Biochemical Pharmacology, Vol. 23, pp. 3259–3265. Pergamon Press, 1974. Printed in Great Britain.

INHIBITION OF DNA POLYMERASES FROM RNA TUMOR VIRUSES BY TILORONE AND CONGENERS: SITE OF ACTION

P. CHANDRA, G. WILL, D. GERICKE and A. GÖTZ

Gustav-Embden-Zentrum der Biologischen Chemie, Abteilung für Molekularbiologie, Theodor-Stern-Kai 7, Universität Frankfurt/Main, Germany

(*Received* 28 *February* 1974; *accepted* 23 *April* 1974)

Abstract—The dihydro-chloride salt of 2,7-bis [2-(diethyl-amino) ethoxy]-fluoren-9-one, referred to as tilorone hydrochloride or bis-DEAE-fluorenone (DEAE-F), has been shown by us to inhibit the DNA polymerase activity in RNA tumor viruses. In this report we examine the mechanism of this inhibition. Product analysis of the DNA-polymerase reaction (Friend Leukemia virus) in the absence and in the presence of tilorone (1×10^{-4}M) showed that it specifically blocks the formation of the double stranded DNA. Studies with tilorone congeners show some specific structural requirements for the biochemical activity of tilorone. Some substitutions lead to a potentiation of its biochemical activity. A direct correlation between the magnitude of antiviral activity and the extent of inhibition of DNA-polymerase activity by tilorone and congeners was observed.

THE DIHYDRO-CHLORIDE salt of 2,7-bis [2-(diethyl-amino)ethoxy]-fluoren-9-one, referred to as tilorone hydrochloride or bis-DEAE-fluorenone (DEAE-F), is a broad spectrum antiviral compound[1] with antitumor activity.[2–4] Tilorone hydrochloride has been shown by us to form a molecular complex with DNA;[5] specifically, with dAT-regions of the double-stranded DNA.[6] On the basis of our hydrodynamic studies with the tilorone–DNA complex we proposed an intercalative mode of binding of tilorone to DNA.[6] These interactions inhibit the DNA template functions in DNA- and RNA-polymerase reactions *in vitro*.[5] Tilorone was also reported to inhibit the DNA-polymerase activity in RNA tumor viruses.[7] The latter inhibition was found to be selectively dependent on the type of primer-template used in the viral enzymatic reaction.

Ting *et al.*[8] were the first to demonstrate a direct correlation between inhibition of focus formation and of reverse transcriptase of murine leukemia-sarcoma virus by rifamycin derivatives. The development of potent inhibitors of the viral enzyme(s) may therefore, provide a means of understanding the biological role of the enzyme, and such inhibitors may find a prophylactic application.[9] The present communication describes the effects of some new congeners of tilorone on the DNA-polymerase activity of a leukemic virus (Friend Leukemia Virus, FLV). Structural modifications in the tilorone molecule lead, in some cases, to a potentiation of its activity. The chemical structures of congeners are shown in Fig. 1. Studies on the site of tilorone action show that it specifically blocks the formation of the double-stranded DNA. In addition, we found that there is a direct correlation between the magnitude of antiviral activity and the extent of inhibition of DNA-polymerase activity by tilorone

