0026-895X/90/010114-07$02.00/0

MOLECULAR PHARMACOLOGY, 38:114-120

Allosteric Interaction of Components of the Replitase Complex Is Responsible for Enzyme Cross-inhibition

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Received December 21, 1989; Accepted April 11, 1990

SUMMARY

The enzymes of DNA polymerization and DNA precursor synthesis are assembled in the replitase complex during the S phase of the cell cycle. Cross-inhibition is a phenomenon shown by enzymes of the replitase complex, in which inhibition of one enzyme of the complex leads to inhibition of a second, unrelated enzyme. This inhibition occurs only *in vivo* and only during S phase. The second enzyme shows no inhibition *in vitro*. In this study, using Chinese hamster embryo fibroblast cells, we have shown that direct allosteric interactions, i.e., structural interaction from a remote site within the replitase complex, is the cause of cross-inhibition of thymidylate synthase activity by the inhibitors of ribonucleotide reductase and DNA polymerase, because disruptions of the deoxynucleotide pools, which would be predicted for alternative explanations, do not occur. Cross-inhibition of DNA polymerase by hydroxyurea is demonstrated by the cessation of DNA synthesis when ribonucleotide reductase block is circumvented by the provision of all four deoxynucleosides. In addition to the cross-inhibition for thymidylate synthase and DNA polymerase, we have also presented evidence, on the basis of alterations of the *in vivo* conversion of deoxyuridine to dUMP, that cross-inhibition also occurs for the enzyme thymidine kinase. This conclusion is further supported by the lack of inhibition of the similar process in RNA synthesis, because enzymes of RNA synthesis are not included in the replitase complex. To facilitate the measurements, we have introduced a novel method of distinguishing between thymidine and deoxyuridine derivatives, making use of the fact that a tritium label placed in the 5′-position of deoxyuridine is removed on conversion to thymidine by methylation, whereas a tritium placed in the 6′-position is not.

Reddy and Pardee (1) showed that, during the period of DNA synthesis, at least eight enzymes relating to deoxynucleotide synthesis and polymerization are associated in a complex called "replitase." The presence of such multienzyme complexes, containing the enzymes of both DNA precursor biosynthesis and DNA replication, is widely reported in mammalian cells (2–8). Furthermore, the *in vivo* catalytic activity of the enzymes, such as TS and DNA polymerase, are confined to S phase, even though the enzyme levels, as measured *in vitro*, remained relatively constant throughout the cell cycle (9–11). *In vivo* activation of these enzymes during S phase is suggested to be due their assembly into the replitase complex (10).

Reddy and Pardee (12) showed that a variety of antimetabolites cross-inhibit TS *in vivo* but not *in vitro*. For instance, HU, which inhibits ribonucleotide reductase, shows an *in vivo* block of TS; TS, when isolated in soluble form, is not affected by HU. Similarly, *in vivo*, aphidicolin, an inhibitor of DNA polymerase α, also blocks TS; *in vitro*, there is no inhibition.

The basis of these cross-inhibitions is of interest because it may yield fundamental insights into the control mechanisms involved in DNA synthesis. *A priori*, there are several mechanisms by which these inhibitions could take place. Firstly, there could be product inhibition. For instance, blocking of DNA polymerase could back the precursor pools and, thereby, cause product inhibition to block up the steps upstream. Secondly, the inhibitors could act through uptake mechanisms, preventing exogenous substrates from reaching their respective enzymes by limiting entry to the cell. This would also explain why the *in vivo* effect was not seen *in vitro*. Thirdly, the cross-inhibition could take place via allosteric structural interactions within the replitase complex itself. The third mechanism would be the most interesting of the three, because it would imply that assembly into the replitase complex confers tight internal controls between enzymes at different stages of DNA precursor synthesis and of DNA polymerization.

Because product inhibition and blockage of transport both would cause major changes in nucleotide and nucleoside pools, we have analyzed pool composition *in vivo* under the conditions where cross-inhibition occurs. In these studies, there are no artifacts of isolation possible, because replitase function is observed in its physiological milieu.

This work is supported by Grant CA 39445 from the United States Public Health Service, National Institutes of Health, National Cancer Institute.

ABBREVIATIONS: TS, thymidylate synthase; dUrd, deoxyuridine; HPLC, high performance liquid chromatography; HU, hydroxyurea.

Materials and Methods

Synchronized Chinese hamster embryo fibroblast (CHEF/18) cells were prepared by 36-hr isoleucine starvation and subsequent restoration of complete medium, as described elsewhere (1).

