# ZINC REQUIREMENT FOR DNA REPLICATION IN STIMULATED HUMAN LYMPHOCYTES

RICHARD O. WILLIAMS and LAWRENCE A. LOEB

From The Institute for Cancer Research, Fox Chase Center for Cancer and Medical Sciences, Philadelphia, Pennsylvania 19111



## ABSTRACT

The requirement for $Zn^{++}$ in DNA replication by phytohemagglutinin-stimulated human lymphocytes was studied. When 6 $\mu$M *o*-phenanthroline, a chelator with a high affinity for $Zn^{++}$, is added to cultures of stimulated lymphocytes a nearly complete inhibition of thymidine incorporation results within a few hours. In contrast, the incorporation of uridine is only slightly reduced and the incorporation of leucine is unaffected. *m*-Phenanthroline, a nonchelating analogue, does not alter the rate of thymidine incorporation even when present in 10-fold greater amounts than *o*-phenanthroline. The inhibition of thymidine incorporation by *o*-phenanthroline could be entirely reversed by the addition of $Zn^{++}$ to the cultures, or could be prevented by the prior addition of either $Zn^{++}$ or $Ni^{++}$. All other divalent cations tested were incapable of reversing the *o*-phenanthroline inhibition of thymidine incorporation.

## INTRODUCTION

Recent studies indicate an obligatory requirement for zinc during DNA synthesis in animal cells. The incorporation of thymidine into DNA is impaired in livers of rats maintained on a zinc-deficient diet (1). $Zn^{++}$ appears to be required for the induction of DNA synthesis in kidney cultures (2) and in liver cells after partial hepatectomy of rats (3) as well as for the continuation of DNA synthesis in cultured chick embryos (4). In addition, zinc is required for catalysis by *Escherichia coli* DNA polymerase I (5). It is present in homogeneous preparations of DNA polymerases from *E. coli*, sea urchin nuclei (6), and T4 bacteriophage (5).

In order to understand the relationship between the requirement for zinc during DNA synthesis in vivo and its function in catalysis by purified polymerase, we have begun an investigation on the role of zinc in DNA replication during lymphocyte transformation. Human peripheral lymphocytes are well-differentiated cells which seldom divide in vivo. When these cells are placed in culture their quiescent behavior is continued. However, the addition of mitogens such as phytohemagglutinin (PHA) to cultures of these cells leads to a series of metabolic alterations culminating in DNA replication with subsequent cell division (7). Since this transformation occurs in culture, one can monitor and manipulate the events leading to DNA replication so as to probe the role of zinc in this replication.

In these studies we made use of the chelating agent, 1,10-phenanthroline (*o*-phen), and its nonchelating analogue, 1,7-phenanthroline (*m*-phen). The use of *m*-phen provides a control for determining which alterations in cellular metabolism can be attributed solely to the chelating properties of *o*-phen (6, 8).

## MATERIALS AND METHODS

### *Lymphocyte Cultures*

Human peripheral lymphocytes were isolated from freshly obtained heparinized blood using nylon columns as described by Bach and Hirschhorn (9). The purified lymphocytes were suspended at a concentration of $7.5 \times 10^5$ cells/ml in Eagle's minimal essential medium (Spinner modification) supplemented with 20% dialyzed fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (1%). All of the constituents of the medium were obtained from Grand Island Biological Company, Grand Island, N. Y. The lymphocytes were cultured in quadruplicate in 5% $CO_2$-95% air for the times indicated. Each culture (1 ml) contained $7.5 \times 10^5$ lymphocytes in 125-mm 16-X Falcon plastic culture tubes (Falcon Plastics, Los Angeles, Calif.). At the start of incubation 0.025 ml of phytohemagglutinin "M" (General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio) was added to each culture. The complete culture medium including serum contained 1–2 μM zinc as determined by atomic adsorption spectroscopy (5).

