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# EVIDENCE FOR ACYLOXYMETHYL ESTERS OF PYRIMIDINE 5′-DEOXYRIBONUCLEOTIDES AS EXTRACELLULAR SOURCES OF ACTIVE 5′-DEOXYRIBONUCLEOTIDES IN CULTURED CELLS

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**Abstract**—Cells commonly resist growth inhibition by purine and pyrimidine bases and nucleosides by restricting intracellular formation of the corresponding 5′-mononucleotides. Nucleotide derivatives that can act as effective membrane-transport precursors of the poorly membrane-permeable nucleotides have not been identified so far. We studied the bis(pivaloyloxymethyl)ester (I) of FdUMP (5-fluoro-dUMP) and a cyclic phosphodiester (II) of FdUMP derived from 1,3-dihydroxy-1-*C*-(pivaloyloxymethyl)propane which are active *in vivo* against a 5-fluoro-2′-deoxyuridine (FUdR)-resistant mouse leukemia and are attacked by carboxylic esterases under physiological conditions to produce FdUMP by elimination of formaldehyde and acrolein respectively. The assay for intracellular FdUMP was the inhibition of DNA synthesis due to inhibition of TMP synthetase in cultured mouse LM(TK$^-$) fibroblasts genetically devoid of thymidine kinase (TK) and thus unable to convert FUdR directly to FdUMP. At $10^{-6}$ M, I, II, or FUdR inhibited DNA synthesis in 2 hr by 99, 80, and 35% respectively; at $10^{-5}$ M, maximal inhibition was attained after < 15, 30 and 90 min respectively. Inhibition of DNA synthesis in TK$^+$ cells by $10^{-5}$ M I, II, or FUdR was reversed completely by $10^{-5}$ M thymidine (TdR) but unaffected by $10^{-5}$ M UdR, confirming TMP synthetase as the locus of inhibition. At $10^{-5}$ M, bis(pivaloyloxymethyl) esters of phenyl phosphate or a *p*-substituted benzylphosphonic acid did not inhibit significantly DNA synthesis in TK$^+$ cells. From this finding, and from effects produced by V (see below), we conclude that pivalic acid and $CH_2O$ arising from I contribute little to its above inhibitory effects. In TK$^-$ cells in which DNA synthesis is prevented by blockade of TMP synthetase with aminopterin, the bis(pivaloyloxymethyl) ester (V) of TMP, at $0.9 \times 10^{-4}$ M, induced a 4-fold faster rate of DNA synthesis than did $10^{-3}$ M TMP, whereas $10^{-3}$ M TdR did not affect the rate. After 3 hr the rate with V was 80% that in the absence of aminopterin. In the above systems the nucleotide diesters I, II and V appear to be acting as effective extracellular sources of active intracellular FdUMP and TMP, in processes that involve loss of the two esterifying groups.

Tumour cells commonly become resistant to growth inhibition by purine and pyrimidine base and nucleoside analogues through loss of the ability to convert them to 5′-mononucleotides, a step required for their inhibitory activity [1]. The nucleotides themselves are inactive against drug-resistant cells, due to their dephosphorylation in extracellular fluids and on cell surfaces [2–4] and to their existence at physiological pH as poorly membrane-permeable phosphodianions [5–7]. To overcome this problem, it would be desirable to devise a strategy to introduce the nucleotides efficiently into the cell. Attempts to do this, using a variety of protective or masking groups for the anionic oxygens, have so far been largely unsuccessful, as exemplified in recent studies [8–11].

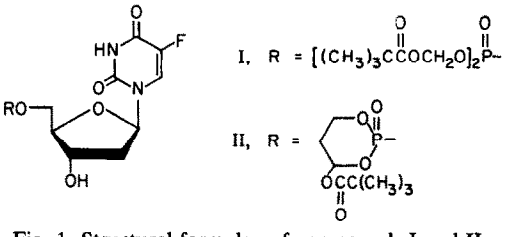


Fig. 1. Structural formulae of compounds I and II.

