In vivo Effect of Nalidixic Acid (NegGram) on the DNA of Human Diploid Cells in Tissue Culture.* (30586)

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Nalidixic acid (1-ethyl-7-methyl-1,8-naphthrydin-4-one-3 carboxylic acid) is currently being used clinically for treatment of urinary tract infections caused by Gram-negative bacteria(1,2). Goss *et al*(3,4) reported that this drug irreversibly inhibited the synthesis of DNA in *E. coli,* thereby presumably causing death of the bacteria. Our laboratory has been interested for several years in the mode of action of agents that interfere with nucleic acid metabolism. A study of nalidixic acid was therefore undertaken, which revealed (Winshell and Rosenkranz, unpublished results) that the DNA isolated from treated bacteria was reversibly denaturable, thus indicating a cross-linkage between the polynucleotide strands (see ref. 5). In this respect the action of nalidixic acid appeared to resemble that of radiomimetic agents such as nitrogen mustard(5) and mitomycin C(6); moreover the "cross-linked" DNA isolated from nalidixic acid-treated bacteria was unstable in solution, as would be expected if the effect had been caused by an alkylating agent(7). When the clinical use of nalidixic acid was considered in the light of previous findings that alkylating agents with antibacterial properties also react with animal cells, it was decided to investigate the effect of nalidixic acid on the DNA of stable human diploid cells.

Materials and methods. A line of stable human amnion cells (FL) was grown at 37°C in Eagle's basal medium with Hanks' balanced salt solution and 10% calf serum. Thirty ml portions of trypsinized cell suspensions (5×10^5 cells/ml) were transferred to 32-oz bottles and incubated for 3 hours, at which time nalidixic acid was added to a final concentration of 50 μg per ml. After 48 hours of incubation the monolayers were washed 4 times with cold 0.15 M NaCl containing 0.015 M sodium citrate (saline-citrate); the cells were then scraped from the glass and resuspended in a small volume of 0.03 M *tris* buffer (pH 8.45) containing 0.015 M sodium citrate. The cells were lysed by adding Dupanol C to a final concentration of 2% and heating at 60°C in a water bath for 3 minutes. The preparations were cooled, adjusted to 1 M $NaClO_4$, and the crude nucleic acids were then precipitated with 2 volumes of 95% ethanol (Procedure I). Cells were also lysed by adding Dupanol C to a concentration of 2% and then an equal volume of 88% phenol. After vigorous shaking the emulsion was heated to 60°C for 1 minute and the aqueous phase was collected. The nucleic acids were brought out of solution by adding 2 volumes of ethanol (Procedure II).

After standing at 4°C for 2 hours the fibers were washed thoroughly with alcohol and re-dissolved in saline-citrate/10. The "crude" nucleic acid prepared by Procedure I occasionally contained insoluble material, which was removed by low-speed centrifugation. The manner in which the nucleic acids were prepared did not influence their buoyant densities.

The various DNA specimens (*ca* 5 μg/ml) to be analyzed by cesium chloride density gradient centrifugation were supplemented with a marker DNA of known density (*Micrococcus lysodeikticus,* 1.731 g/cm^3) and adjusted to a density of 1.70 g/cm^3 with solid cesium chloride (optical grade, Harshaw Chemical Co.). The samples were spun at 44,770 r.p.m. in a Spinco Model E analytical ultracentrifuge. After centrifugation for 24 hours, pictures of the DNA bands at their equilibrium positions were taken through the ultraviolet optical system. The pictures were scanned with a Joyce-Loebl Mark III B microdensitometer and from the tracings so