### *Isotope Incorporation*

Rates of RNA, DNA, and protein synthesis were measured by adding 0.025 ml of a solution containing 2.5 μCi of either [$^3$H]uridine, [*methyl*-$^3$H]thymidine, or L-[$^3$H]leucine (New England Nuclear, Boston, Mass.) to each culture 1 h before harvesting the cells. Incorporation of the radioactive precursor was terminated by adding 0.1 ml of a solution containing 10 μmol of the corresponding unlabeled compound and chilling the cultures in an ice-water bath. The lymphocytes were harvested by centrifugation for 10 min at 2,500 *g*. Then they were washed by centrifugation in 2 ml of 0.15 M potassium chloride and centrifuged again. The cell pellet was suspended in 1 ml of 0.15 M potassium chloride, frozen, thawed, and mixed with 2 ml of 0.5 N perchloric acid. The acid-insoluble precipitate was collected on glass fiber filters (GF/C) and counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) as previously described (10).

## RESULTS

### *Effect of o-Phen on DNA, RNA, and Protein Synthesis*

The transformation of nondividing human lymphocytes into actively replicating lymphoblasts is accompanied by increases in the rate of RNA, protein, and DNA synthesis. The effects of *o*-phen and its nonchelating analogue, *m*-phen, on the rates of incorporation of labeled thymidine, uridine, and leucine are given in Table I. Cultures of lymphocytes were stimulated with PHA and 20 h later were exposed to either *o*-phen (5 μM) or *m*-phen (50 μM). Subsequently, 3, 6, and 12 h later, the cells were incubated with each of the labeled precursors for 1 h and the rate of incorporation into an acid-insoluble precipitate was determined. The rate of thymidine incorporation was inhibited by 80% in 3 h by *o*-phen; a 10-fold greater concentration of *m*-phen was without effect.

TABLE I

*Effects of o-Phen and m-Phen on DNA, RNA and Protein Synthesis*

| Compound added | Exposure hour | [$^3$H]thymidine incorporation | [$^3$H]uridine Incorporation | [$^3$H]leucine incorporation |
|---|---|---|---|---|
| | | *cpm/culture* | *cpm/culture* | *cpm/culture* |
| *m*-phen | 3 | 5,005 | 239 | 3,884 |
| *o*-phen | 3 | 858 | 281 | 3,987 |
| *m*-phen | 6 | 8,432 | 197 | 3,032 |
| *o*-phen | 6 | 1,310 | 181 | 2,855 |
| *m*-phen | 12 | 6,526 | 1,106 | 7,061 |
| *o*-phen | 12 | 1,194 | 712 | 4,985 |

Lymphocytes were purified and cultured with PHA as described in Materials and Methods. Either *o*-phen (5 μM) or *m*-phen (50 μM) was added 20 h after PHA stimulation. The rate of incorporation of the radioactive precursor was determined at the indicated times (see Materials and Methods). Each value represents the average of four cultures after subtracting incorporation of unincubated controls. Incorporation of [$^3$H]thymidine in cultures not stimulated by PHA was 225 cpm. Incorporation of thymidine in PHA-stimulated cultures which were not exposed to *o*-phen or *m*-phen at 23, 26, and 32 h was 6,420, 8,132 and 8,793 cpm, respectively.

Only after 12 h was the rate of incorporation of labeled uridine or leucine diminished in cultures treated with *o*-phen. Thus, the alteration brought about by *o*-phen seems to be associated with the chelating properties of the molecule and is primarily manifested as an inhibition of DNA synthesis.

