted to determine if the inhibition of these two activities also correlated with the time of onset of inhibition. The results shown in Fig. 4 indicate that the inhibition of TdR incorporation occurred earlier than the inhibition of thymidylate synthase (Fig. 2). When we examined [6-$^3$H]UdR incorporation into DNA (Fig. 5), the time of onset of inhibition was similar to the results found with TdR, i.e. an early onset of action. This last experiment also provides additional evidence against deoxyuridine kinase as the site of action. Since this enzyme is common to both assays, it is unlikely that the time of onset of inhibition of [6-$^3$H]UdR incorporation and inhibition of the release of [$^3$H]water using the [5-$^3$H]UdR *in situ* assay would be different.

The time of onset of the inhibition of DNA synthesis does, however, agree with the results found with the [5-$^3$H]uridine *in situ* assay. Specifically, UdR and TdR incorporation, at 1 mM 3,4-DHBA, was

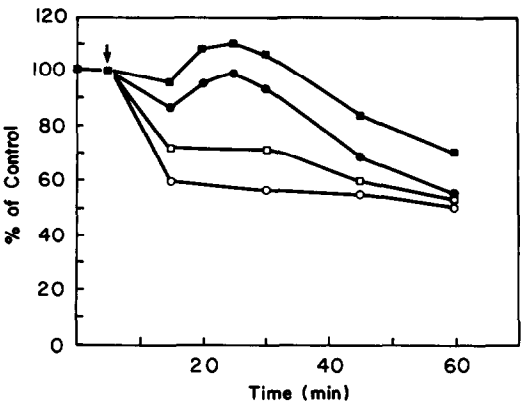


Fig. 2. Time course for the inhibition of the *in situ* release of [$^3$H]water using [$^3$H]deoxyuridine as the labeled precursor (control activity was 19,896 cpm/2 × $10^5$ cells/hr) in the presence of 0.2 mM (■) and 1.0 mM (●) 3,4-DHBA; and [$^3$H]uridine as the labeled precusor (control activity was 36,630 cpm/2 × $10^5$ cells/hr) in the presence of 0.2 mM (□) and 2.0 mM (○) 3,4-DHBA. The drug was added at the time indicated by the arrow.

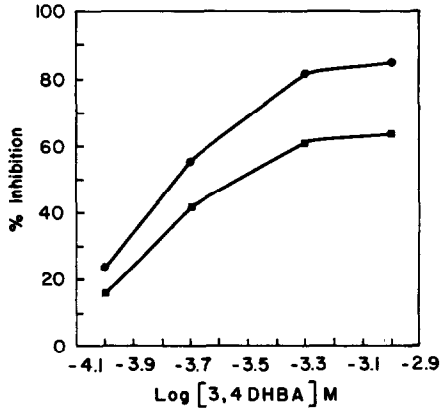


Fig. 1. Effect of 3,4-DHBA on the *in situ* release of [$^3$H] water using [5-$^3$H]deoxyuridine (●) and [5-$^3$H]uridine (■) as the labeled precursor. The control activities were 14,472 cpm/2 × $10^5$ cells/hr and 33,402 cpm/2 × $10^5$ cells/hr respectively. The cells were preincubated with 3,4-DHBA for 1 hr prior to the addition of the labeled substrate.

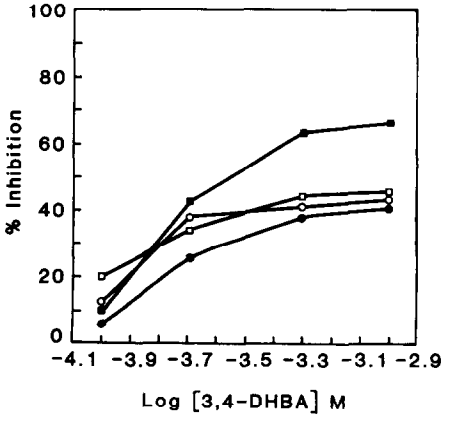


Fig. 3. Effect of time on the inhibition of thymidylate synthase. The inhibitory effect was determined for the cells treated with 3,4-DHBA for 60 min (●) and 25 min (■) using the [$^3$H]deoxyuridine *in situ* assay (control activities were 10,860 cpm/2 × $10^5$ cells/hr and 6,111 cpm/2 × $10^5$ cells/hr respectively); and for the cells treated with 3,4-DHBA for 60 min (○) and 25 min (□) using the [$^3$H]uridine *in situ* assay (control activities were 28,938 cpm/2 × $10^5$ cells/hr and 7,236 cpm/2 × $10^5$ cells/hr respectively).