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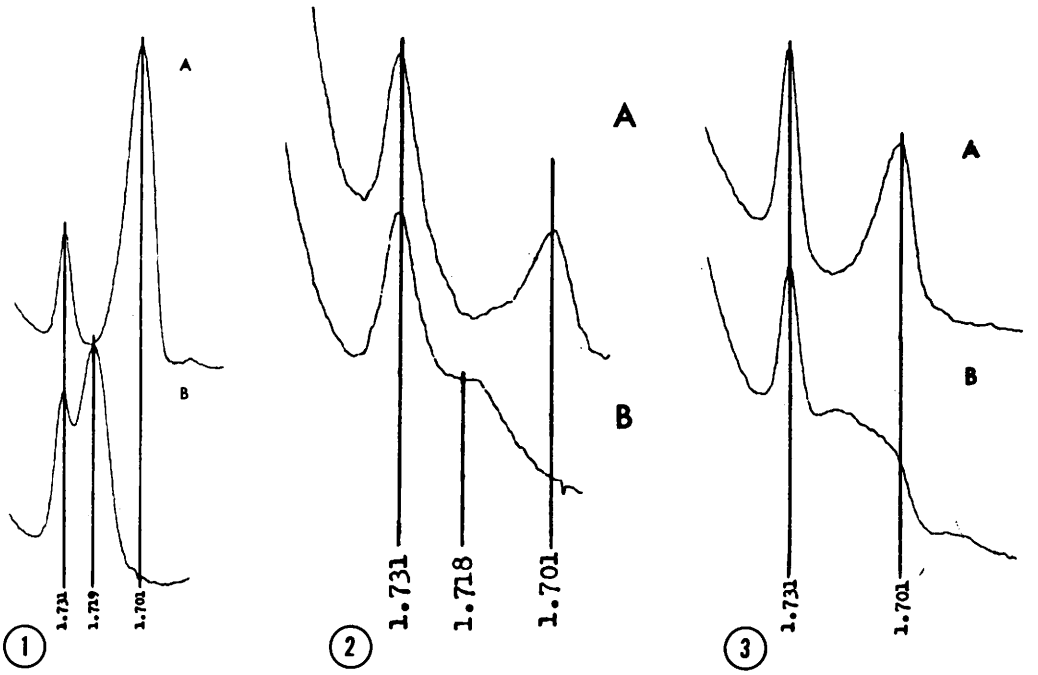

FIG. 1. Cesium chloride density gradient centrifugation of DNA isolated from FL-cells by Procedure I. Fig. 1A, Unheated specimen; Fig. 1B, Specimen after heat denaturation. Band on the left represents position of the marker DNA *(Micrococcus lysodeikticus)*.

FIG. 2. Cesium chloride density gradient centrifugation of DNA isolated by Procedure I from FL-cells treated with nalidixic acid (Preparation I). Fig. 2A and 2B represent the properties of unheated and thermally denatured DNA, respectively.

FIG. 3. Cesium chloride density gradient centrifugation of DNA isolated by Procedure II from FL-cells treated with nalidixic acid (Preparation II). Fig. 3A, Unheated DNA; Fig. 3B, Heat-denatured DNA.

obtained the buoyant densities of the samples were calculated by comparison with the position of the marker DNA(8).

Nalidixic acid was donated by Mr. E. E. Radigan, Winthrop Laboratories. In accordance with directions provided by the manufacturer, 10 mg were dissolved in 0.5 ml of 0.1 N NaOH and diluted to a concentration of 500 μg/ml with distilled water. Only fresh solutions of the drugs were used.

Thermal denaturation of the DNA specimens was accomplished by heating dilute solutions (*ca* 20 μg/ml of saline-citrate) in a boiling water bath for 10 minutes and then chilling them in an ice bath.

Results and discussion. Normally, the buoyant density of a double-stranded DNA preparation increases by approximately 0.015 g/cm^3 when the specimen is rendered single-stranded (*i.e.*, denatured) as by heating(9). This was indeed found to be the behavior exhibited by the DNA isolated from untreated FL-cells (Fig. 1). The banding behavior of 3 different preparations of DNA derived from cells treated with nalidixic acid (50 μg/ml) is shown in Fig. 2-4 (upper portions). The values of the buoyant densities of the unheated DNA's from treated cells are identical with those calculated for the nucleic acids of normal cells (1.701 g/cm^3). The greater width of the bands describing the behavior of the DNA's of cells exposed to the drug should be noted; this change in width indicates lowered molecular size(10). The cesium chloride density gradient analyses of these same DNA's after heat denaturation are illustrated in the lower sections of Fig. 2-4. There is considerable overlap between the densities of the heated and unheated materials and in Fig. 4 two bands can actually be seen. These results presumably mean that the procedure of heating and quick chilling does not yield a uniformly denatured molecular population, but that

