# Miracil D: An Inhibitor of Ribonucleic Acid Synthesis in *Bacillus subtilis*

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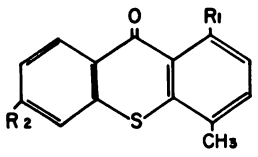
SUMMARY

Miracil D (1-diethylaminoethylamino-4-methyl-10-thiaxanthenone) is effective against schistosomiasis in man and against experimental tumors in mice, but its mode of action had not been defined. The present study indicates that at 20 μg/ml the drug arrests the growth of *Bacillus subtilis*. This antibacterial action is also exhibited by a tumor-inhibitory derivative, but not by a derivative devoid of carcinostatic activity. Incubation of *B. subtilis* with $^{14}$C-uracil, thymidine, or leucine in the presence of 20 μg/ml of Miracil D indicated a complete inhibition of RNA synthesis, immediate but less severe inhibition of protein synthesis, and no inhibition of DNA synthesis. The drug has absorption maxima at 442 and 330 mμ. In the presence of native DNA there is quenching of absorption at these wavelengths, with a shift in the maxima to 450 and 337 mμ. In heat-denaturation studies, Miracil D raised the $T_m$ of DNA by 15°. Similar studies gave evidence for a lesser affinity between Miracil D and sRNA. These results indicate that the major action of this drug in *B. subtilis* is to complex with DNA, thereby blocking DNA-dependent RNA synthesis.

## INTRODUCTION

Miracil D, a 10-thiaxanthenone, is an effective agent in the treatment of schistosomiasis (1). In addition, studies by Hirschberg *et al.* (2) have established the cytotoxicity of this drug in tissue culture and the antitumor effect in mice bearing a variety of transplantable neoplasms. Despite clinical interest in this drug and its derivatives, the mode of action of this class of compounds has not been previously elucidated.

The structures of this drug and two of its analogs are shown in Fig. 1. Miracil D and a cytotoxic analog, compound II, have in the $R_1$ position the dialkylaminoalkylamino side chain which is characteristic of several series of biologically active compounds (2, 3). Compound III, by contrast, has an $R_1$ side chain which does not have a terminal-substituted amino group and it is not cytotoxic for animal cells (2). The fact that Miracil D shares certain structural features with the acridines and actinomycin D, com-




| | $R_1$ | $R_2$ |
|---|---|---|
| I (MIRACIL D) | $-NHCH_2CH_2N(CH_2CH_3)_2$ | H |
| II | $-N(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$ | Cl |
| III | $-N(CH_2CH_3)CH_2CH_2OH$ | H |

FIG. 1. *Structure of Miracil D and two related analogs (compound II and compound III)*

pounds which are known to interact with DNA (4, 5), suggested to us the possibility that its mode of action might be similar to that of these compounds.

We have found that Miracil D is an effective inhibitor of the growth of *Bacillus subtilis*, and this organism has been utilized to study DNA, RNA, and protein synthesis in the presence of the drug. Though the synthesis of all three of these macromolecules is eventually inhibited and the cells lyse, the initial effect is a dramatic inhibition in the synthesis of RNA. There is also an immediate but less profound inhibition in protein synthesis. Under the same conditions DNA synthesis remains uninhibited for at least 20 min. In addition, evidence has been obtained for a physical interaction between Miracil D and DNA and also between Miracil D and RNA. A preliminary report of these studies has been presented (6).

## METHODS

Miracil D (Nilodin, Lucanthone) was obtained from Burroughs-Wellcome Company through the courtesy of Dr. George Hitchings, and compounds II and III were kindly supplied by Dr. Edward Elslager of Parke, Davis and Company. The following materials were obtained from commercial sources: calf thymus DNA (Worthington Biochemical Corporation), *E. coli* B sRNA (General Biochemicals, Inc.); uracil-2-$^{14}$C and thymidine-2-$^{14}$C were products of New England Nuclear Corporation, and randomly labeled leucine-$^{14}$C was prepared by Schwarz BioResearch, Inc. Polyadenylic acid (poly A), polyuridylic acid (poly U), and polycytidylic acid (poly C) were purchased from Miles Laboratories. *Bacillus subtilis* strain no. 168 i$^-$ was obtained from Dr. Steven Zamenhof and grown in Penn-assay Broth (Difco) supplemented with 0.5 g % glucose. *Escherichia coli* strain no. 47 was obtained from Dr. Salvadore Luria and grown in LB broth containing 0.2 g % glucose. Sidearm growth flasks of 125 ml volume, containing 10 ml of culture medium, were inoculated with 0.4 ml of a 16-hr culture and incubated at 37° on a gyrorotary shaker. Growth was measured by reading turbidity in a Klett-Summerson colorimeter (filter no. 42). Aqueous solutions of the drugs were prepared at 1 mg/ml on the day of the experiment and, unless indicated otherwise, were added to the culture at mid-exponential phase. Subsequent growth readings were corrected for absorption by the drug.