### *Effect of o-Phen on DNA Synthesis*

The influence of *o*-phen (6 μM) on the incorporation of thymidine during lymphocyte transformation may be seen in Fig. 1. As previously indicated (10) stimulation by PHA of human lymphocytes for 60 h results in a 140-fold increase in the rate of incorporation of thymidine into DNA (Fig. 1, control). Adding *o*-phen 40 h after PHA markedly reduces thymidine incorporation. In this experiment inhibition was nearly maximal in 4 h after the addition of *o*-phen and remained in effect until the experiment was terminated at 72 h. The effect of *o*-phen is primarily a function of the divalent chelating properties of this molecule since *m*-phen at a concentration about eightfold greater produced a much smaller effect on the incorporation of thymidine (Fig. 2). The composition and aromaticity of the two isomers of phenanthroline are similar with the exception that *m*-phen cannot chelate metals while *o*-phen is able to do so effectively. We interpret the slight immediate delay in thymidine incorporation to manipulation of the cultures upon adding the phenanthroline; after this delay the increase in the rate of thymidine incorporation in cultures containing *m*-phen parallels that in cultures without *m*-phen.

### *Reversal by Zinc*

The extent of inhibition of thymidine incorporation relative to the amount of added *o*-phen is seen in Fig. 3. The addition of 5 μM *o*-phen 20 h after stimulation by PHA leads to a fivefold decrease in the rate of thymidine incorporation while the addition of 10 μM *o*-phen almost completely abolishes thymidine incorporation. As shown in Fig. 1, this diminution in the rate of thymidine incorporation can be reversed by the addition of $Zn^{++}$ (50 μM). Reversal by zinc has been observed in cultures of lymphocytes at concentrations of zinc as low as 10 μM. If the effect of zinc were antagonistic to that of *o*-phen it might be possible to prevent the action of *o*-phen by the prior addition of zinc to the lymphocyte cultures. The addition of $Zn^{++}$ (50 μM) 24 h after PHA-stimulation markedly decreases the inhibi-

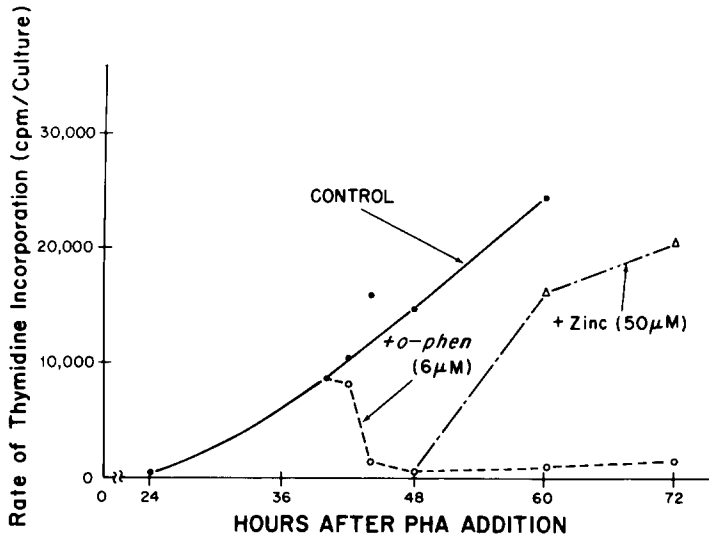


FIGURE 1 The inhibition of thymidine incorporation by *o*-phen and the reversal by zinc. Lymphocytes were isolated and cultured as described in Materials and Methods. The cultures were exposed to [$^3$H]thymidine for 1 h before harvesting. PHA was added at the start of the experiment, *o*-phen at 40 h, and zinc at 48 h. The concentrations given are that in the final culture medium. Each point represents the average incorporation of thymidine in five cultures. In cultures incubated without PHA for 60 h the incorporation of [$^3$H]thymidine was 140 cpm/culture.