Measurement of *in vivo* TS activity and DNA synthesis. TS activity in intact cells was measured by the tritium-release assay, in which dUrd labeled with tritium in the 5-position was administered to the cells. When [5-^{3}H]dUMP, formed from [5-^{3}H]dUrd, is converted to dTMP by TS inside the cell, tritium in the 5-position is released to form tritiated water (see Fig. 1A). The rate of formation of tritiated water reflects the rate of intracellular (*in vivo*) TS activity. The procedures for measuring *in vivo* activity of TS and DNA polymerase were as described by Reddy (10).

Preparation of cell extracts for measurement of dUrd and thymidine nucleotide pools formed from [^{3}H]dUrd. Ten hours after release from isoleucine block, CHEF/18 cells were treated with antimetabolites and, 15 min later, [5- or 6-^{3}H]dUrd (5 μM) with a specific activity of 2460 cpm/pmol was added. After 1 hr, the monolayers of cells in the culture dishes were washed twice with 2 ml of ice-cold solution containing 2 mM $HgCl_2$, 1.2 mM NaI, and 150 mM NaCl. This washing procedure stops the movement of nucleotides and nucleosides across the cell membrane in either direction (13). The nucleotide and nucleoside pools were then extracted by bathing of the monolayer in 1 ml of ice-cold 10% trichloroacetic acid. The acid-soluble material was then extracted four or five times with an equal volume of ether to remove trichloroacetic acid (14). Each sample (0.6 ml) was ultrafiltered, using an Amicon MPS-1 micropartition system. The ultrafiltered aqueous solution (0.5 ml) was lyophilized and the residue was taken up in 50 μl of 7 mM KH_2PO_4, pH 4.2.

Measurement of endogenous deoxynucleoside triphosphate pools. dTTP, dCTP, dGTP, and dATP levels in acid extracts prepared from HU- or aphidicolin-treated and control cells were determined by a DNA polymerase assay, as described by North *et al.* (15).

dUrd and thymidine nucleotide pool analysis. Because there is no simple chromatographic method for the separation of dUrd nucleotides from thymidine nucleotides when they exist in a mixture, we developed a novel and effective approach to determine the conversion of radioactive dUrd to the corresponding dUrd or thymidine nucleotides. This approach is based upon the principle that, when cells are labeled with [5-^{3}H]dUrd, the radioactivity will be incorporated only into dUrd nucleotides because of the removal of this hydrogen upon methylation (see Fig. 1A), whereas the radioactivity from [6-^{3}H]dUrd will be incorporated into both dUrd and thymidine nucleotides (see Fig. 1B).

Deoxynucleoside mono-, di-, and triphosphates of uridine or thymidine in the acid-soluble material of cells were separated by HPLC. HPLC analyses were performed on a Gilson System 42 HPLC equipped with a variable wavelength UV detector (Gilson, model 116). Samples were chromatographed on a 4.6 × 250 mm Partisil 10 SAX (Whatman) column at an eluent flow rate of 1.5 ml/min. The column effluent was monitored at 254 nm. Concentrated cell extract (10 μl) along with 0.25