In isotope incorporation experiments, uracil-2-$^{14}$C (1.8 mC/mmole) and leucine-$^{14}$C (6 mC/mmole) were added to the medium at a final concentration of 0.1 $\mu$C/ml and thymidine-2-$^{14}$C (29 mC/mmole) at a final concentration of 0.2 $\mu$C/ml. Isotopes were added at the mid-exponential phase of growth to control cultures or to cultures which had received drug 30 sec previously. One-milliliter aliquots were removed at subsequent times and processed for incorporation of radioactivity into RNA, DNA, or protein essentially as described by Roodyn and Mandel (7). Optical density profiles were determined at room temperature (22°) on a Cary model 14 recording spectrophotometer, and heat denaturation studies were performed essentially as described by Marmur and Doty (8). The latter studies were done in a buffer of low ionic strength, which had the following composition: $3.3 \times 10^{-4}$ M sodium phosphate, pH 6.8; $1 \times 10^{-4}$ M disodium ethylenediamine tetraacetate; $3 \times 10^{-3}$ M sodium chloride.

## RESULTS

### *Effect of Miracil D on the growth of* B. subtilis and E. coli

The effect of Miracil D on growth when added at mid-logarithmic phase of a culture of *B. subtilis* is indicated in Fig. 2a. At a final concentration of 5 $\mu$g/ml the drug produced no detectable growth inhibition, but at 10 $\mu$g/ml it produced a marked inhibition in growth rate. At 20 $\mu$g/ml growth was arrested, and after 20–30 min lysis of the culture, manifested by a loss of turbidity, became apparent.

Figure 2b indicates that compound II, which differs from Miracil D by having a chlorine group in position 7 and a 3-diethylaminopropylmethylamino rather than

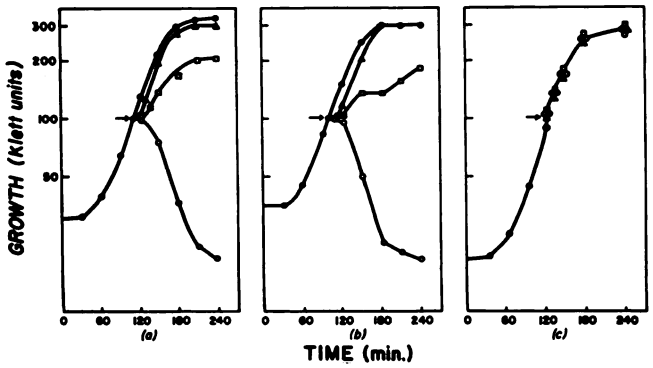



FIG. 2. *Effect of Miracil D (a), compound II (b), and compound III (c) on growth of B. subtilis* Drug added at Klett 100. ●——● Control; △——△ 5 μg/ml of drug; □——□ 10 μg/ml of drug; ○——○ 20 μg/ml of drug.

a diethylaminoethylamino side chain (see Fig. 1), was somewhat more active than the parent compound. In contrast to Miracil D and compound II, compound III, which has an ethyl(2-hydroxyethyl)amino side chain, did not inhibit growth, even at 20 μg/ml.

In additional studies the drug was introduced at the time of inoculation of the flask and the subsequent growth curve was plotted. Miracil D, at 10 μg/ml, delayed the onset of exponential growth for 150 min beyond the control flask. Thereafter there was "escape" with growth at approximately the same rate as the control culture. At 20 and 45 μg/ml there was complete inhibition of growth. Under the same conditions, compound II caused complete growth inhibition at 10 μg/ml. Compound III was totally inactive at 10 and 20 μg/ml; at 45 μg/ml exponential growth proceeded at approximately 90% of the control rate.