B.P. 23/23 C

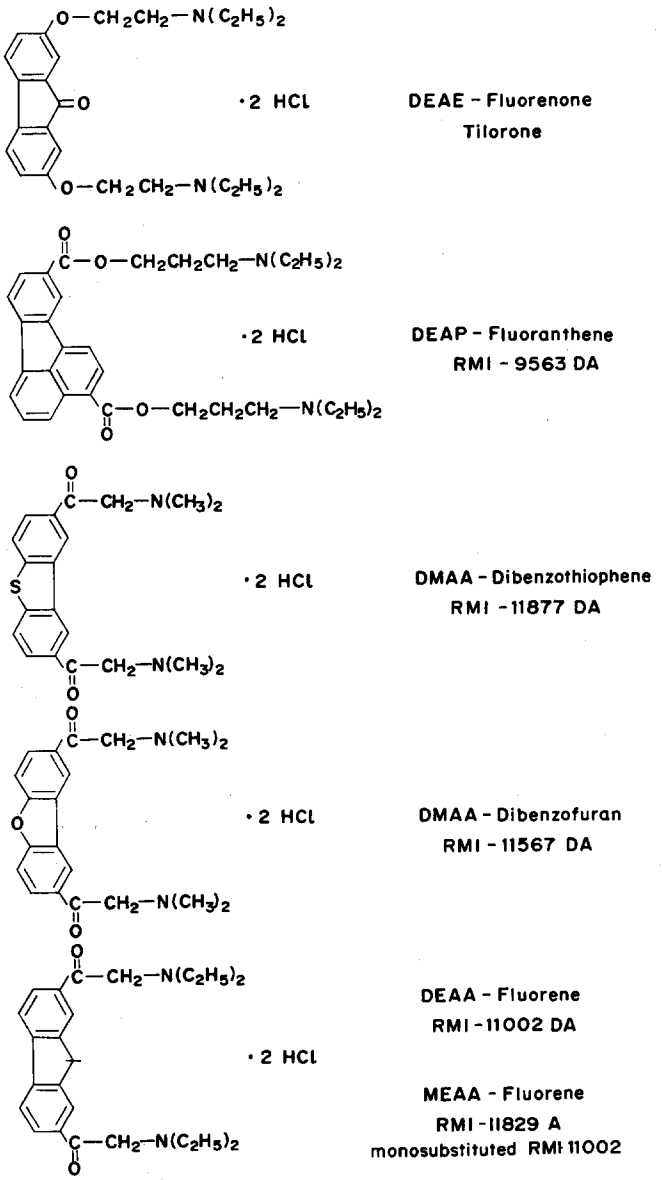

FIG. 1. Chemical structures of tilorone congeners.

and congeners. The importance of some structural parameters required for this activity will be discussed.

MATERIALS AND METHODS

^{3}H-Labeled deoxynucleoside triphosphates were obtained from NEN-Chemicals GmbH, Germany. Unlabeled deoxynucleoside triphosphates were purchased from Nutritional Biochem. Corp., Ohio, U.S.A. Poly (dA-dT) and poly rA. $(dT)_{12}$ were supplied by Miles Chemical Laboratories, Elkhart, Indiana, U.S.A.

MSV(M), kindly supplied by Dr. J. B. Moloney (NCI, Bethesda, Md.) was passed by intramuscular injection into suckling Swiss mice, and extracted from the tumor tissue. FLV was isolated from infected spleens (AKR mice) and purified by sucrose density gradient centrifugation. The viral extracts used to assay for DNA-polymerase activity were stored at $-70°$ before use.

DNA-polymerase assay. DNA-polymerase activity was assayed essentially by the method of Ross *et al.*[10] The reaction mixture, regardless of the template, was similar to that of Ross *et al.*,[10] except that we used 0·04 M Tris and the final concentration of Nonidet P-40 (Shell Chemie, Hamburg) was 0·2 per cent. The reaction mixture contained 0·25 μg of the template used. The reaction mixture (0·25 ml) containing virions (28 μg of protein) was incubated at 37° for 90 min. Acid precipitable material was counted on Millipore filters (HAWP 02500) in a liquid scintillation counter. Protein was estimated by the method of Lowry *et al.*[11]

Biological studies. The effect of prior treatment of MSV(M) with tilorone and congeners on MSV(M)-induced sarcoma was studied in 3–5 day old AKR mice. The viral suspension was preincubated with the compound (5×10^{-7}moles/ml) for 1 hr at 37°; the control studies were carried out by preincubation without the added compound. 0·2 ml of this suspension was injected intraperitoneally into mice.

The leukemogenic potential of cell-free spleen extracts from mice infected with FLV (LD_{90}) was determined by measuring spleen weights of the recipient mice. The FLV suspensions at the same dilutions were preincubated with the compound ($5 + 10^{-7}$ moles/ml) at 37° for 1 hr. In control experiments the suspension was incubated without the added compound. The experimental and the control animals received 0·2 ml of the suspension, preincubated with, or without the compound. Spleen weights were determined on the 12th day after infection.