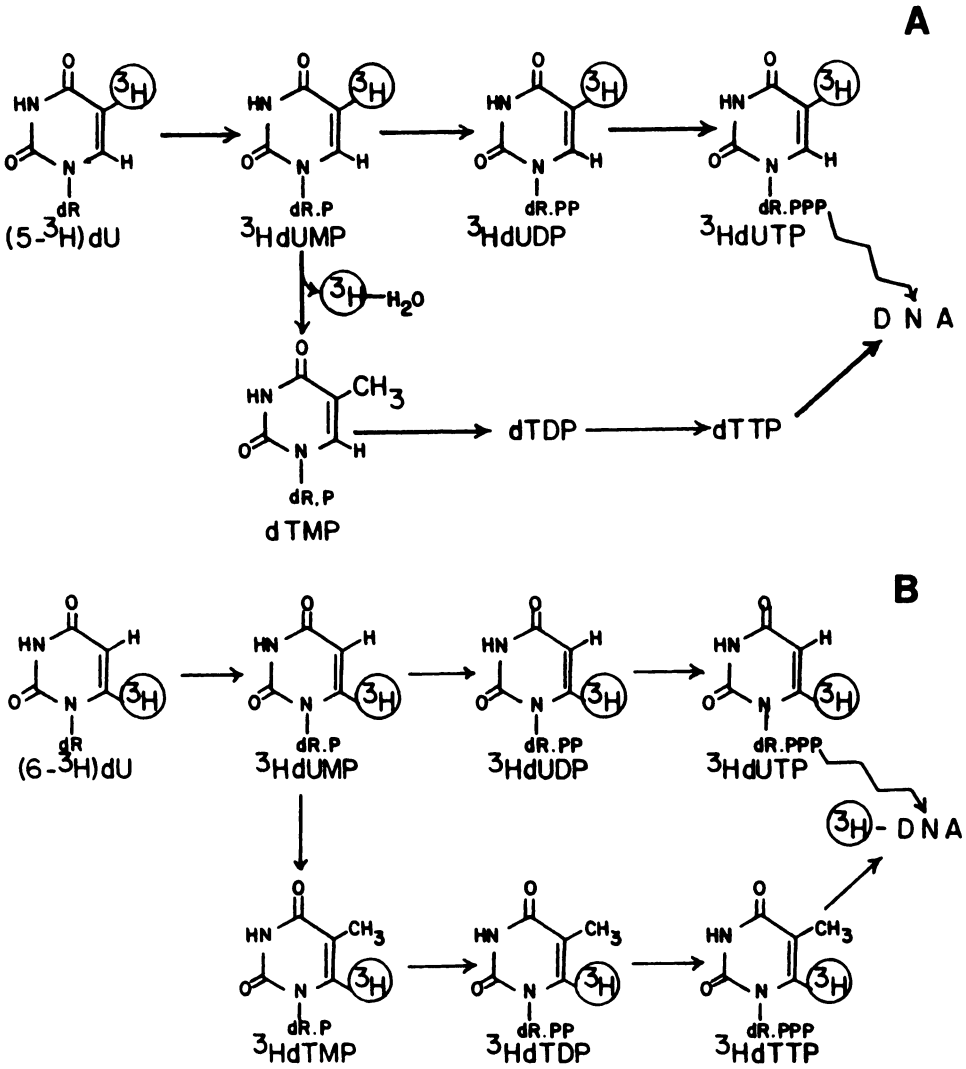

Fig. 1. Fate of radioactivity from [5-^{3}H]dUrd [*(5-^{3}H)dU*] (A) and [6-^{3}H]dUrd [*(6-^{3}H)dU*] (B). →, Effective conversion, ⇝, ineffective conversion.

nmol of each unlabeled marker nucleotide were injected onto the column. At the time of the injection, the column was equilibrated with 95% buffer A (7 mM KH_2PO_4, pH 4.2) and 5% buffer B (0.6 M $NH_4H_2PO_4$, pH 4.7). The column was then eluted with a linear gradient of buffer A and buffer B. The gradient was constructed to obtain 30% buffer B at 5 min and 100% buffer B at 10 min; then an isocratic period of 10 min of buffer B was used. Before the injection of each sample onto the column, the column was regenerated by washing with 50 ml of buffer B.

In experiments employing [5-^{3}H]dUrd, all radioactivity recovered was considered to be in dUrd nucleotides. In parallel experiments using [6-^{3}H]dUrd, the radioactivity was distributed between dUrd and thymidine nucleotides. Therefore, radioactivity in deoxythymidine nucleotides was determined by subtracting radioactivity in individual nucleotides (mono-, di-, or triphosphates) of [5-^{3}H]dUrd-labeled material from that of [6-^{3}H]dUrd-labeled material.

Materials. [6-^{3}H]dUrd was purchased from Dupont NEN Research Products. [5-^{3}H]dUrd, [^{3}H]thymidine, [5,6-^{3}H]uridine, [^{3}H]dATP, [8-^{3}H]dGTP, [5-^{3}H]dCTP, and [*methyl*-^{3}H]TTP were from ICN Radiochemicals. Poly[d(A-T)], poly[d(I-C)], and *Escherichia coli* DNA polymerase I (endonuclease free) for deoxynucleoside triphosphate pool measurements were obtained from Boehringer Mannheim. HU, dUrd, uridine, and other nucleotides and nucleosides were purchased from Sigma Chemical Company. Aphidicolin was from Wako Pure Chemicals (Japan). 3,4-Dihydroxybenzamidoxime (amidox) was generously provided by Dr. H. Elford, Molecules for Health, Inc. (Richmond, VA).

Results

Cross-inhibition of TS activity by the inhibitors of ribonucleotide reductase and DNA polymerase. In S phase CHEF/18 cells, 3,4-dihydroxybenzamidoxime (amidox), an inhibitor of ribonucleotide reductase (16), caused concentration-dependent parallel inhibition of TS activity and DNA synthesis, as measured *in vivo* (Fig. 2). However, *in vitro* (as measured in soluble extracts of S phase CHEF/18 cells), neither TS nor DNA polymerase activities were sensitive to this drug (data not shown). Similarly, as reported earlier (12) and presented in Table 1, HU and aphidicolin also caused cross-inhibition of TS activity only when measured *in vivo* but not *in vitro*.