The results obtained in a similar study utilizing *Escherichia coli* are indicated in Fig. 3. It is apparent that Miracil D and compound II were inhibitory, whereas compound III was not. *E. coli* were more resistant than *B. subtilis* since 50 μg/ml of Miracil D and compound II were required to inhibit the former and, in contrast to the latter, the cells did not lyse with Miracil D. Compound II, at 50 μg/ml, did cause some lysis of *E. coli*.

The growth-inhibitory activity of Miracil D and compound II in the *B. subtilis* and *E. coli* systems and the relative inactivity

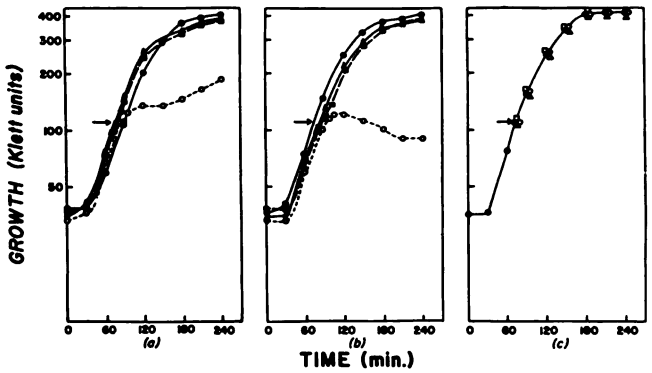



FIG. 3. *Effect of Miracil D (a), compound II (b), and compound III (c) on growth of E. coli* Drug added at Klett 100. ●——●, Control; ▲——▲, 10 μg/ml of drug; ■——■, 20 μg/ml of drug; ○——○, 50 μg/ml of drug.

of compound III parallel previous results obtained with mammalian systems (2).

*Effects of Miracil D on RNA, DNA, and protein synthesis in* B. subtilis

When added at a final concentration of 20 μg/ml, Miracil D caused an almost complete inhibition of the incorporation of uracil-$^{14}$C into total cellular RNA (Fig. 4a). The effect was apparent within 1 min without at the same time inhibiting DNA synthesis, is reminiscent of the effects of actinomycin D (5, 9). The delayed inhibition of protein synthesis which occurs with actinomycin D has been attributed to a consequence of the inhibition of RNA synthesis (5, 9). However, the immediate inhibition of incorporation of leucine-$^{14}$C obtained with Miracil D suggests that, in addition to inhibiting RNA synthesis, this

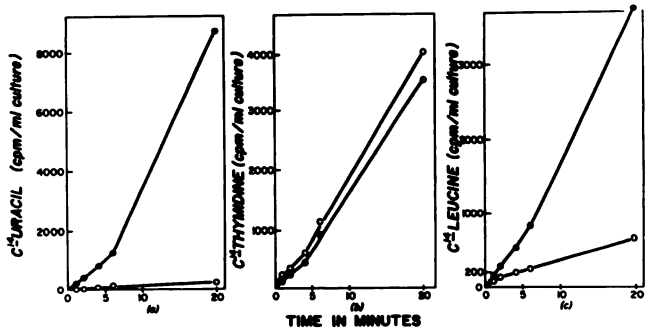



FIG. 4. *Effect of Miracil D on RNA (a), DNA (b), and protein (c) synthesis* ●——●, Control; ○——○, 20 μg/ml of drug added at time zero.

after addition of the drug and, during the subsequent 20 min, incorporation by the treated culture was never greater than 2% of the control culture. Because of subsequent lysis of the cells in the treated culture, experiments were terminated at 20 min. In contrast to its effects on uracil-$^{14}$C incorporation into RNA, Miracil D did not inhibit the incorporation of thymidine-$^{14}$C into DNA (Fig. 4b). Indeed, when compared to the control culture, the drug caused a slight stimulation of thymidine-$^{14}$C incorporation. The lack of inhibition by Miracil D was observed consistently; the stimulation of thymidine incorporation was variable, ranging from 0 to 13% of the control value.

Under the same conditions, Miracil D caused a partial inhibition of the incorporation of leucine-$^{14}$C into protein (Fig. 4c). This inhibition was less profound than that occurring in RNA synthesis but increased with time, from 46% at 2 min to 84% at 20 min. The ability of Miracil D to cause an immediate and almost complete inhibition of RNA synthesis in *B. subtilis*, drug may exert a direct effect on protein synthesis.