Recently, Farquhar and colleagues prepared certain pivaloyloxymethyl esters of FdUMP§ (Fig. 1, compounds I and II) that were converted by carboxylic esterases to unstable intermediates that rapidly yielded free FdUMP by elimination of formaldehyde and acrolein respectively. Compounds I and II inhibited the growth of cells resistant of FU *in vitro* and were active *in vivo* against transplantable tumors resistant to FU and FUdR [12–15]. These findings leave open the question of whether I and II form FdUMP via degradation to FU or FUdR, or whether they diffuse into the cells and then yield free FdUMP by loss of their esterifying groups. To address this question, we have examined effects of I and II on

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§ Abbreviations: BUdR, 5-bromo-2′-deoxyuridine; CdR, 2′-deoxycytidine; FdUMP, 5-fluoro-dUMP; FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; HAT, cell culture medium supplemented with glycine (15 μg/ml), hypoxanthine (15 μg/ml), aminopterin (1 μg/ml) and thymidine (4 μg/ml); PBS, phosphate-buffered saline; TdR, thymidine; and TK, (EC 2.7.1.21), the cytosolic form of thymidine kinase.

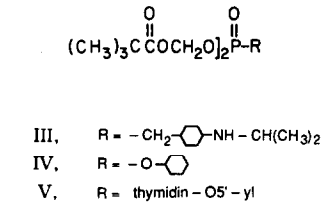

Fig. 2. Structural formulae of compounds III, IV and V.

cells genetically devoid of cytosolic thymidine kinase (TK) and incapable, therefore, of converting FUdR tc FdUMP or converting TdR to TMP. Because TMP is essential for synthesis of nuclear DNA, and because FdUMP powerfully inhibits TMP synthetase, the sole alternative source of TMP [16], we studied the effects of I and II on the rate of DNA synthesis, measured as the ability to incorporate [$^3$H]CdR, a DNA-specific label, into acid-insoluble material.

## MATERIALS AND METHODS

*Reagents.* Compounds I, II, III, IV and V (Figs 1 and 2) were prepared at the M.D. Anderson Hospital; the methods used have been described [12, 13]. These compounds were prepared as stock solutions at 10 mM in 50% ethanol–water and stored at −20°. No decomposition of compounds I or II was detected under these conditions by thin-layer chromatography on silica gel in ethyl acetate–isopropanol–water (4:2:1), methanol–chloroform (1:9) or acetone–methylene dichloride (2:3) (the $R_f$ values of I were 0.78, 0.38, and 0.39 and of II were 0.68, 0.30, and 0.20 respectively). The solutions were diluted into cell growth medium just before each experiment.

FU, FUdR and TMP were obtained from the Sigma Chemical Co. (St Louis, MO), [$^3$H]CdR ([5-$^3$H]deoxycytidine, 18 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). All other chemicals were reagent grade.

*Cell cultures.* Thymidine kinase deficient mouse cell line LM(TK$^-$) was a gift of Dr Richard Axel; the phenotype was confirmed by selection in medium with BUdR or HAT. Thymidine kinase positive cells used as controls were mouse cell line L929, obtained from the American Type Culture Collection. The cells were routinely propagated at 37° in 5% $CO_2$ as monolayers in Dulbecco's modified Eagle medium (GIBCO high glucose) supplemented with 10% fetal bovine serum. Freedom from mycoplasmas was confirmed by Hoechst 33258 staining [17] and by a hybridization assay (Gen-Probe, Fisher Scientific, Fallstown, NJ).