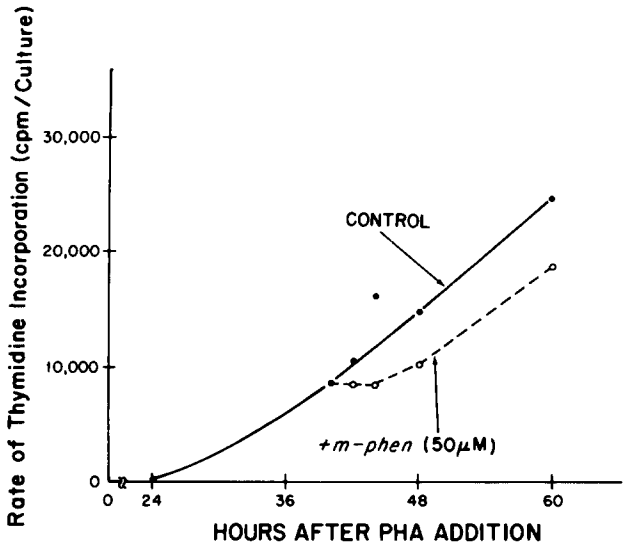


FIGURE 2 Lack of inhibition of thymidine incorporation by the nonchelating isomer *m*-phen. Culture conditions are those used in Fig. 1 except the *m*-phen at an eightfold greater concentration was added in place of *o*-phen.

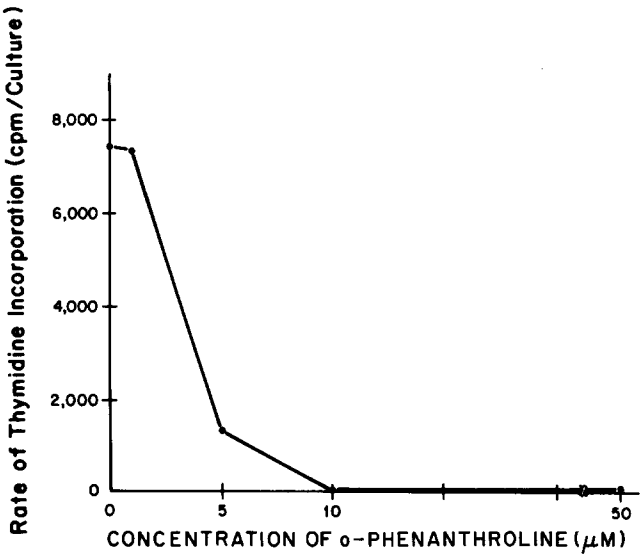


FIGURE 3 The effect of *o*-phen concentration on thymidine incorporation. In this experiment *o*-phen was added only 20 h after PHA stimulation and incorporation of thymidine was determined at 28 h. Thus overall incorporation is lower than the previous experiments in which *o*-phen was added at 40 h.

tion of thymidine incorporation by *o*-phen when added at 40 h (Fig. 4).

The degree of specificity of zinc in reversing the inhibition of *o*-phen was defined by measuring the effect of other divalent cations. Of those cations tested (Table II) only $Zn^{++}$ and $Ni^{++}$ were effective antagonists of *o*-phen. $Cu^{++}$ and $Co^{++}$ had little effect on the ability of this chelator to inhibit the incorporation of thymidine by the lymphocytes, even though divalent cations such as these have exceptionally high binding affinities for *o*-phen in vitro.