In additional experiments, uridine-2-$^{14}$C and arginine-$^{14}$C were used instead of uracil-2-$^{14}$C and leucine-$^{14}$C, with qualitatively similar results.

*Effect of nucleic acids on the optical density profile of Miracil D*

In view of the *in vivo* effects of Miracil D on nucleic acid and protein synthesis, it was of interest to seek evidence for a physical interaction between the drug and nucleic acids. With respect to the drug itself, it was found that free Miracil D (10 μg/ml) in buffer had absorption maxima at 330 and 442 mμ (Fig. 5). The drug had a third peak at 255 mμ, but because this peak overlaps with the absorption maximum of nucleic acids it was not used in the optical density profile studies. In the presence of native DNA (50 μg/ml), there was an appreciable change in the optical density profile of Miracil D with a shift in the previous absorption maxima to 337 and 450 mμ. The DNA alone gave no detect-

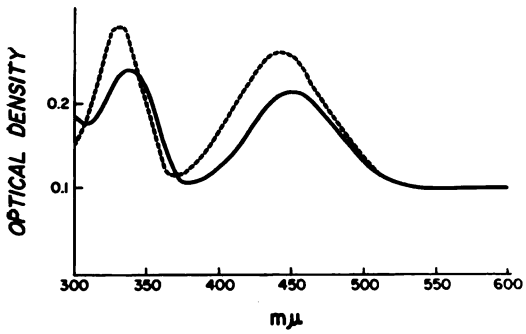



FIG. 5. *Effect of DNA (50 μg/ml) on optical density profile of Miracil D (10 μg/ml)*
-----, Miracil D; ——, Miracil D + DNA.

able absorption in the 300–600 mμ range. When Miracil D and DNA were placed in separate cuvettes and the light was allowed to pass sequentially through both cuvettes, the absorption profile was the same as that of Miracil D alone, indicating that the observed change required physical interaction between the two components. Quenching of absorption at 330 and 442 mμ was approximately proportional to the concentration of DNA added in the range of 2 to 25 μg/ml; there was no significant difference between native and denatured DNA (Fig. 6).

In view of the fact that *in vivo* the drug inhibited not only RNA but also protein synthesis, the effect of RNA on the spectral properties of Miracil D was examined. It was found that several RNA's, both natural and synthetic, produced changes in the absorption profile of Miracil D which were qualitatively similar to those obtained with DNA. Quantitative differences between nucleic acids were found, however, when they were compared with respect to their ability to induce a shift in the absorption maximum of Miracil D from 330 to 337 mμ. At 25 and 50 μg of nucleic acid per milliliter, the relative effectiveness of nucleic acids (in decreasing order) was: DNA > sRNA > polyadenylic acid > polyuridylic acid ≫ polycytidylic acid (Fig. 7).

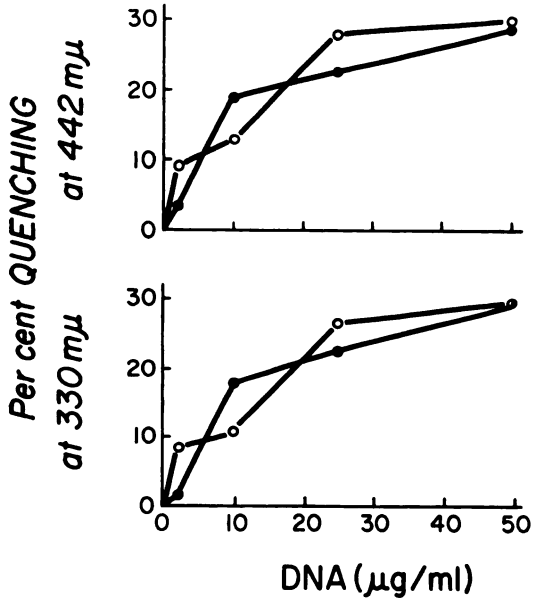



FIG. 6. *Effect of DNA on Miracil D (10 μg/ml) absorption at 330 and 442 mμ*
●——●, Native calf thymus DNA; ○——○, denatured calf thymus DNA.