Steady state levels of various deoxynucleotide pools labeled by [^{3}H]dUrd in the presence of HU, amidox, and aphidicolin. We measured deoxynucleotide pools to evaluate their role in the observed cross-inhibition of TS activity by the

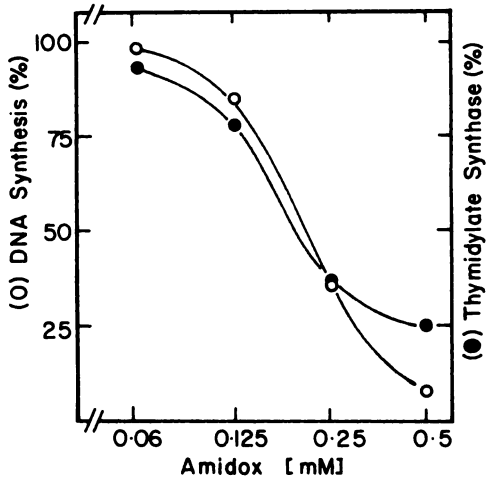

Fig. 2. Effect of amidox on *in vivo* TS activity and DNA synthesis in S phase CHEF/18 cells.

TABLE 1

Effect of HU, amidox, and aphidicolin on DNA synthesis, TS activity, and deoxynucleotide pools in intact S phase cells

Mean values obtained from two independently repeated experiments, in each of which individual samples were analyzed in duplicate, are presented. Variation between individual samples was less than 6%. Deoxynucleoside and deoxynucleotide pool levels are expressed as pmol/10^8 cells after incubation with radioactive precursor for 1 hr. The specific activities of 2500 cpm/pmol for [5-^{3}H]dUrd and 2460 cpm/pmol for [6-^{3}H]dUrd were used in calculating the molar concentration of each of the deoxynucleosides and deoxynucleotides presented in the table. These are the specific activities of the radioactive precursors that were included in the media for the assay of TS activity, DNA synthesis, and deoxynucleotide pools. Experimental procedures are as described in Materials and Methods section.

	Control	HU (1 mM)	Amidox (1 mM)	Aphidicolin (1 μg/ml)
			pmol/10^8 cells/hr	
DNA synthesis	581	2	6	13
TS activity	649	ND[a]	ND	34
			pmol/10^8 cells	
dUrd	7	14	11	11
dUMP	51	23	23	19
dThd	6	10	6	5
dTMP	50	52	48	43
dTDP	64	93	100	61
dTTP	8	9	9	7

[a] ND, the levels were below the detection capability of the assay.

inhibitors of ribonucleotide reductase and DNA polymerase. As shown in Table 1, we observed no significant difference in thymidine nucleotide pool levels, in the presence or absence of the drugs, large enough to account for the inhibitory effect on TS activity. Of the nucleotides measured, only TDP is increased by about 50% with HU and amidox. But even this modest increase in TDP is not significant, because aphidicolin, whose cross-inhibitory effect is the same, showed no change in TDP level (Table 1).

Table 1 shows a 55 to 60% decrease in dUMP in the presence of each of the drugs used in this study. Because dUrd was actually raised in the presence of all the drugs, this suggests that thymidine kinase, which is involved in converting dUrd to dUMP, must have been inhibited by these drugs in intact cells. Furthermore, as in the case of TS activity (12), inhibition of thymidine kinase activity was not observed for any of these drugs when we tested thymidine kinase activity in soluble cell extracts *in vitro* (Fig. 3). Therefore, this is another case of cross-inhibition.

In Table 1, one can also make two additional observations that, at the outset, appear paradoxical but actually point toward important regulatory aspects (see Discussion) in the biosynthesis of deoxynucleotides responsible for nuclear DNA replication. These observations are that 1) although TTP levels remained unchanged in the presence of all of the antimetabolites tested, their levels were 8–10-fold and 5–6-fold smaller than TDP and TMP levels, respectively, and 2) inhibition of TS activity by antimetabolites did not completely prevent the accumulation of radioactivity from [^{3}H]dUrd in thymidine nucleotide pools.