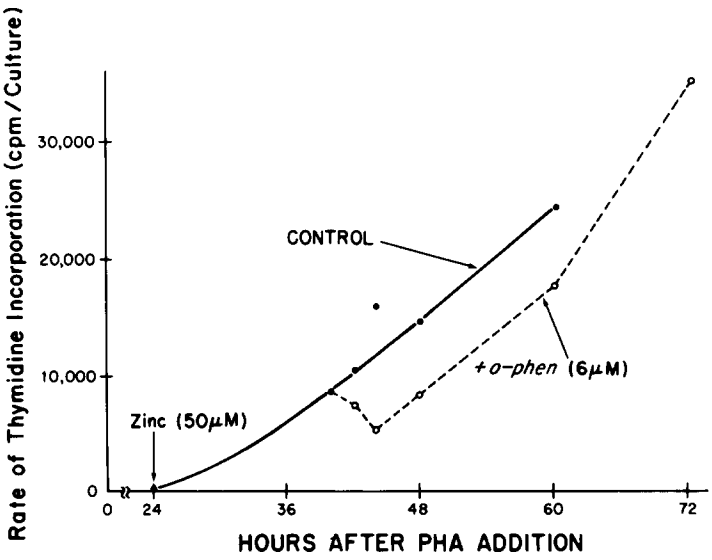


FIGURE 4 Pretreatment with $ZnCl_2$. To cultures of lymphocytes, PHA was added at time zero, $ZnCl_2$ at 24 h, and *o*-phen at 40 h. Each point represents the average rate of thymidine incorporation (cpm/hr/culture) of quintuplet cultures as outlined in Materials and Methods. The observed initial drop in the rate of thymidine incorporation could indicate some systematic error in the treatment of the cultures; a similar decrease in thymidine incorporation rate is observed immediately after the cultures are treated with nonchelated *m*-phen (see Fig. 2).

### *Inhibition of Deoxycytidine Incorporation*

It is possible that the inhibition of thymidine incorporation by *o*-phen does not represent a diminution in DNA synthesis but rather a specific effect on thymidine metabolism such as a decrease in the transport of thymidine into the cell or an inhibition in the specific phosphorylation of thymidine before its incorporation into DNA. However, the effect of *o*-phen is not restricted to thymidine; it also inhibits the rate of incorporation of deoxycytidine (Fig. 5) and deoxyguanosine (data not shown) to the same extent as thymidine. Each of these precursors for DNA synthesis may have different cellular transport mechanisms and appears to be phosphorylated by distinct kinases (12). The inhibition by *o*-phen suggests that it effects a step in DNA synthesis common to the incorporation of all three precursors.

### *Effect on Uridine Incorporation*

The possibility of RNA serving as an initiator for DNA replication (13, 14) prompted us to study the effects of *o* phen on uridine incorporation in greater detail. In other studies it has been shown that the increase in the rate of uridine incorporation after PHA stimulation of lymphocytes begins as early as 2 h (15) and reaches a maximum by about 3 days (16). Fig. 6 shows the effect of exposing the lymphocytes to *o*-phen 40 h after PHA stimulation. The presence of 6 μM *o*-phen reduces the rate of uridine incorporation about 20% at 44 h, while at the same concentration *o*-phen inhibits thymidine incorporation by 80% at 44 h (Fig. 1). At 3 μM *o*-phen there is little if any alteration in the rate of uridine incorporation (Fig. 6), but the rate of thymidine incorporation was reduced 54% at 44 h (results not shown). Even though synthesis of both RNA and DNA, as measured by the rate of incorporation of appropriate nucleosides, can be reduced by *o*-phen, DNA synthesis is much more susceptible to low concentrations of the compound.

## DISCUSSION

Our studies indicate that the chelator, *o*-phen, can inhibit DNA synthesis in PHA-stimulated human lymphocytes and that this inhibition can be prevented or reversed by zinc. In contrast, *m*-phen, a nonchelating analogue at a 10-fold greater concentration is without effect. The most direct explanation is an obligatory participation of free zinc or zinc complexes in DNA replication.

$Zn^{++}$ has been shown to be required for the

increase in DNA replication when rat kidney cells (2) are explanted into culture and when rat liver cells are stimulated to divide by partial hepatectomy (3). The time at which the cells are sensitive to the chelator, EDTA, suggests that $Zn^{++}$ is required for an event before the onset of DNA replication. A recent report of Chesters (17) using PHA-stimulated pig lymphocytes indi-