some of the molecules are either resistant to denaturation or are reversibly denaturable. Iyer and Szybalski(11) have shown that reversible denaturation of cross-linked DNA molecules could be inhibited by heating the specimens in the presence of formaldehyde, thereby preventing the orderly reformation of hydrogen bonds between complementary base sequences. As anticipated, when DNA from nalidixic acid-treated cells was denatured in the presence of formaldehyde, a much greater proportion of the molecules remained single-stranded (Fig. 5).

These data indicate that the DNA isolated from nalidixic acid-treated cells is structurally modified. There are other agents which have been shown to have similar effects on the physicochemical properties of DNA, including ultraviolet light, alkylating agents and mitomycin C, a potential alkylating agent. Although the mode of action of nalidixic acid is not yet understood, the widening clinical use of the drug suggested that these preliminary findings of its effect on the DNA of a human cell line might be of general interest.

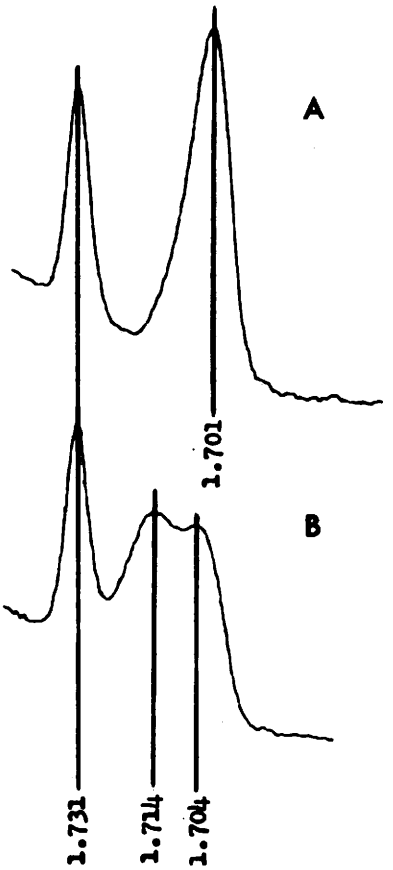

FIG. 4. Cesium chloride density gradient centrifugation of DNA isolated by Procedure I from FL-cells treated with nalidixic acid (Preparation III). Fig. 4A, Unheated DNA; Fig. 4B, Thermally-denatured DNA.

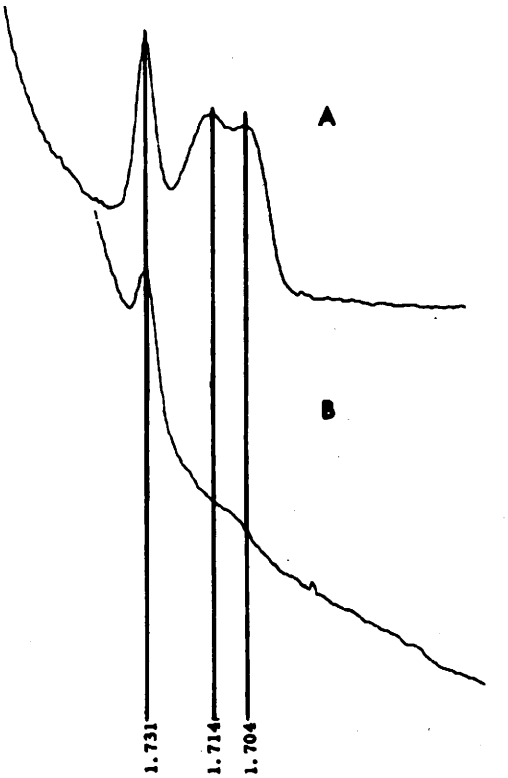

FIG. 5. Effect of heating in formaldehyde on the properties of DNA isolated from FL-cells treated with nalidixic acid (Preparation III). Fig. 5A, Heated in absence of formaldehyde; Fig. 5B, Heated in presence of formaldehyde (1%).