Product analysis of the DNA-polymerase reaction. The product analysis of the DNA-polymerase reaction of FLV in the absence or in the presence of tilorone was carried out, as described by Kotler and Becker.[12] The reaction mixtures were dissolved with Na-dodecyl sulfate [1% (w/w) final concn.], loaded on a hydroxyl-apatite column (1 g, Bio-Rad Lab., Richmond, Calif.), eluted with a sodium phosphate gradient (0·05–0·4 M), collected into about 40 tubes (total volume 100 ml) and the TCA insoluble radioactivity was collected on GF/C filters (Whatman) and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

To measure the effect of various tilorone congeners (Fig. 1) on the oncogenic activity of MSV(M), viral suspensions were incubated with 5×10^{-7}moles/ml of each compound at 37° for 1 hr. In the control group, where no compound was used, virus was preincubated with the solvent, Tris-buffer, 0·01 M, pH 7·4. 0·2 ml of this mixture, containing 1×10^{-7}moles of the drug, was injected intraperitoneally. The amount of compound introduced this way had no direct physiological effect in the host (unpublished results). The mortality and the survival period were significantly influenced by tilorone and two of its congeners, DEAP-fluoranthene and DEAA-fluorene (Table 1). Viral suspensions, pretreated with MEAA-fluorene, were as active as the untreated viral suspensions. It is important to note that at the time of writing this report 30 per cent of the animals in the tilorone and DEAP-fluoranthene groups, and 20

TABLE 1. *In vitro* EFFECT OF TILORONE AND CONGENERS ON THE ONCOGENIC ACTIVITY OF MSV(M), AND LEUKEMOGENIC POTENTIAL* OF CELL-FREE SPLEEN EXTRACTS FROM MICE INFECTED WITH FLV

Compound	MSV(M)-Oncogenesis No. of survivors per No. of infected animals	MSV(M)-Oncogenesis Survival time†	FLV-Oncogenesis Spleen weight (g)‡
Control	0/10	13·2 ± 1·4	1·34 ± 0·27
DEAE-fluorenone (tilorone)	3/10	24·3 ± 1·2	0·86 ± 0·15
DEAP-fluoranthene (RMI-9563 DA)§	3/10	24·4 ± 1·6	0·82 ± 0·17
DEAA-fluorene (RMI-11002)	2/10	20·9 ± 0·7	1·06 ± 0·13
MEAA-fluorene (RMI-11829 A)	0/10	14·2 ± 0·4	1·32 ± 0·11

* Determined by measurement of spleen weights, 12 days after FLV infection.

† Mean ± S.E. derived from ten animals in the control group and 10−n no. of animals in the experimental groups, where n is the no. of survivors.

‡ Mean ± S.E. derived from ten animals in the control group, and five animals in each of the experimental groups.

§ Pharmaceutical code numbers of Merrell-National Laboratories, Ohio, U.S.A.

Abbreviations: MSV(M), Murine Sarcoma Virus (Moloney); FLV, Friend Leukemia Virus.

per cent of the animals in DEAA-fluorene group were still alive. Thus, the mean survival period in these groups depicts a mean of 6–7 animals only.

The effect of prior treatment of cell-free spleen extracts from FLV-infected mice with tilorone congeners to induce splenomegaly is shown in Table 1. The experimental conditions are described under Table 1 and in the text. Under these conditions, only tilorone and DEAP-fluoranthene showed a significant inhibition of splenomegaly induced by FLV. DEAA-fluorene showed only slight activity. It is interesting to note that MEAA-fluorene does not show any activity in this system.

Since none of the compounds at the concentrations used showed a complete suppression of splenomegaly, one would expect a residual viral activity in spleen extracts of mice, which received FLV suspensions preincubated with these compounds. Studies are now in progress to evaluate the leukemogenic activity of cell-free spleen extracts, prepared from mice inoculated with pretreated suspensions. Wu *et al.*[13] have carried out such studies with RLV and rifamycin derivatives. They reported that the inoculation of mice with inocula from mice infected with RLV pretreated with AF/ABDP and AF/DNF1 did not cause splenomegaly.

If the suppression of biological activity of RNA tumor viruses is due to a block of some molecular event(s) involved in oncogenesis, one would expect an inhibition of DNA polymerases by these compounds. That tilorone does inhibit the DNA-polymerase activity of oncornaviruses has been shown already by us.[7] An attempt was made to correlate the biological response of various congeners with their inhibitory activity in the DNA-polymerase system on oncornaviruses. These studies were done using purified FLV, since in our hands this system showed a good endogenous activity.