TABLE II

*Effect of Cations on Inhibition of Thymidine Incorporation by o-Phen*

| Cation added | % thymidine incorporation in PHA-stimulated cultures | Stability constants (log $K_1$) |
|---|---|---|
| None (control) | 100 | — |
| + *o*-phen (6 μM) | 9 | — |
| " " " + $Zn^{++}$ (10 μM) | 89 | — |
| " " " + $Zn^{++}$ (50 μM) | 105 | 6.4 |
| " " " + $Fe^{+++}$ (50 μM) | 15 | 6.5 |
| " " " + $Cd^{++}$ (50 μM) | 10 | 6.4 |
| " " " + $Mg^{++}$ (50 μM) | 12 | 1.2 |
| " " " + $Mn^{++}$ (50 μM) | 9 | 3.9 |
| " " " + $Co^{++}$ (50 μM) | 22 | 7.3 |
| " " " + $Cu^{++}$ (50 μM) | 12 | 9.1 |
| " " " + $Ca^{++}$ (50 μM) | 10 | 0.7 |
| " " " + $Ni^{++}$ (50 μM) | 83 | 8.6 |

Control cultures were stimulated by PHA, incubated for 60 h, and labeled with [$^3$H]-thymidine for 1 h before harvesting. Incorporation (100%) was 43,500 cpm culture. 40 h after the addition of PHA, *o*-phen was added at a final concentration of 6 μM. The indicated metals were added as chlorides at 46 h and the rate of thymidine incorporation was determined at 60 h. Each value represents the average incorporation of five cultures relative to that obtained by PHA stimulation alone. The stability constant (log $K_1$) are those of 1:1 complexes of the metals and *o*-phen (11).

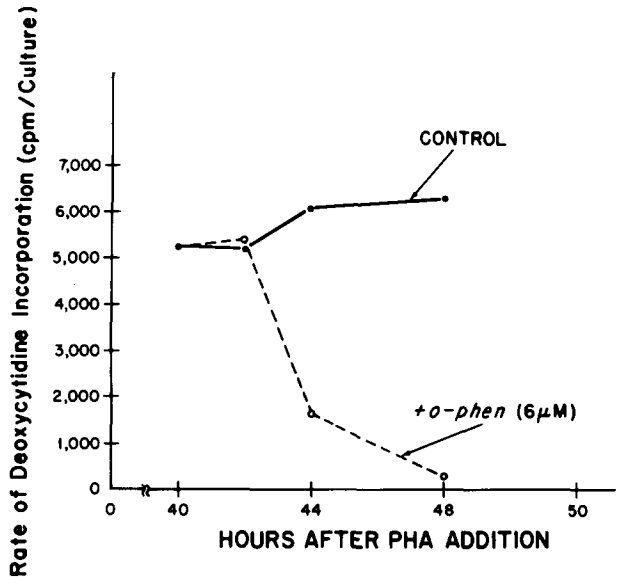


FIGURE 5 Inhibition of deoxycytidine incorporation. 6 μM of *o*-phen was added to cultures of human lymphocytes 40 h after PHA stimulation. 1 h before harvesting 2.5 μCi of [$^3$H]deoxycytidine was added to each culture and the amount of incorporation into an acid-insoluble precipitate was determined as described for thymidine incorporation with the exception that unlabeled deoxycytidine was used for terminating incorporation.

