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Action of Hydroxyurea on *Anabaena variabilis*

II. Effects of Pre- and Post-Treatment with Chloramphenicol and Base Analogues

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Summary. Low concentrations of hydroxyurea stimulated the growth of the blue-green alga *Anabaena variabilis* that had been pretreated with sublethal concentrations of chloramphenicol or of certain nucleic acid base analogues. When supplemented to the culture medium, hydroxyurea also counteracted the growth inhibitory effect of chloramphenicol on this organism. In contrast, when *A. variabilis* cells grown in the presence of hydroxyurea were subsequently treated with chloramphenicol, they were found to have become highly susceptible to the growth inhibitory effects of chloramphenicol. The growth of hydroxyurea pretreated cells in basal medium was attended by a lag that was shorter than that of untreated controls; on the other hand, when hydroxyurea pretreated cells were inoculated into chloramphenicol-supplemented medium, they exhibited a longer lag than that shown by untreated cells in chloramphenicol.

The results obtained are discussed in terms of the probable effects of hydroxyurea and chloramphenicol on certain enzyme systems.

In a previous paper (SINGH and KUMAR, 1968) hydroxyurea, the antileukaemic drug, was reported to exert growth stimulatory effects in lower concentrations and inhibitory effect in higher concentrations in the blue-green alga *Anabaena variabilis*. The survival curve of the alga for this drug was of a biphasic type. The inhibition was stronger when the drug was added during or soon after the lag-phase. Further, exponentially growing cells showed least lag in 10 μg hydroxyurea/ml, whereas cells in the lag-phase or the stationary phase of growth grew after long lags. In this paper we report the effects of pre- and posttreatment of the alga with chloramphenicol or base analogues on its response to hydroxyurea. Hydroxyurea specifically affects DNA metabolism and inhibits DNA synthesis (ROSENKRANZ and JACOB, 1967). Chloramphenicol is known to inhibit protein synthesis (see KUMAR, 1964; SOMPOLINSKY and SAMRA, 1968) while base analogues inhibit nucleic acid synthesis. The objective of the present study was to gain an insight into the mechanism of action of hydroxyurea in blue-green algae.

Material and Methods

The strain of the alga used and general methods for its culture and growth measurement have been reported in the previous paper (SINGH and KUMAR, 1968). Stock solutions of chloramphenicol (Parke Davis & Co.) were prepared by aseptically dissolving it in sterile distilled water and appropriate volumes were added into cold sterile medium to obtain the desired concentrations. Stock solutions of 5-aminouracil, 8-azaguanine (both supplied by Nutritional Biochemical Corporation, Cleveland) and 2-aminopurine nitrate (Calbiochem, Los Angeles) were prepared by dissolving 10 mg of analogue in 50 ml of absolute ethyl alcohol. Serial dilutions were prepared in distilled water and appropriate volumes added to the medium which was then sterilized by autoclaving.

Pretreatments

The alga was grown in 0.5 μg chloramphenicol/ml and exponentially growing cultures of such pretreated cells were centrifuged, washed and suspended in fresh basal medium followed by incubation for four days to bring them into log-phase. These cells were washed by centrifugation, diluted appropriately and inoculated into triplicate culture tubes containing basal medium supplemented with nil, 1.0, 10, 50 and 100 μg hydroxyurea/ml. Similar sets of hydroxyurea tubes (controls) were also inoculated with cells that had not been pretreated with chloramphenicol. Growth in treated and control cultures was followed by regular counts on alternate days.

Post-Treatments

The alga was grown in 10 μg hydroxyurea/ml of culture medium and exponentially growing cultures were centrifuged, washed and resuspended in fresh basal medium. This suspension was incubated three days to bring to log-phase and the algae were then washed by centrifugation, diluted appropriately and inoculated into triplicate tubes containing culture medium supplemented with nil and 1.0 μg chloramphenicol/ml. A similar set of tubes was inoculated with the alga grown in basal medium to serve as controls. Cell numbers were counted on alternate days.

Similar pre- and post-treatments were also performed in the highest growth-permitting concentrations of the base analogues.

Effect of Post-Treatment on Lag

Inocula prepared in the manner described in post-treatment experiments were transferred into triplicate sets of tubes containing nil, 0.1, 0.5, 1.0, 1.5 and 2.0 μg chloramphenicol/ml. Cell numbers were counted daily and the lag-phase in each culture was determined by extrapolation of the exponential part of the growth curve on the abscissa.

Direct Supplementation

Duplicate culture tubes containing nil and 10 μg hydroxyurea/ml were supplemented with 1.0 μg chloramphenicol/ml. One such set of duplicate tubes was left unsupplemented to serve as control. Exponentially growing cells were centrifuged, washed and inoculated into these tubes and cell numbers counted on alternate days.