Influence of preincubation of intact cells with 5 μM [^{3}H]dUrd on the inhibitory effect of HU on DNA synthesis and TS activity. To test for a possible effect of the order of addition, the experiment described above was repeated with antimetabolites added to the cells 15 min before the radioactive precursors were added. This was to test whether antimetabolites could have modified the endogenous deoxynucleotide pools and could, thereby, influence our observations made with added

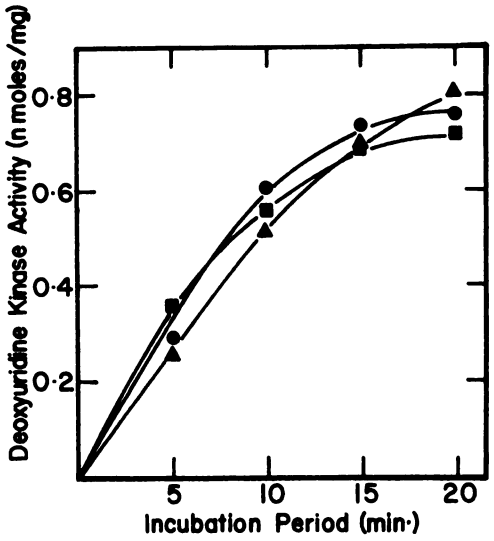

Fig. 3. Effect of HU and aphidicolin on thymidine kinase activity in soluble extracts of S phase CHEF/18 cells. Soluble extracts of S phase cells were prepared as described elsewhere (12) and thymidine kinase activity in the lysates was measured as described by Reddy and Pardee (1), except that [^{3}H]dUrd was used as substrate. Control (●) and assayed in the presence of HU (1 mM) (■) and aphidicolin (▲).

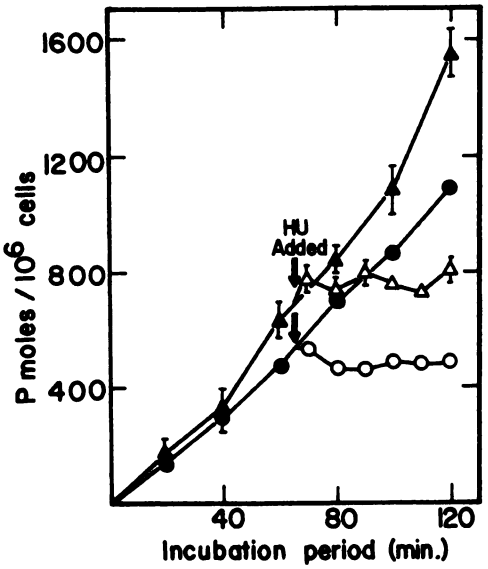

Fig. 4. Inhibitory effect of HU (1 mM) on *in vivo* TS activity and DNA synthesis in the presence of exogenous 5 μM dUrd in S phase CHEF/18 cells. *Triangles*, TS activity; *circles*, DNA synthesis; *open symbols*, after addition of HU; *closed symbols*, without HU.

radioactive precursor by changing its specific activity. In this control experiment, we allowed exogenous radioactive precursor to first equilibrate with endogenous nucleotide pools before addition of the drug, HU. In four independent observations, radioactivity from dUrd reached steady state levels inside the cells within 20 min after its addition. Consistent with our observation in Table 1, when HU was added 60 min after the addition of radioactive precursor to the cultures with S phase cells, both the DNA synthesis and the TS activity were still immediately inhibited, as determined by our assay method (Fig. 4). Furthermore, the inhibition of TS activity occurred almost simultaneously with the inhibition of DNA synthesis by HU.

In the experiment of Fig. 4, we examined the levels of intracellular dUrd and thymidine nucleotide pools before and after exposure to the drug. These results are shown in Table 2. The standard deviations indicate that the nucleotide levels did not vary significantly when measured from 20 to 120 min in control cells and from 70 to 120 min in cells to which HU was added at 60 min. This observation once again suggests that, shortly after its addition, the radioactivity from exogenous dUrd reaches a steady state level inside the cells after incorporating into dUrd and thymidine nucleotide pools. Under these conditions we observed that the addition of HU increased the level of TDP by about 54% and decreased the level of dUMP by about 60%. These observations are very similar to those presented in Table 1. Therefore, it is clear that nucleotide pools equilibrate rapidly with exogenous precursor, both before and after drug addition, and consequently there are no artifacts of specific activity.