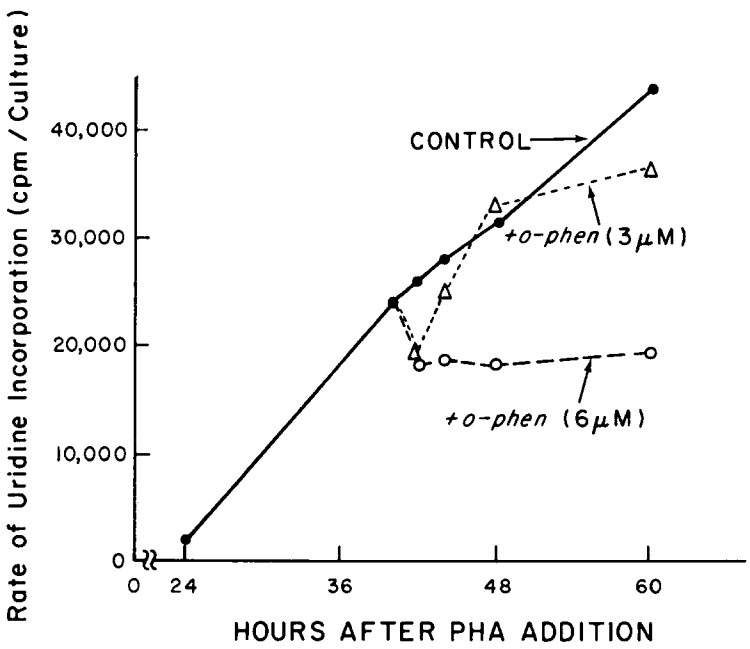


FIGURE 6 Insensitivity of uridine incorporation to inhibition by *o*-phen. The rate of [$^3$H]uridine incorporation was determined in cultures of lymphocytes which were exposed to 3 μM (△) and 6 μM (○) *o*-phen 40 h after PHA stimulation.

cates that EDTA can inhibit thymidine incorporation and that this inhibition is most effective immediately before DNA synthesis. However, in our studies *o*-phen at a low concentration (6 μM) is completely able to prevent thymidine incorporation at a time when most of the lymphocytes are actively dividing, thus implying a direct interaction of the chelator with a target critical to DNA replication. In agreement with the latter interpretation are the experiments of Rubin with chick embryo cells (4) in which thymidine incorporation is markedly inhibited with high concentrations of EDTA (600 μM), an inhibition which is reversed uniquely by zinc. These inhibitor studies demonstrate a zinc requirement for DNA replication but by themselves do not establish the exact nature of this requirement.

The concentration of *o*-phen used in these studies is at least one order of magnitude less than that reported to be effective with other chelators in a variety of different cells (2–4, 17). This could indicate that the structure of *o*-phen may be important for its effects with lymphocytes. However, since the inhibition of thymidine incorporation is not immediate, one can postulate that *o*-phen is concentrated within the lymphocytes. Our previous data on the inhibition of DNA synthesis in vitro (6) is compatible with the latter interpretation. In order to achieve a 50% reduction of DNA polymerase activity with extracts from stimulated lymphocytes we needed an excessive concentration of *o*-phen (1 mM), one which is much higher than that used in these studies to obtain complete inhibition of thymidine incorporation in cultures.

The ability of zinc to reverse the inhibition of *o*-phen could indicate that free zinc is a cofactor in some enzymatic reaction involved in the synthesis of DNA or that *o*-phen binds to an active site on an enzyme or structural protein presumably containing zinc. The ability of $Ni^{++}$ to prevent the inhibition of thymidine incorporation by *o*-phen would support the latter concept. $Ni^{++}$ could act by complexing with *o*-phen and thus displacing it. However, in other studies $Ni^{++}$ is reported ineffective in diminishing the effects of EDTA on DNA synthesis in pig lymphocytes (17). We are unaware of evidence for a nickel metalloenzyme functioning in any capacity in DNA synthesis.

Even though the primary effect of *o*-phen is to inhibit the incorporation of thymidine, it also diminishes uridine incorporation. However, DNA synthesis can be entirely prevented in experiments

under conditions in which total uridine incorporation is only marginally affected. The simplest explanation for the decrease in uridine incorporation is that it represents a separate effect at a different cellular site because of the lower sensitivity. Zinc has been found in *E. coli* RNA polymerase (18). A similar enzyme may be present in the lymphocytes and could be inhibited by *o*-phen.