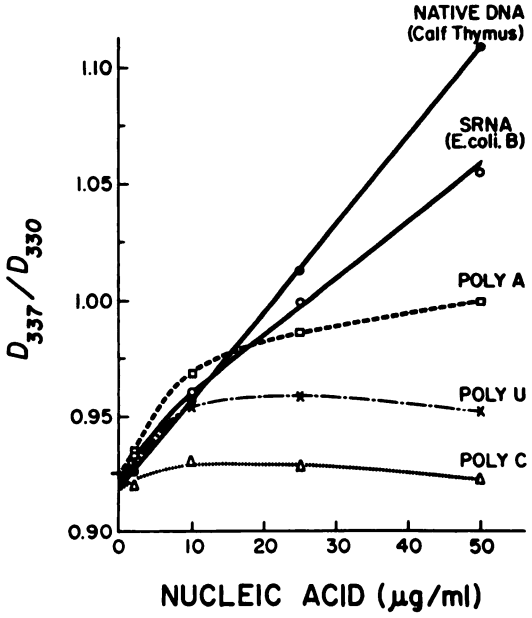



FIG. 7. *Shift in absorption maximum of Miracil D (10 μg/ml) induced by various nucleic acids*

At a lower concentration (10 μg/ml) polyadenylic acid appeared to be the most effective. This suggests that, in terms of nucleotide specificity, the drug may have a high affinity for adenylic acid residues. This

finding is of interest in view of previous studies by Hirschberg *et al.* which indicated that the *in vivo* cytotoxicity of Miracil D to tumor cells was reversed by adenine (10). The relative effectiveness of nucleic acids which differ markedly with respect to guanine content remains to be determined. When nucleic acids were compared with respect to their effect on the shift of the 442 mμ absorption peak of Miracil D to 450 mμ, the differences were not as striking as those shown in Fig. 7. At 10, 25, and 50 μg of nucleic acid per milliliter, the relative effectiveness (in decreasing order) was: DNA = sRNA > polyadenylic acid ⩾ polyuridylic acid ⩾ polycytidylic acid.

When the 2′,3′-mononucleotides of adenine, guanine, cytosine, and uridine were tested in amounts equivalent (with respect to moles of nucleotide residues) to 2, 10, 25, and 50 μg of nucleic acid, they did not significantly affect the absorption of Miracil D at 330, 337, 440, or 450 mμ. These results indicate the importance of the polynucleotide structure for effective interaction with Miracil D.

### *Effect of Miracil D on the heat denaturation profile of DNA and sRNA*

The above studies provided evidence for a physical interaction between Miracil D and nucleic acids. It was therefore of interest to determine the effect of this interaction on the secondary structure of the nucleic acid. When assayed in buffer alone, the heat denaturation profile of calf thymus DNA (25 μg/ml) revealed a $T_m$ (8) (50% of total hyperchromicity) of 56°. The presence of only 2 μg/ml of Miracil D caused a marked increase in heat stability with a shift in $T_m$ to approximately 71° (Fig. 8). Miracil D at 4 μg/ml increased the $T_m$ to 79°. The slope of the melting curve was considerably less steep in the presence of the drug. In the presence of Miracil D, the total hyperchromicity was also somewhat greater than in its absence. In part, this may simply reflect the contribution by the drug to the total absorption at 260 mμ. However, when the 260 mμ absorption of the same amount of free Miracil D (measured at 90°) was subtracted from the absorption at 260 mμ obtained at the end of the melt of DNA plus Miracil D, this effect persisted. It is possible, therefore, that at high temperatures this compound causes an actual increase in the hyperchromicity of DNA. The above studies were done in a buffer of low ionic strength. When studied in standard saline citrate (8) (0.15 M sodium chloride; 0.015 M sodium citrate, pH 7.0), Miracil D did not appreciably affect the