Effect of antimetabolites on endogenous deoxynucleoside triphosphate pools. In the studies described so far, the rates of DNA synthesis or TS activity and the levels of deoxynucleotides derived from [^{3}H]dUrd were calculated based on the specific activity of [^{3}H]dUrd provided in the medium. However, if TTP formed from [^{3}H]dUrd were to mix with endogenous TTP pools, then the specific activity of the radioactive TTP formed from [^{3}H]dUrd inside the cells would depend upon the level of endogenous TTP pools. For this reason, endogenous TTP levels, along with the levels of dCTP, dGTP, and dATP, were measured in the cells treated with HU or aphidicolin and the same level of precursor (5 μM unlabeled dUrd) as in the experiments with radioactive precursor ([^{3}H]dUrd). As shown in Table 3, TTP levels in cells treated with aphidicolin were similar to levels in control cells. However, in

TABLE 2

Changes in dUrd and thymidine nucleotide pools after treatment with HU in the experiment of Figure 4

Each value is an average of two independent samples and there was less than 5% variation between the duplicate samples. The values presented following ± represent standard deviations between the measurements made at 20-, 40-, 60-, 80-, 100-, and 120-min intervals for the control experiment and at 70-, 80-, 90-, 100-, and 120-min intervals for the HU-treated experiment.

Deoxyribonucleotides	Pool size: Before addition of HU (control)	Pool size: After addition of HU (1 mM)
	pmol/10^6 cells	
dUMP	61 ± 7.0	25 ± 5.7
dUDP	2 ± 0.5	4 ± 0.6
dUTP	2 ± 0.6	4 ± 0.7
dTMP	34 ± 9.3	42 ± 12.6
dTDP	48 ± 4.5	74 ± 7.3
dTTP	4 ± 2.0	5 ± 3.3

TABLE 3

Effect of HU and aphidicolin on endogenous deoxynucleoside triphosphate pools in S phase CHEF/18 cells

Endogenous deoxynucleoside triphosphate (dNTP) pools in cells exposed to 5 μM dUrd (unlabeled) and treated with HU or aphidicolin were measured as described in Material and Methods. Specific activity of TTP is derived by combining the observed endogenous TTP levels with the radioactivity (cpm) incorporated into TTP pools from exogenous [^{3}H]dUrd in the experiment of Table 1.

Cells	Specific activity of TTP	Endogenous dNTP pools			
		TTP	dCTP	dGTP	dATP
	cpm/pmol	*pmol/10^6 cells*			
Control	226	87	103	12	18
HU-treated	157	141	80	≤0.5	≤0.5
Aphidicolin-treated	239	72	55	4	9

HU-treated cells TTP levels were about 60% higher than controls. Based on the levels of these endogenous TTP pools, the specific activity of TTP derived from [^{3}H]dUrd in HU-treated cells was about 30% lower than that present in control cells. In aphidicolin-treated cells it was similar to that in control cells.

Thus, the observed inhibitory effect of the antimetabolites on TS activity cannot be ascribed to an increase in the level of thymidine nucleotides synthesized either endogenously (Table 3) or from exogenous precursor (Tables 1 and 2) in intact cells, and some cause other than pool alteration must be sought to explain this cross-inhibition.

Influence of deoxynucleosides on the inhibitory effect of HU on DNA synthesis and TS activity. If HU were to inhibit DNA synthesis solely by inhibiting ribonucleotide reductase, as reflected by the decrease in endogenous dCTP, dGTP, and dATP pools (Table 3), one would expect that the provision of moderate concentrations of deoxycytosine, deoxyguanosine, and deoxyadenosine, along with dUrd or thymidine, in the medium would circumvent this block. We examined this possibility by testing the incorporation of [^{3}H]dUrd (5 μM) or [^{3}H]thymidine (5 μM) into DNA in the presence of the other three deoxynucleosides (5 μM each). As shown in Table 4, neither the inhibitory effect of HU on DNA synthesis nor that on TS activity could be circumvented by the provision of deoxynucleosides in the medium. These results strongly indicate that, in addition to ribonucleotide reductase, other enzymes in the pathway of deoxynucleoside triphosphate and DNA biosynthesis are being inhibited by HU. As shown in Tables 1 and 2, these steps include at least thymidine kinase and TS.

Effect of HU and aphidicolin on RNA synthesis and uridine nucleotide pools in S phase CHEF/18 cells. Because much of our deoxynucleotide pool data indicated that thymidine kinase activity is inhibited in the presence of HU or aphidicolin in intact cells, we then inquired whether other nucleoside kinases (other than those associated with DNA replication) are similarly affected by these antimetabolites. We tested this possibility using [5,6-^{3}H]uridine incorporation into acid-precipitable material (RNA) in intact cells in the presence of HU and aphidicolin. As shown in Table 5, we observed that there was no significant inhibitory effect of these two drugs on RNA synthesis. Furthermore, incorporation of [^{3}H]uridine into the combined pool of uridine nucleotides (i.e., UMP plus UDP plus UTP) remained virtually unchanged in the presence of these drugs, as compared with the control.