The inhibition of the endogenous activity of FLV-DNA polymerase by tilorone and congeners is shown in Fig. 2. A maximum inhibition was obtained with DEAP-fluoranthene. The inhibitory responses of tilorone (DEAE-fluorenone), DMAA-dibenzothiophene and DEAA-fluorene were of the same magnitude; whereas, DMAA-dibenzofuran showed a weak response. It is interesting to note that the monosubsti-

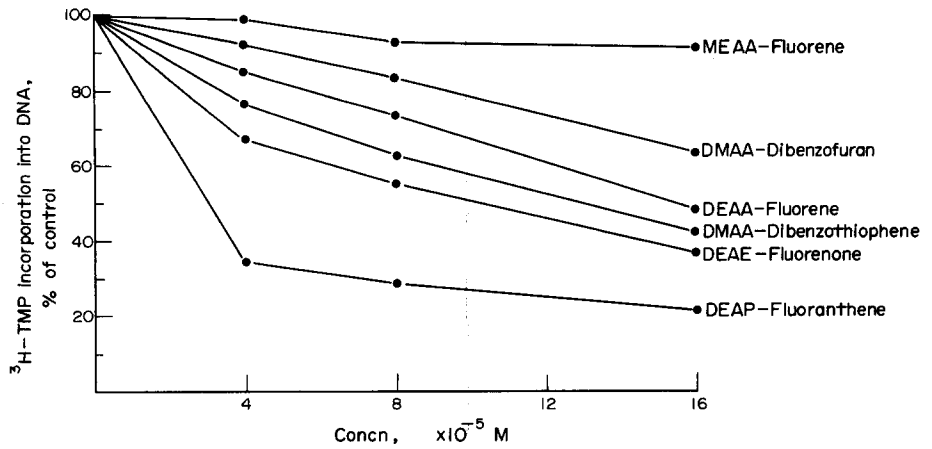

FIG. 2. Inhibition of endogenous RNase sensitive FLV-DNA-polymerase activity by tilorone and congeners. Experimental details are described under Materials and Methods.

tuted congener MEAA-fluorene did not inhibit the endogenous reaction at any concentration.

A similar inhibitory response by the tilorone cogeners was exhibited in the DNA-polymerase system of FLV, catalyzed by the template poly rA. $(dT)_{12}$, as shown in Fig. 3. The effect of tilorone and congeners on the FLV-DNA-polymerase reaction, catalyzed by poly (dA-dT), is shown in Fig. 4. This reaction was more sensitive towards tilorone and its congeners than the endogenous, or poly rA. $(dT)_{12}$-catalyzed reactions. This is in accordance to our findings on tilorone action, reported earlier.[7]

The data reported in Table 1 and Figs. 2–4 shows that tilorone congeners, which inhibit the DNA polymerases of the virus, suppress also the oncogenic activity of the virus. The structure–activity relationship is exhibited by the congeners in both the biological, as well as in FLV-enzymatic systems. A similar structure–activity rela-

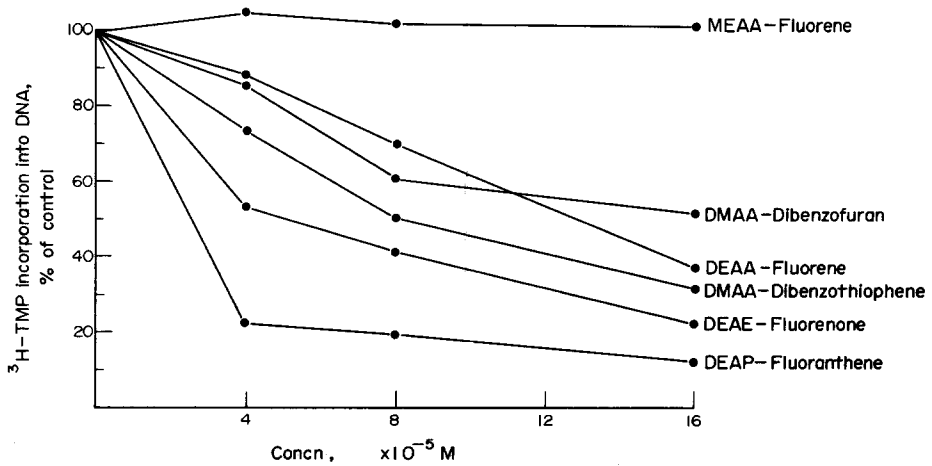

FIG. 3. Inhibition of the poly rA. $(dT)_{12}$-catalyzed FLV-DNA-polymerase activity by tilorone and congeners. Experimental details are described under Materials and Methods.