*DNA synthesis assay.* The rate of DNA synthesis was estimated from the uptake of [$^3$H]CdR. Replicate cultures for each assay were set up by seeding $5 \times 10^5$ cells per 6-cm Falcon TC petri dish in the above growth medium. After 24 hr, the medium was replaced with 2 ml of test medium, which contained the test compounds and heat-inactivated (1 hr, 55°) serum to reduce potential enzymatic degradation of those compounds. After 2 hr of incubation, [$^3$H]CdR (2 μCi) was added for an additional hour to label newly synthesized DNA. The cells were then harvested by rinsing with 0.02% EDTA in Puck's saline A followed by 15-min incubation with 0.04% trypsin in the same solution. In some experiments, in which short exposures to test medium were required, labeling time was reduced to 15 min and cells were detached from the dishes with a rubber policeman; the results were similar. The harvested cells were collected on 25 mm diameter Millipore filters (HA, 0.45 μm pore size), and the dish and filter were washed with 10 ml Dulbecco's PBS. The filters were washed three times with 5 ml of cold 5% trichloroacetic acid, once with 5 ml of 70% ethanol, and dried. The dry filters were placed in counting vials with 5 ml Cytoscint (Fisher Scientific), and $^3$H was counted with approximately 10% efficiency.

For each experiment, the number of cells per replicate culture was estimated by counting duplicate petri dishes using a Coulter $ZB_1$ electronic cell counter.

To measure release of blocked DNA synthesis by compound V, the cells were first exposed to growth medium to which was added aminopterin (1 μg/ml), glycine (15 μg/ml) and hypoxanthine (15 μg/ml). After 3 hr, when [$^3$H]CdR uptake had fallen to minimal levels, the test compound was added. Following addition of [$^3$H]CdR (1 μCi/ml), the cell samples were harvested at various times and counted as described above.

In the experiments reported below, duplicate assays were carried out for each experimental point. The mean coefficient of variation for such duplicate determinations was 8.4% (SD 9.8%).

The significance of differences discussed in the text or figure legends was estimated using Student's *t*-test.

## RESULTS

*Inhibition of DNA synthesis in TK$^-$ cells by FU derivatives.* When DNA synthesis was assayed using [$^3$H]CdR incorporation as described above, control cultures typically gave an uptake of about 10,000 cpm per culture ($5 \times 10^5$ cells recovered per dish). In cultures to which compounds I or II was added, the [$^3$H]CdR incorporation was reduced greatly; for each experiment these results were expressed as a percentage of the control value. The effects of compounds I and II were assayed at various concentrations and compared to the effects of FU and FUdR, with the results summarized in Fig. 3. At concentrations of $10^{-5}$ M and higher, all four agents nearly completely blocked [$^3$H]CdR uptake after the 2-hr exposure. At $10^{-6}$ M, the block to uptake was substantially greater for compounds I and II than for FU or FUdR. Compound I was significantly more effective than compound II.

The time required to establish the block to [$^3$H]CdR incorporation was studied, as shown in Fig. 4. Compound I completely blocked uptake at the earliest time measurable (15 min), whereas for compound II complete inhibition was only observed after 30 min. At that time, incorporation in the presence of FUdR remained at about half the control level.

*Requirement for FdUMP for inhibition.* To investigate whether the above inhibition by I and II could

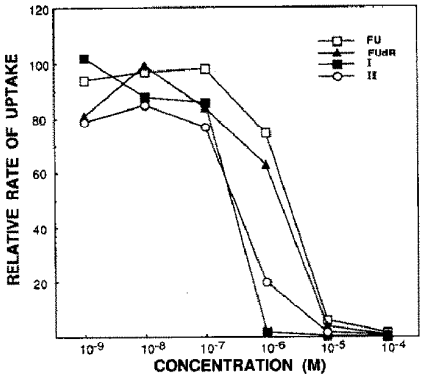


Fig. 3. Inhibition of DNA synthesis in TK⁻ cells by FU derivatives as a function of concentration. Duplicate cell cultures were exposed to the compounds shown for 2 hr, incubated with the addition of 1 μCi/ml [³H]CdR for an additional hour, then harvested and counted. Uptake of acid-insoluble radioactivity is expressed as a percentage of that in control cultures to which no compound was added (mean value 18,400 cpm, SD 1,200 cpm). The curves differed significantly only as follows: at $10^{-6}$ M, FU or FUdR were less effective than I or II ($P \ll 0.01$) and II was less effective than I ($P < 0.01$).