The stimulus for these studies was the kinetic experiments of Chang and Bollum suggesting a role for zinc in catalysis by the terminal deoxynucleotidyltransferase of calf thymus (19) and our direct demonstration of the presence of zinc in homogeneous DNA polymerases from *E. coli*, sea urchin nuclei (6), and bacteriophage T4 (5). These enzymes can be inhibited by *o*-phen (6). With the sea urchin the inhibition by *o*-phen is partly competitive with DNA (unpublished data) suggesting that both *o*-phen and zinc interact at a common site on the enzyme (6). Most important, enzyme-bound zinc (1 g atom per mol of enzyme) is required for catalysis by *E. coli* DNA polymerase I (5). The removal of $Zn^{++}$ is accompanied by a proportional loss of polymerase activity, an effect which can be entirely reversed by incubating the apoenzyme with zinc (5). Other DNA polymerases from chicken embryos (20) and from Rous' sarcoma virus (21) have been reported to be inhibited by *o*-phen. These findings suggest that all DNA polymerases are zinc metalloenzymes. Thus, the inhibition of DNA synthesis by *o*-phen during lymphocyte transformation may reflect the direct interaction of this chelator with a DNA polymerase in these cells. The concentration of zinc at the site of DNA synthesis could be of importance in regulating the onset of this synthesis during the cell cycle.

The authors gratefully acknowledge the assistance of Sylvia Bugbee and thank Drs. Albert S. Mildvan and Clark Springgate for generous counsel.

This work was supported by grants from The National Institutes of Health (CA-12818, CA-11835) and The National Science Foundation (GB-36812), by grants to this Institute from the National Institutes of Health (CA-06927, RR-05539), by an appropriation from the Commonwealth of Pennsylvania, and by a post-doctoral fellowship (to R. O. Williams) from the Damon Runyon Memorial Fund for Cancer Research.

*Received for publication 8 March 1973, and in revised form 21 May 1973.*

## REFERENCES

1. Sandstead, H. H., and R. A. Rinaldi. 1969. *J. Cell Physiol.* **73**:81.
2. Lieberman, I., R. Abrams, and P. Ove. 1963. *J. Biol. Chem.* **238**:2,141.
3. Fujioke, M., and I. Lieberman. 1964. *J. Biol. Chem.* **239**:1,164.
4. Rubin, H. 1972. *Proc. Natl. Acad. Sci. U.S.A.* **69**:712.
5. Springgate, C., A. S. Mildvan, R. Abramson, J. L. Engle, and L. A. Loeb. *J. Biol. Chem.* In press.
6. Slater, J. P., A. S. Mildvan, and L. A. Loeb. 1971. *Biochem. Biophys. Res. Commun.* **44**:37.
7. Nowell, P. C. 1966. *Cancer Res.* **20**:462.
8. Villafranca, J. J., and A. S. Mildvan. 1971. *J. Biol. Chem.* **246**:772.
9. Bach, F., and K. Hirschhorn. 1964. *Science (Wash. D. C.)* **143**:813.
10. Loeb, L. A., S. S. Agarwal, and A. M. Woodside. 1968. *Proc. Natl. Acad. Sci. U.S.A.* **61**:827.
11. Sillen, L. G., and A. E. Martell. 1964. *Chem. Soc. Spec. Publ.* **17**.
12. Griffith, T. J., and C. W. Helleimer. 1965. *Biochim. Biophys. Acta.* **108**:114.
13. Brutlag, C., R. Schekman, and A. Kornberg. 1971. *Proc. Natl. Acad. Sci. U.S.A.* **68**:2,826.
14. Lark, K. G. 1972. *J. Mol. Biol.* **64**:47.
15. Cooper, H. L., and A. D. Rubin. 1965. *Blood J. Hematol.* **25**:1,014.
16. Loeb, L. A., and S. S. Agarwal. 1971. *Exp. Cell Res.* **66**:299.
17. Chesters, J. K. 1972. *Biochem. J.* **130**:133.
18. Scrutton, M. C., C. W. Wu, and D. A. Goldthwait. 1971. *Proc. Natl. Acad. Sci. U.S.A.* **68**:2,497.
19. Chang, L. M. S., and F. J. Bollum. 1970. *Proc. Natl. Acad. Sci. U.S.A.* **65**:1,041.
20. Stavrianpoloulos, J. G., J. D. Karkas, and E. Chargaff. 1972. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1,781.
21. Levinson, W., A. Faras, B. Woodson, J. Jackson, and J. M. Bishop. 1973. *Proc. Natl. Acad. Sci. U.S.A.* **70**:164.