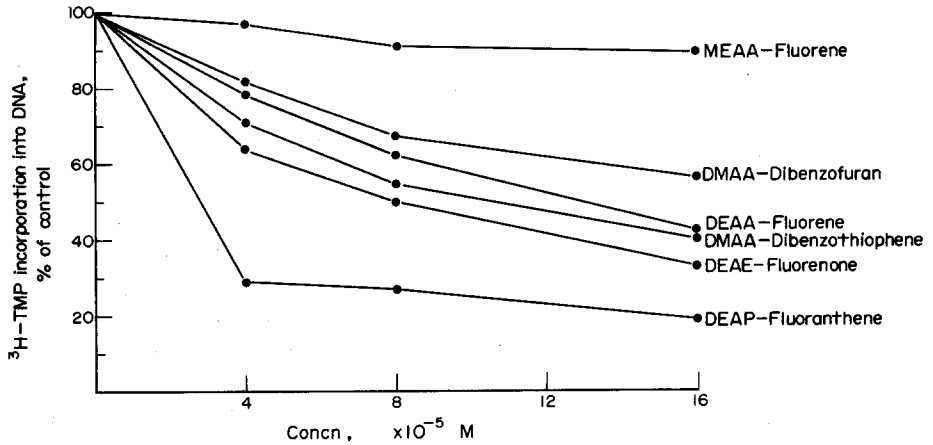

FIG. 4. Inhibition of the poly (dA-dT)-catalyzed FLV-DNA-polymerase activity by tilorone and congeners. Experimental details are described under Materials and Methods.

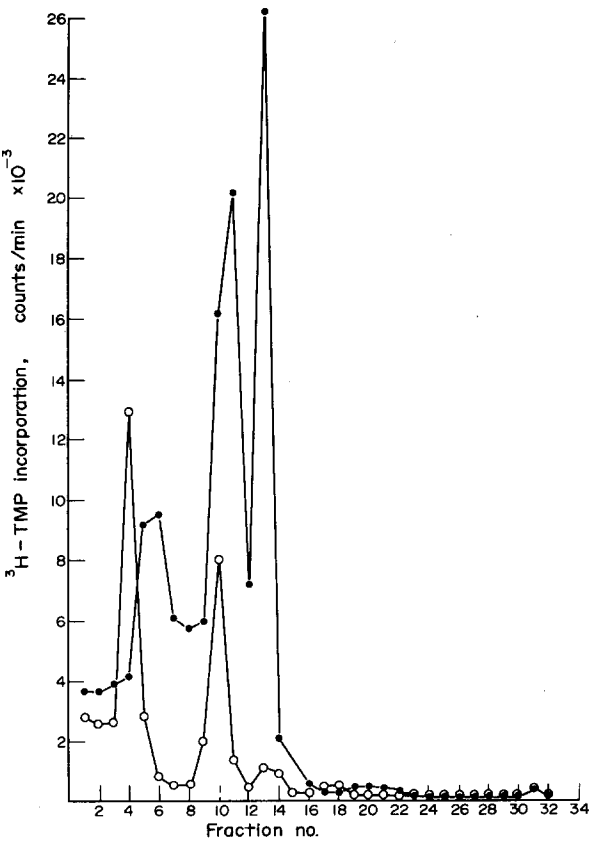

FIG. 5. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxylapatite column. Each column was filled with 1 g of hydroxylapatite and carefully washed with 0·05 M sodium phosphate (approx. 50 ml). The columns were loaded with the reaction products, as described under Materials and Methods. The columns were washed with 0·05 M sodium phosphate buffer, pH 6·8, until equilibrium was reached. Macromolecules were eluted from the columns by a linear gradient of sodium phosphate (0·05 M–0·4 M). ●——●, DNA species synthesized in the absence of tilorone; ○——○, DNA species synthesized in the presence of 10^{-4}M tilorone.

tionship was observed in the MSV(M)-polymerase system, except that the MSV(M)-endogenous system was more sensitive than the FLV-endogenous system towards these compounds (manuscript in preparation).