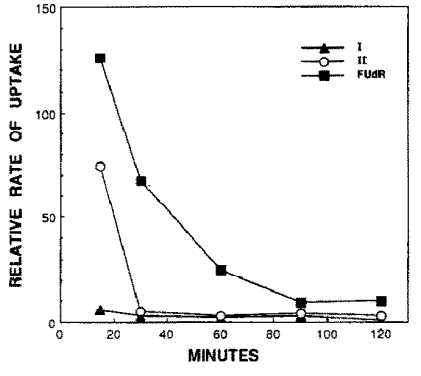


Fig. 4. Time course of inhibition of DNA synthesis in TK⁻ cells by FU derivatives at $10^{-5}$ M. Duplicate cultures were exposed from time zero to the test compounds and 3.3 μCi/ml [³H]CdR was present during the last 15 min of incubation. The cells were harvested using a rubber policeman at the times shown and counted. Uptake of acid-insoluble radioactivity is expressed as a percentage of that in control cultures to which no compound was added (mean value 2400 cpm, SD 500 cpm). The curve for FUdR differed significantly ($P \ll 0.01$) from those for I and II through 60 min. At 15 min, II differed significantly ($P < 0.02$) from I or FUdR.

Table 1. Rate of DNA synthesis in mouse LM(TK⁻) cells after exposure to compounds containing FdUMP (I) or lacking this group (III, IV).

| Compound | Concentration (M) | Rate of DNA synthesis |
|---|---|---|
| None | | 100 ± 13 |
| I | $10^{-5}$ | 1.1 ± 0.04* |
| I | $10^{-6}$ | 1.3 ± 0.03* |
| III | $10^{-5}$ | 72 ± 9.3 |
| III | $10^{-6}$ | 83 ± 1.3 |
| IV | $10^{-5}$ | 72 ± 4.3 |
| IV | $10^{-6}$ | 61 ± 10.1 |

The rate of DNA synthesis was estimated as a percentage of the uptake by control cultures (mean value 15,000 cpm). Values are averages ± SD. Duplicate cell cultures were exposed to the test compounds at the concentrations shown for 2 hr, then 1 μCi/ml [³H]CdR was added and incubation was continued for an additional hour. Cells were harvested, and acid-insoluble radioactivity was counted as described in Materials and Methods.

* Significantly different from controls ($P < 0.05$).

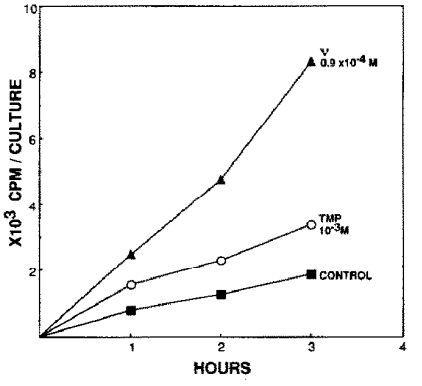


Fig. 5. Rate of DNA synthesis in TK⁻ cells released from an aminopterin-induced block by addition of the TMP ester, compound V or by free TMP. Duplicate cultures were exposed to medium supplemented with glycine, hypoxanthine and aminopterin for 3 hr to block DNA synthesis, compounds were added to the concentrations shown and incubated for an additional 2 hr, 1 μCi/ml [³H]CdR was added, and incubation was continued for the times shown. Cells were then harvested, and acid-insoluble radioactivity was counted. The three curves differed significantly ($P < 0.05$) at all time points.

be caused by the intracellular liberation of formaldehyde and pivalic acid, we evaluated the effects of compounds III and IV (Fig. 2) on [³H]CdR uptake. As shown in Table 1, these compounds did not reduce the rate of [³H]CdR uptake significantly ($P > 0.05$) and were far less effective than compound I ($P < 0.01$).