These results suggest that the inhibitory effect of HU and aphidicolin on thymidine kinase is a highly specific one, specific to that fraction of dUrd or thymidine kinase that is involved in DNA replication.

Discussion

Cross-inhibition is the phenomenon in which an inhibitor of one enzyme in a complex blocks a second enzyme also, whereas the second enzyme is unaffected by the inhibitor when not associated with the complex. Allosteric interaction, of which cross-inhibition is one example, is a more general concept of changes at a protein's active center modulated by effectors that are bound at a remote site and that act through structural coupling. In an aggregated multienzyme complex, such as replitase, the catalytic activation or inactivation of the constituent enzymes, such as the antimetabolite cross-inhibition demonstrated here, is a function of allosteric interaction between them.

In the studies reported here, we have confirmed and extended an earlier demonstration of cross-inhibition between enzymes of the replitase complex and have investigated its basic mechanism. In addition to confirmation of the earlier demonstration that TS is cross-inhibited by aphidicolin and HU, we have extended the observations of cross-inhibition to an additional drug, amidox (an inhibitor of ribonucleotide reductase), and an additional enzyme, thymidine kinase. In addition, we have carried out extensive measurements of nucleoside and nucleotide pools and their changes, in order to elucidate the mechanism of cross-inhibition. By ruling out many of the suggested explanations of cross-inhibition, we have shown it is likely that cross-inhibition occurs by allosteric interaction within the structure of the replitase complex.

Several possible explanations have been put forth to explain cross-inhibition. It has been suggested 1) that product inhibition blocked pathways (17, 18), 2) that there is inhibition of the uptake mechanisms of the cell, or 3) that the specific activity of deoxynucleotides derived from [^{3}H]dUrd in the cells is greatly decreased due to the changes in corresponding endogenous pools (19). All three of these suggested explanations lead to specific predictions that are not observed. For instance, blocking of uptake mechanisms implies depletion of nucleotide pools, whereas product feedback inhibition implies significant increases in deoxythymidine nucleotides and their immediate precursors. These predicted pool changes do not occur; in fact, the pools and their specific activities are virtually unchanged, ruling out these two suggestions.

The third suggestion was that the observed inhibitions are misinterpretations based on failure to properly correct for specific activity changes from mixing with the endogenous pools or for specific activity alterations of the pools due to the actions

TABLE 4

Effect of HU on DNA synthesis and TS activity in intact S phase CHEF/18 cells in the presence of deoxynucleosides

Petri dishes (35 mm) containing about 2 × 10^6 cells were synchronized to S phase. Ten hours after release from isoleucine block, 0.5 mM HU was added to the culture dishes. Fifteen min later radioactive precursors with or without unlabeled deoxyadenosine (dA), deoxycytosine (dC), and deoxyguanosine (dG) (5 μM each) were added. After incubation for 1 hr at 37° in a humidified incubator containing 10% CO_2, culture dishes were processed for measurement of rates of DNA synthesis and TS activity.

Condition	Rate of radioactive precursor incorporation into DNA	Rate of *in vivo* TS activity
	pmol/10^6 cells/hr	*pmol/10^6 cells/hr*
Control	504	532
HU (0.5 mM)	11	17
HU (0.5 mM) plus dA, dG, dC, and dUrd[a]	15	<5
HU (0.5 mM) plus dA, dG, dC, and dThd[a]	19	

[a] Radioactive precursors; when any drug alone was tested for its effect, [^{3}H]dUrd was used as radioactive precursor.

TABLE 5

Effect of HU and aphidicolin on the incorporation of [5,6-^{3}H]uridine into acid-precipitable material and acid-soluble uridine nucleotides

Molar concentrations are calculated based on the specific activity of the radioactive precursor (818 cpm/pmol) included in the media. Each value is an average of duplicate samples. There was less than 5% variation between the duplicate samples.