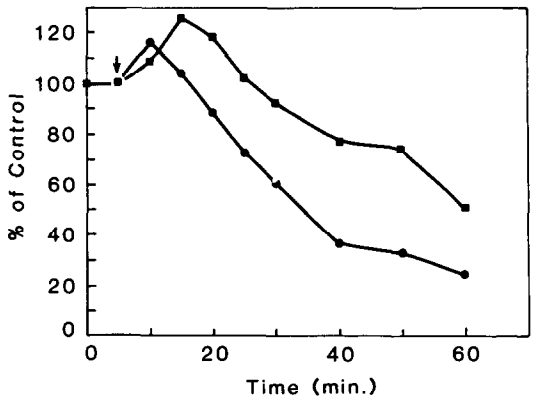


Fig. 4. Time course for the inhibition of [$^3$H]TdR incorporation in the presence of 0.2 mM (●) and 1.0 mM (■) 3,4-DHBA added at the time indicated by the arrow (control activity was 28,695 cpm/2 × $10^5$ cells/hr).

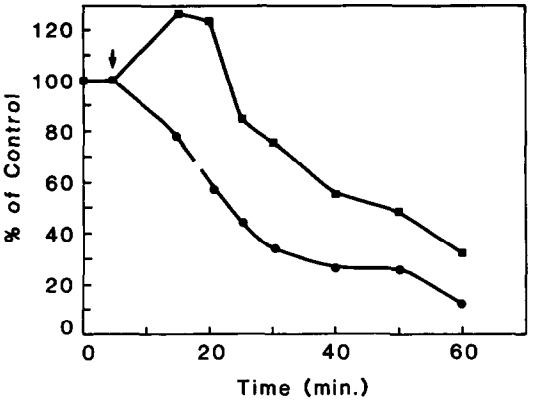


Fig. 5. Time course for the inhibition of [6-$^3$H]UdR incorporation in the presence of 0.2 mM (●) and 1.0 mM (■) 3,4-DHBA added at the time indicated by the arrow (control activity was 12,210 cpm/2 × $10^5$ cells/hr).

inhibited immediately as was the release of [$^3$H]water measured using the [$^3$H]uridine *in situ* assay. At low drug concentrations, the inhibition of UdR and TdR incorporations was delayed by approximately 15 min, whereas the inhibition of the [5-$^3$H]uridine was not delayed (Fig. 2). We feel that this correlation is the most significant link between the inhibition of DNA synthesis and the site of action of 3,4-DHBA.

## DISCUSSION

The dihydroxybenzene derivatives related to levodopa and dopamine have been shown to inhibit three enzymes related to DNA synthesis; ribonucleotide reductase, DNA polymerase and thymidylate synthase. From our results using the permeabilized cell technique and from our *in situ* assays, it would appear that the most likely initial event is the inhibition of ribonucleotide reductase. If the inhibition of ribonucleotide reductase were sufficient, perhaps resulting in a reduction in deoxynucleotide pools below a critical threshold level, then the inhibition of DNA synthesis would be immediate. The results presented in this paper as well as previous results using the permeabilized cell system appear to indicate that 3,4-DHBA need not inhibit ribonucleotide reductase completely in order to totally supress DNA synthesis.

At lower concentrations of 3,4-DHBA, the inhibitory effect on DNA syntheis was delayed (*ca.* 15 min at 0.2 mM), possibly until the deoxynucleotide pools were reduced sufficiently. In contrast, the inhibition of thymidylate synthase was delayed regardless of the drug concentration for about 20 min at 1 mM and 30 min at 0.2 mM (Fig. 2). The inhibition of DNA polymerase is also delayed at each of the drug concentrations [6]. The time of onset of inhibition of these two enzymes did not correlate with the time of onset of inhibition of DNA synthesis, whereas the time of onset of inhibition of ribonucleotide reductase either coincided or preceded the inhibition of DNA synthesis.

The results of our investigation suggest that the inhibition of thymidylate synthase and DNA polymerase involves an indirect mechanism. Recently Reddy and Pardee [8] have shown that several inhibitors of DNA synthesis that have no direct effect on thymidylate synthase inhibit the enzyme's activity *in vitro*, and a similar mechanism may be operative here.

Another possible explanation for the delayed inhibitory effect on DNA polymerase and thymidylate synthase is that the drug must be activated to the quinone in order to inhibit these enzymes. We have shown previously that the oxidized form is a very potent inhibitor of DNA polymerase [9], and the results in Table 2 indicate that the quinone is also a very potent inhibitor of thymidylate synthase. The evidence presented in the drug reversibility experiments that showed that the inhibitory effect of 3,4-DHBA against thymidylate synthase in intact cells was reversible, unlike the irreversible inhibition observed with the preformed quinone (Table 2), support the former explanation. In cells of high oxidative potential, e.g. melanoma, the quinone-mediated mechanism may be uniquely important.

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