*Restoration of DNA synthesis by a protected derivative of TMP.* We examined the effects of compound V (Fig. 2), an analog of compound I. As shown in Fig. 5, compound V induced the synthesis of DNA at a faster rate than did the parent compound, TMP. The low rate of ³H incorporation in the control cultures is presumed to reflect mitochondrial DNA synthesis that utilizes the mitochondrial deoxypyrimidine kinase that remains unimpaired in these cells [18].

*Effect of TdR or UdR on inhibition of DNA synthesis by I and II.* TK⁺ L-cells (strain L929) were treated with I, II, FU or FUdR, alone or in the presence of various concentrations of TdR or UdR. In TK⁺ cells, both TdR and UdR can be phosphorylated. As shown in Table 2, TdR produced significant reversal of inhibition by the four FdUMP precursors. Treatment with UdR had little effect

Table 2. Effects of pyrimidine nucleosides on the inhibition of DNA synthesis in mouse L929 (TK$^+$) cells exposed to FU, FUdR or FdUMP derivatives I or II

| | Relative rates of incorporation of [$^3$H]CdR into DNA (% of control) | | | | | | |
|---|---|---|---|---|---|---|---|
| | | +TdR (concn, M) | | | +UdR (concn, M) | | |
| Inhibitor | −TdR, −UdR | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| None | 100 | | | | | | |
| FU | 23 ± 0.7 | 192 ± 4.7* | 129 ± 14.7* | 24 ± 0.6 | 57 ± 0.02* | 29 ± 0.8* | 18 ± 0.3 |
| FUdR | 10 ± 0.5 | 158 ± 0.8* | 52 ± 5.5* | 21 ± 2.5* | 9 ± 0.3 | 10 ± 0.1 | 10 ± 1.0 |
| I | 3 ± 0.6 | 131 ± 2.5* | 76 ± 3.4* | 7 ± 0.4* | 5 ± 1.8 | 4 ± 0.2 | 3 ± 0.1 |
| II | 11 ± 3.3 | 148 ± 8.3* | 95 ± 1.8* | 15 ± 0.7 | 12 ± 0.3 | 9 ± 0.1 | 7 ± 0.3 |

L929 (TK$^+$) cell cultures were exposed to the inhibitors at $10^{-5}$ M for 2 hr in the presence or absence of nucleoside supplements as shown. [$^3$H]CdR (1 $\mu$Ci/ml) was added and incubation was continued for 1 hr. The cells were then harvested, and acid-insoluble radioactivity was counted as described in Materials and Methods. The data shown are rates of uptake expressed as a percentage of the uptake (27,000 cpm mean value) in cultures free of both inhibitor and supplement. Averages for duplicate determinations ± SD are shown.

* Significantly different ($P < 0.05$) from the values obtained with the test compound alone, i.e. showed significantly reduced inhibition.

Table 3. Inhibition of DNA synthesis by compounds I and II in cell culture media prepared with native or heat-inactivated serum

| Serum | Compound | Rate of DNA synthesis |
|---|---|---|
| Heat-inactivated | None | 100 ± 0.04 |
| | I | 1.5 ± 0.07 |
| | II | 44 ± 0.7 |
| Native | None | 89 ± 2.8* |
| | I | 1.7 ± 0.08 |
| | II | 23 ± 0.2* |

The rate of DNA synthesis was estimated as a percentage of the uptake by control cultures. Duplicate cultures of LM(TK$^-$) cells were exposed to the test compounds at $10^{-6}$ M for 2 hr, 1 $\mu$Ci/ml [$^3$H]CdR was added, and incubation was continued for an additional hour. Cells were harvested, and acid-insoluble ratioactivity was counted as described in Materials and Methods. Control cultures in heat-inactivated serum incorporated 13,000 cpm. Values are averages ± SD.

* Significantly ($P < 0.05$) reduced by native serum relative to heat-inactivated serum.

except in inhibition induced by FU, in which partial reversal was found with UdR at $10^{-6}$ or $10^{-5}$ M.