	Control	Hydroxyurea (1 mM)	Aphidicolin (1 μg/ml)
		pmol/10^6 cells/hr	
Acid-precipitable material (RNA)	560[a]	475	502
		pmol/10^6 cells	
rUridine	43[b]	37	47
rUMP	543	486	578
rUDP	641	596	593
rUTP	522	604	519

[a] Values for the rate of RNA synthesis are expressed in pmol/10^6 cells/hr.
[b] Nucleoside and nucleotide pool levels are expressed as pmol/10^6 cells after incubation with radioactive precursor for 1 hr.

of the drugs (19). As described above, the measurements of specific activity with normal and reversed order of addition of inhibitors and of labeled nucleotides demonstrate that there are no major alterations in specific activity of the nucleotide pools in the presence of the inhibitors. Furthermore, as has been shown elsewhere (20), at low levels of exogenous nucleoside precursors, they are preferentially transferred to the replication-active pool with very little dilution by the remainder of the endogenous pools. Therefore, in this study, where we have made direct measurements of specific activity, we can rule out specific activity artifacts as the source of observed inhibitions.

Some of the earlier work in this area may have been complicated by failure to carefully distinguish between replication-active and replication-inactive pools (20, 21). In fact, we observed that raising of the level of exogenous dUrd above 0.5 μM leads to more uptake into pools that seem less subject to cross-inhibition.[1] This is probably because, at high dUrd levels, passive diffusion becomes a more significant factor in influx across the cell membrane (22, 23). Some of this passive influx goes to replication-inactive pools not associated with replitase. The enzymes acting on the replication-inactive pools are probably free soluble enzymes and not subject to allosteric inhibition. Cross-inhibition seems to be solely associated with the replitase complex.

DNA synthesis, which is associated with replitase, cannot be supported by exogenous deoxynucleosides, which might be expected to circumvent a ribonucleotide reductase block due to HU. This observation, which matches that of Scott and Forsdyke (24) with thymus cell DNA synthesis, cannot be explained by pool alterations and points to inhibition by allosteric interaction between the tightly coupled enzymes within the replitase complex.

Although the studies of pool sizes do not show the alterations predicted by the alternative explanations for cross-inhibition, they do show an important change in pool size, namely, that in the presence of inhibitors there is a pile up of dUrd relative to control values. This indicates that thymidine kinase, the enzyme that converts dUrd to dUrd monophosphate is inhibited by inhibitors of DNA polymerase and ribonucleotide reductase. Free thymidine kinase is unaffected by these inhibitors, so this represents an entirely new case of cross-inhibition within the replitase complex.

One reason why the results here can point more strongly toward control mechanisms within replitase itself than can some of the earlier literature is because of the high level of synchronization in CHEF/18 cells obtained with isoleucine block removal. Because the cells in this study are highly enriched in S phase, the replitase complex is present uniformly throughout the cell population. The data are not confused by the metabolism of cells in other phases of cell cycle. Many of the studies in the literature are carried out on logarithmically growing cells, with the internal controls in the replitase complex obscured by the fact that a majority of the cells are out of the synthesis phase and consequently do not have fully assembled replitase complexes.

In the present studies with S phase cells, the levels of TDP derived from exogenous [^{3}H]dUrd (5 μM) are about 5 times larger than the levels of TTP (Table 2). This observation is paradoxical, because there is a high level of nucleoside diphosphate kinase, which converts TDP to TTP, in these cells (1). Broad specificity for the phosphorylation of nucleotides of RNA and DNA by highly purified preparations of nucleoside diphosphate kinase from a variety of mammalian cells (25, 26) and the presence of high levels of this enzyme in the cells have led some to wonder whether there are any conditions under which this enzyme activity is regulated *in vivo* (27). However, based on related observations in prokaryotic cells, it is suggested that only a small fraction of nucleoside diphosphate kinase may be available, via complex formation with the other enzymes of DNA biosynthesis, to participate in deoxynucleoside triphosphate biosynthesis (28). In this regard, it is interesting to note that only a similar small but specific fraction of total cellular nucleoside diphosphate kinase is associated with the multienzyme complex replitase that is responsible for nuclear DNA replication in mammalian cells (1). This small but important fraction of nucleoside diphosphate kinase associated with the replitase complex may be responsible for all the incorporation of radioactivity from [^{3}H]dUrd into replication-active TDP.

Why does it make sense to have allosteric controls in the replitase complex? Two reasons stand out. The first, as in other cellular multienzyme complexes, is the kinetic advantage of delivering substrate to subsequent enzymes in a metabolic pathway at short diffusion distance and at high local concentration. This very proximity, a requirement of efficient function, makes structural interaction possible. The second reason is that a cellular process as important to life as DNA synthesis needs tight control in order to maintain proper synchronization of all the concurrent processes leading to cell division. From this study and other related studies, it seems that one important mechanism for this control is allosteric interaction within the highly structured framework of the replitase complex.

Acknowledgments

We are thankful to Jill A. Sfetko for her invaluable technical assistance throughout the entire study.

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