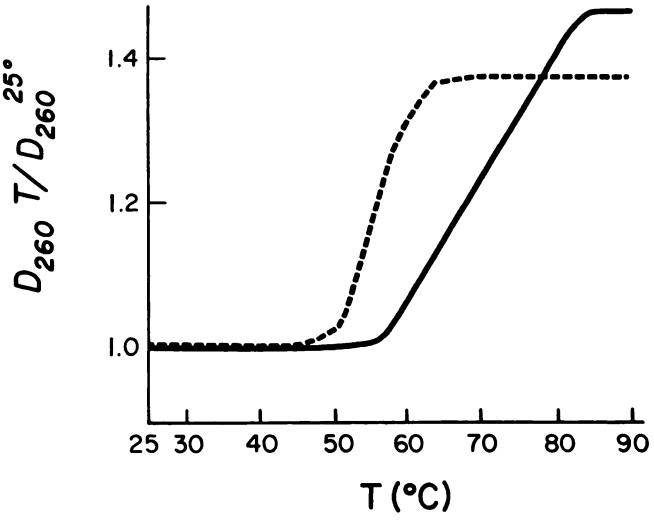



FIG. 8. *Effect of Miracil D on heat denaturation of native calf thymus DNA* -----, DNA (25 μg/ml); ———, DNA (25 μg/ml) + Miracil D (2 μg/ml).

heat denaturation curve of calf thymus DNA. This suggests that Miracil D may not interact with DNA in a medium of moderate salt concentration, perhaps because of competing ions. Alternatively, since the $T_m$ of calf thymus DNA is 88° in standard saline citrate as compared to 56° in low-ionic-strength buffer, it is possible that the drug forms a heat-labile complex with DNA and that in the presence of saline-citrate this complex dissociates prior to the critical temperature at which melting occurs.

Figure 9 indicates the effects of Miracil D on the heat denaturation of *E. coli* sRNA. The drug causes a small increase in $T_m$ of sRNA as well as an increase in total hyperchromicity. These results support the previous evidence that the drug can complex with RNA as well as DNA. The effect of Miracil D on the $T_m$ of sRNA is not nearly as great as that observed with DNA. The significance of this difference is not clear since interpretation of heat denaturation profiles of a mixture of sRNA's is considerably more complicated than in the case of DNA (11).

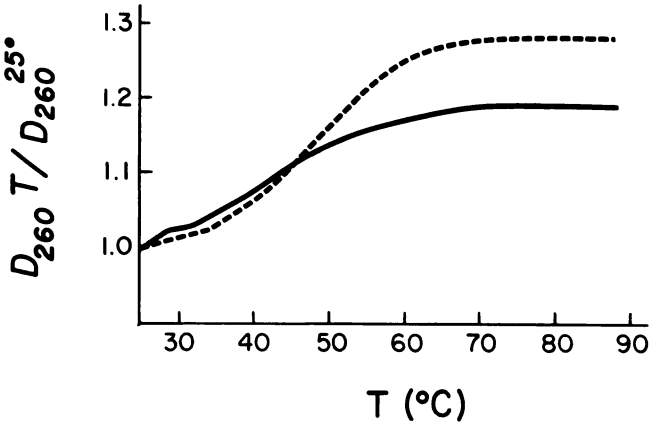



FIG. 9. *Effect of Miracil D on heat denaturation of sRNA*
———, *E. coli* B sRNA (25 μg/ml); - - - - -, sRNA (25 μg/ml) + Miracil D (2 μg/ml).

### *Effect of compounds II and III on heat denaturation of DNA*

Figure 10 indicates the effects of compounds II and III on the heat denaturation profile of calf thymus DNA in low-ionic-strength buffer. Like Miracil D, compound II caused a marked increase in $T_m$, a decrease in the slope of the melting curve, and an increase in total hyperchromicity. On the other hand, compound III, which had little or no biologic activity, raised the $T_m$ of DNA by only three degrees, only slightly decreased the slope of the melting curve, and did not produce an increase in total hyperchromicity.

## DISCUSSION

These results suggest that the mode of action of Miracil D involves an ability to complex with nucleic acids and thereby inhibit the template activity of DNA with respect to RNA synthesis and, to a lesser extent, inhibit the function of RNA's in protein synthesis. As an inhibitor of RNA synthesis, this drug is considerably less potent than actinomycin D (5, 9). The immediate effect of Miracil D on protein synthesis and the *in vitro* experiments indicating that Miracil D complexes with RNA's as well as DNA suggest that the drug may not be as specific in its interaction with nucleic acids as is actinomycin D.