*Effectiveness of compounds I and II in native serum.* The experiments described above were carried out using heat-inactivated serum in the cell culture medium, in order to prevent extracellular enzymatic degradation of the test compounds. To learn whether this precaution is necessary, we performed comparable experiments using native serum, i.e. as customarily supplied for cell culture use. As shown in Table 3, the use of native serum produced small but significant decreases in the measured incorporation; native serum did not reverse the inhibition produced by I or II. Thus, enzymes present in serum seem to have little effect on the protected compounds during experiments of the duration reported here.

## DISCUSSION

A general rationale for the preparation of I and II was as a model system to evaluate acyloxymethyl ester protective groups as a means to promote intracellular delivery of anionic molecules. In I and II, the desired active intracellular product was FdUMP. The hope was that I and II would permeate the plasma membrane, be deacylated by the carboxylate esterases commonly found in cells, and that the products would then furnish biologically effective levels of FdUMP by spontaneous elimination of formaldehyde and acrolein respectively. Initial evidence consistent with such a membrane-transport precursor role was the ability of I and II to inhibit the growth of FU-resistant cell cultures and a mouse tumor resistant to FU and FUdR [14, 15]. The present study further investigates, in cell culture systems, the mechanism of action of these compounds. The experiments employed primarily the cell line LM(TK$^-$), an extensively studied genetic mutant entirely deficient in expression of TK, the cytosolic form of thymidine kinase. Since no revertants have been isolated from this line, it is presumed to represent a genetic deletion. The mitochondrial form of deoxypyrimidine kinase is retained, but is present at a low level and does not provide precursor for nuclear DNA synthesis [18]. In these cells, FUdR that might arise from extracellular and/or intracellular degradation of the test compound can thus not be directly phosphorylated in the cytoplasm. To estimate the rate of DNA synthesis in these cells, we measured incorporation of [$^3$H]CdR. CdR is a specific precursor of DNA [19], most of the label appearing in DNA-thymine, a lesser amount in DNA-cytosine [20].

The experiments provide evidence that I and II can serve as efficient extracellular sources of intracellular FdUMP, since both compounds inhibited DNA synthesis more rapidly (Fig. 4) and nearly ten times more effectively (Fig. 3) than did FUdR. As discussed below, these effects appear to be due to

inhibition of TMP synthetase by intracellular FdUMP. I and II could supply FdUMP in the $TK^-$ cells either via loss of their two FdUMP esterifying groups, or via their degradation to intracellular FUdR and conversion of this to FU followed by four or five metabolic steps known to furnish FdUMP [16]. The first of these routes of FdUMP is favored because V, the TMP analogue of I, readily furnishes intracellular TMP by a mechanism that is unlikely to involve TdR formation (see below).

Compound I reduced the rate of DNA synthesis more rapidly and at a lower concentration than did compound II (Figs 3 and 4 and Table 3). The basis for this difference remains to be determined. It may reflect a difference in the rates at which I and II permeate the plasma membrane; it may also relate to the findings that compound I (half-life, 17 hr) is hydrolytically more stable than compound II (half-life, 6 hr) in pH 7.4 buffer at 37° and that it is more rapidly attacked by a hog liver esterase [15].

Intracellular degradation of the protective groups following esterase attack on I and II would liberate pivalic acid and formaldehyde in the case of I, and pivalic acid and acrolein in the case of II [12, 13, 15]. We tested for possible inhibition of DNA synthesis by pivalic acid and formaldehyde by using compounds III and IV, analogues of I that cannot yield FdUMP as a product. These compounds inhibited the rate of DNA synthesis weakly if at all (Table 1), although they would be expected to passively diffuse into cells more rapidly than I and to yield pivalic acid and formaldehyde at a rate comparable to that of I [12, 13]. Further, as discussed below, compound V at $10^{-4}$ M could restore most or all of DNA synthesis blocked by aminopterin, a finding which implies that pivalic acid and formaldehyde are unlikely to contribute significantly to the inhibition of DNA synthesis mediated by compound I at $10^{-6}$ M (Fig. 3) within a similar period of time.