Since tilorone and the congeners exhibit a specific mode of interaction with DNA,[5,6,15] and the poly (dA-dT)-catalyzed reaction of viral DNA-polymerase is most sensitive to these compounds, it is possible that the biological activity of these compounds is due to their interaction with the hybrid RNA–DNA (hy-DNA), single stranded DNA (ss-DNA), or the DNA–DNA duplex (ds-DNA). It was, therefore, interesting to locate the site of action of tilorone in the viral DNA-polymerase system. It is still not clear whether a particular site or target in the DNA-polymerase system, other than the true RNA-dependent reaction, can be correlated with the biological role of the oncornaviruses. The key role of the RNA-directed reaction in *in vivo* leukemogenesis using purified enzyme and rifamycin derivatives, has been nicely demonstrated by Wu *et al.*[13]

We have conducted some model studies to analyze the products of the FLV-DNA-polymerase reaction under the influence of tilorone. The procedure we adopted was based on a recent report by Kotler and Becker[12] on distamycin A, which has been shown to react with ss-DNA and ds-DNA.[14]

The product analysis of the DNA-polymerase reaction (FLV) in the absence and in the presence of tilorone (1×10^{-4}M) is depicted in Fig. 5. The products of the viral DNA-polymerase reaction were, under these conditions, eluted in three species. The first species to be eluted from the column contained ss-DNA, the second contained the RNA-DNA hybrid molecules (hy-DNA) and finally, the ds-DNA, eluted in the last species. Analysis of products synthesized in the presence of tilorone showed that the ss-DNA and the hybrid species, but not the ds-DNA species, were synthesized. This indicates that tilorone has a low affinity to viral RNA, but can block the synthesis of ds-DNA by interacting with ss-DNA or hy-DNA.

Acknowledgements—Tilorone and congeners were prepared by the Merrell-National laboratories, Ohio, U.S.A. We thank Dr. R. F. Krueger, Merrell-National Laboratories, for providing useful information on the congeners. Finally, we thank Mr. M. Woltersdorf for his help in some spectrophotometric studies. One of us (P. C.) is grateful to Dr. Robert C. Gallo for valuable suggestions, and useful comments.

REFERENCES

1. R. F. Krueger and S. Yoshimura, *Fedn Proc.* **29,** 635 (1970).
2. R. H. Adamson, *J. natn. Cancer Inst.* **46,** 431 (1971).
3. A. E. Munson, J. A. Munson and W. Regelson, *Int. Colloq. on Interferone and Interferone Inducers*, Leuven, Belgium, abstr. no. 27 (1971).
4. A. E. Munson, J. A. Munson, W. Regelson and G. L. Wampler, *Cancer Res.* **32,** 1397 (1972).
5. P. Chandra, F. Zunino, A. Zaccara, A. Wacker and A. Götz, *FEBS Lett.* **23,** 145 (1972).
6. P. Chandra, F. Zunino, V. P. Gaur, A. Zaccara, M. Woltersdorf, G. Luoni and A. Götz, *FEBS Lett.* **28,** 5 (1972).
7. P. Chandra, F. Zunino and A. Götz, *FEBS Lett.* **22,** 161 (1972).
8. R. C. Ting, S. S. Yang and R. C. Gallo, *Nature, New Biol.* **236,** 163 (1972).
9. R. C. Gallo, R. G. Smith and A. Wu, in *Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacology*, San Francisco 1972, Vol. 3, p. 411. Karger, Basel (1973).
10. J. Ross, E. M. Scolnick, G. J. Todaro and S. A. Aaronson, *Nature, New Biol.* **231,** 163 (1971).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
12. M. Kotler and Y. Becker, *FEBS Lett.* **22,** 222 (1972).
13. A. M. Wu, R. C. Y. Ting and R. C. Gallo, *Proc. natn. Acad. Sci. U.S.A.* **70,** 1298 (1973).
14. P. Chandra, Ch. Zimmer and H. Thrum, *FEBS Lett.* **7,** 90 (1970).
15. P. Chandra and M. Woltersdorf, *FEBS Lett.* **41,** 169 (1974).