It must be emphasized that thus far the evidence that Miracil D inhibits RNA and protein synthesis is restricted to the fact that it blocks the incorporation of $^{14}$C-labeled precursors; the possibility

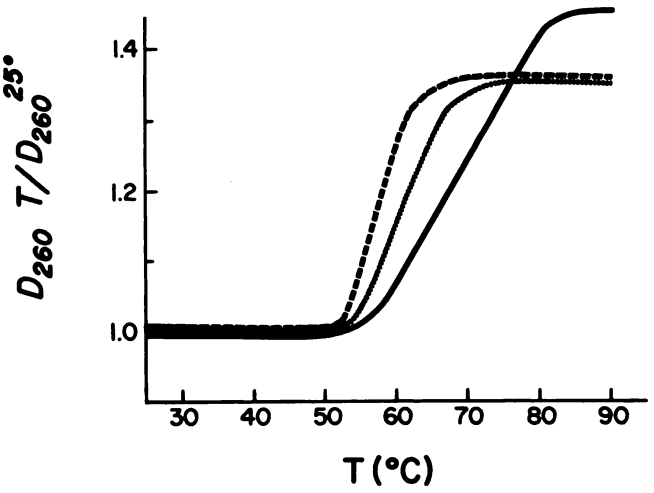



FIG. 10. *Effect of Miracil D analogs on heat denaturation of native calf thymus DNA*
-----, DNA (25 μg/ml); ———, DNA (25 μg/ml) + compound II (2 μg/ml); ....., DNA (25 μg/ml) + compound III (2 μg/ml).

that the drug directly affects precursor utilization has not been excluded. Further studies on the effects of Miracil D on macromolecular synthesis in intact bacterial and mammalian cells and in subcellular systems are in progress.

In a recent communication, Cohen and Yielding reported that chloroquine and a series of related antimalarial 4-aminoquinolines inhibit both the DNA and RNA polymerase reactions when studied *in vitro* with purified enzymes (12). These authors have reviewed previous evidence that these compounds inhibit the incorporation of radioactively labeled precursors in DNA and RNA of intact cells and bind both to DNA and RNA *in vitro*. Allison *et al.* have presented additional evidence for the formation of a complex between chloroquine and double-stranded DNA (13) and Lerman has described the interaction between the acridine derivative quinacrine and DNA (4). All these compounds have structural features which are grossly similar to the biologically active members of the Miracil D series, i.e., a dialkylaminoalkylamino side chain attached to a heterocyclic ring system (2, 3). Further studies on the molecular basis of interaction of Miracil D and related 10-thiaxanthenones with nucleic acids will be required to clarify the role of these functional groups and to provide information with respect to certain structural features of the nucleic acids which are necessary for biologic activity.

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REFERENCES

1. J. Newsome, *Trans. Roy. Soc. Trop. Med. Hyg.* **44**, 611 (1951).
2. E. Hirschberg, A. Gellhorn, M. R. Murray, and E. F. Elslager, *J. Natl. Cancer Inst.* **22**, 567 (1959).
3. L. S. Goodman and A. Gilman, *in* "The Pharmacological Basis of Therapeutics," 2nd Ed. Macmillan, New York, 1955.
4. L. S. Lerman, *J. Cellular Comp. Physiol.* **64**, Suppl. 1, 1 (1964).
5. E. Reich, *Science* **143**, 684 (1964).
6. I. B. Weinstein, R. Chernoff, I. Finkelstein and E. Hirschberg, *Proc. Am. Assoc. Cancer Res.* **6**, 267 (1965).
7. D. B. Roodyn and H. G. Mandel, *Biochim. Biophys. Acta* **41**, 80 (1960).
8. J. Marmur and P. Doty, *J. Mol. Biol.* **5**, 109 (1962).

9. C. Levinthal, A. Keynan and A. Higa, *Proc. Natl. Acad. Sci. U.S.* **48**, 1631 (1962).
10. E. Hirschberg, S. D. Brindle and G. Semente, *Cancer Res.* **24**, 1733 (1964).
11. J. Fresco, *in* "Informational Macromolecules" (H. J. Vogel, V. Bryson, and J. O. Lampen, eds.), p. 121. Academic Press, New York, 1963.
12. S. N. Cohen and K. L. Yielding, *Proc. Natl. Acad. Sci. U.S.* **54**, 521 (1965).
13. J. L. Allison, R. L. O'Brien and F. E. Hahn, *Science* **149**, 1111 (1965).