If compounds I and II liberated FdUMP in a suitable cell compartment, it would bind to and inactivate thymidylate synthetase, as normally observed in inhibition of cell growth by FUdR [16]. Such a block could be overcome by TMP but not by its precursor, dUMP. In $TK^+$ cells treated with I, II, FU or FUdR, it was found (Table 2) that TdR restored DNA synthesis whereas UdR did so only in the presence of FU, consistent with thymidylate synthetase as the locus of inhibition by I and II. UdR may reverse the inhibition of DNA synthesis by FU through competition at the uridine kinase reaction or other step in the conversion of FU to FdUMP [16].

Inhibition of cell growth by FU is sometimes associated with incorporation of FUMP into RNA, leading to a failure of maturation of ribosomal RNA presursor [16]. Such effects are predictably small in the relatively short times of the present experiments. In confirmation, addition of 0.04 $\mu$g/ml of actinomycin D, a concentration that blocks transcription of ribosomal RNA, had no measurable effect on [$^3$H]CdR incorporation into DNA in our system (unpublished experiments).

Findings with compound V, the TMP analogue of I, provide a second line of evidence that bis(pivaloyloxymethyl) esters of 2′-deoxynucleoside 5′-phosphates can furnish, intracellularly, the free, biologically active nucleotide via loss of the two esterifying groups. These studies employed $TK^-$ cells in which cytosolic TMP synthesis from TdR was blocked genetically while TMP synthesis from dUMP, the only alternative route, was blocked with aminopterin. Previous studies had shown that under these conditions the cells retain ability to synthesize DNA when a suitable source of TMP (extracellular TMP or TTP, though not TdR) is provided [11]. Compound V induced DNA synthesis at a rate 4-fold faster than produced by an 11-fold higher level (1 mM) of TMP (Fig. 5). The intracellular TMP responsible for this effect is unlikely to arise from degradation of V to TdR, because 1 mM TdR produces no effect on the rate of DNA synthesis under these conditions [11]. It is also unlikely to arise from degradation of V to thymine, because it is difficult to demonstrate thymine as a precursor of DNA thymine, even in rapidly dividing mammalian cells [21]. Compound V acted as an effective source of intracellular TMP insofar as within the 3-hr period shown in Fig. 5 it established a rate of DNA synthesis that was approximately 80% that of control cultures of $TK^-$ cells not blocked with aminopterin.

The present study thus suggests that compounds I and II each lose their two phosphate esterifying groups and release the parent nucleotide FdUMP in a cell compartment in or from which it is able to inhibit thymidylate synthetase. Compound V appears to furnish biologically active intracellular free TMP by the same mechanism. The principal cell-permeating species are mostly likely the nucleotide phosphodiesters I, II, and V, although the present findings do not exclude from consideration the corresponding nucleotide monoesters or indeed the free nucleotides. These more polar species would clearly enter cells less efficiently than the triesters were the process one of passive diffusion. However, the mode of entry is not established. Compounds I and II produced complete inhibition of DNA synthesis within 0.5 hr under conditions wherein their half-lives, resulting from nonenzymatic hydrolysis at pH 7.4, are 17 and 6 hr, respectively, at 37° [15]. Thus, phosphotriesters comprised the predominant extracellular species of nucleotide precursor molecule in the studies reported here.

Earlier work has suggested that acyloxymethyl esters of carboxylic acids also can function as membrane-transport precursors. Thus, in 1965 it was reported that acyloxymethyl esters of penicillin may promote tissue penetration of penicillin [22], and in 1981–1982 it was reported that acetoxymethyl esters of carboxylic calcium chelators can be used to load the corresponding nonesterified chelators into living cultured cells [23, 24]. Extension of this general approach to nucleotides (as in the present work), and possibly to other types of anionic phosphate derivatives, may provide a valuable tool in future drug design.

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