Summary. DNA isolated from human amnion cells after exposure to nalidixic acid (NegGram) for 48 hours was structurally modified. The altered DNA possessed properties resembling those of nucleic acids isolated from cells treated with alkylating agents.

1. Campbell, J. L., Thomley, M. W., Parsons, R. L., J. Urol., 1964, v92, 549.

2. Akbari, A., Ward, J. N., Hilf, M. M., Lavengood, R. W., Draper, J. W., ibid., 1964, v92, 552.

3. Goss, W. A., Deitz, W. H., Cook, T. M., J. Bacteriol., 1964, v88, 1112.

4. ———, ibid., 1965, v89, 1068.

5. Geiduschek, E. P., Proc. Nat. Acad. Sci. U. S., 1961, v47, 950.

6. Iyer, V. N., Szybalski, W., ibid., 1963, v50, 355.

7. di Mayorca, G., Rosenkranz, H. S., Polli, E. E., Korngold, G. C., Bendich, A., J. Nat. Cancer Inst., 1960, v24, 1309.

8. Schildkraut, C. L., Marmur, J., Doty, P., J. Mol. Biol., 1962, v4, 430.

9. Doty, P., Marmur, J., Eigner, J., Schildkraut, C. L., Proc. Nat. Acad. Sci. U. S., 1960, v46, 461.

10. Meselson, M., Stahl, F. W., Vinograd, J., ibid., 1957, v43, 581.

11. Szybalski, W., Iyer, V. N., Fed. Proc., 1964, v23, 946.

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Role of a Plasma Factor in Porphyrin Biosynthesis.* (30587)

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The presence of a circulating erythropoietic-stimulating factor ("erythropoietin") in the plasma of anemic animals has been demonstrated by a number of investigators(1,2). Evidence for its presence is derived from studies with animals made anemic by bleeding, or by hemolytic agents such as phenylhydrazine(3,4,5), or by other methods. Linman *et al*(6) have suggested that there are probably 2 factors in plasma affecting erythropoiesis, one a thermostable factor which stimulates the division of erythroblasts and another relatively thermostable factor which enhances hemoglobin synthesis. Thomas(7) showed that normal rabbit serum markedly enhanced the rate of incorporation of C^{14} labeled glycine into heme by rabbit bone marrow. There is, however, no general agreement as to the presence of a heme-stimulating factor in the plasma. For example, Goldberg *et al*(8) could not find any difference in the uptake of radioiron into heme between deproteinized plasmas of normal chickens and of chickens made anemic by either bleeding or by phenylhydrazine administration. These workers found that the uptake of radioiron into heme was much greater when saline was substituted for any of these plasma extracts. Other workers(9,10) have shown that polycythemia causes the appearance in the plasma of an active substance which is antagonistic to erythropoietin. This inhibitory factor "erythropoiesis inhibitor"(10) is thermostable. More recently, Bruns and London (11) have shown that hemin is inhibitory to the biosynthesis of the heme moiety of hemoglobin but stimulates the formation of the globin moiety. Because of the foregoing conflicting evidence for erythropoietic stimulating and inhibitory factors in blood plasma or serum, the present investigation was undertaken to study further the effects of different plasmas and plasma preparations on the biosynthesis of porphyrins, as an index of erythropoietic activity.

Materials and methods. The effects of several different plasmas and plasma or serum preparations on porphyrinogenesis were determined by adding these substances to porphyrin-synthesizing systems, chicken erythrocytes or beef liver acetone powder, under controlled conditions. The chicken erythrocyte preparations were made as previously described(12). Normal mongrel dogs were made anemic by the administration (on 2 successive days) of 40 mg phenylhydrazine per kg body weight to obtain the anemic plasma. The blood was taken when the hematocrit reached 20% or less. Beef liver acetone powder was obtained from General Biochemicals Co., Chagrin Falls, Ohio. The incubation procedures, extraction, and determination of porphyrins were essentially the same as described previously(12).

Results and discussion. In preliminary experiments, the effect of several types of incubation media on the biosynthesis of porphyr-

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