Results

Pretreatments

The growth in basal medium of chloramphenicol-grown cells is slower with longer lag as compared to the growth and lag of untreated controls

(Fig. 1). The growth in 10 μg hydroxyurea/ml of both chloramphenicol-grown and basal-grown cells is more or less identical, with a slightly higher growth rate in the former. But when growth enhancement due to hydroxyurea is considered, the enhancement in chloramphenicol-pretreated cells is significantly greater than in basal grown cells. No growth occurred in the presence of 50 μg and 100 μg hydroxyurea/ml.

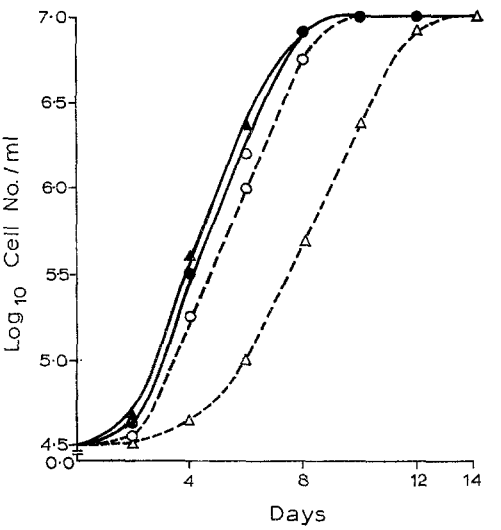

Fig. 1

Effect of chloramphenicol pretreatment on the subsequent growth of *A. variabilis* in 10 μg hydroxyurea/ml of culture medium. o----o untreated, no hydroxyurea; Δ----Δ chloramphenicol-pretreated, no hydroxyurea; •——• untreated, 10 μg hydroxyurea/ml; ▲——▲ chloramphenicol pretreated, 10 μg hydroxyurea/ml

Similar results were also obtained with base analogues, the maximum concentrations permitting growth being 5.0, 20 and 30 μg/ml for 5-aminouracil, 8-azaguanine and 2-aminopurine nitrate respectively.

Post-Treatments

The growth in basal medium of cells grown in 10 μg hydroxyurea/ml for 6 days is slightly better than hydroxyurea-free controls although the duration of the lag is the same in both cases (Fig. 2). Chloramphenicol inhibits the growth of the alga in both cases and such inhibition by chloramphenicol is much more apparent in hydroxyurea pretreated cells than in basal grown cells. The extent of inhibition increases with

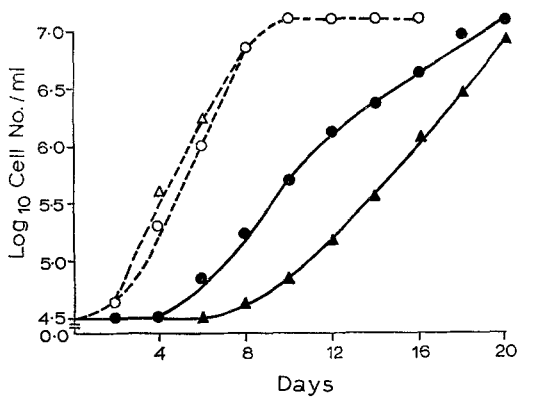

Fig. 2

Effect of post-treatment with chloramphenicol on the growth of cells grown in 10 μg hydroxyurea/ml. o——o basal grown, no chloramphenicol; Δ——Δ hydroxyurea pretreated, no chloramphenicol; •——• basal grown, 1.0 μg chloramphenicol/ml; ▲——▲ hydroxyurea pretreated, 1.0 μg chloramphenicol/ml

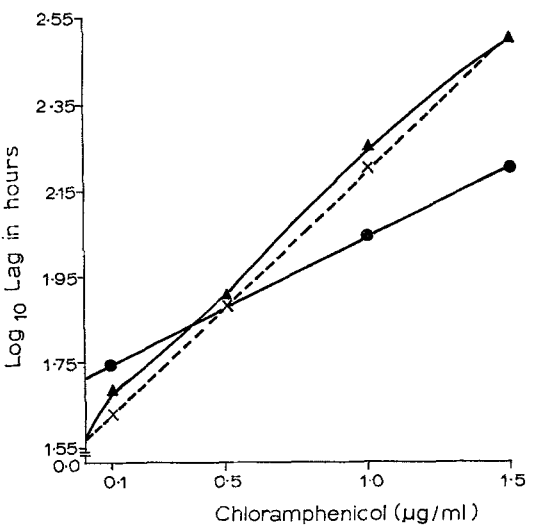

Fig. 3

Effect of post-treatment with different concentrations of chloramphenicol on the lag-phase of cells grown in hydroxyurea. •——• basal grown; ▲——▲ hydroxyurea pretreated; ×——× regression line ($y = mx + c$, where $m = 0.61$ and $c = 1.6$)

increasing concentrations of the antibiotic (Fig.3) and the lag-phase becomes correspondingly longer.

All three base analogues used in this investigation have the same effect on the growth as a post-treatment factor. They exert a growth inhibitory effect in both basal grown and hydroxyurea grown cells, though the inhibition is lesser in the case of hydroxyurea pretreated cells than in basal grown cells. Such effects of base analogues are in striking contrast to the chloramphenicol effect in *A. variabilis*.

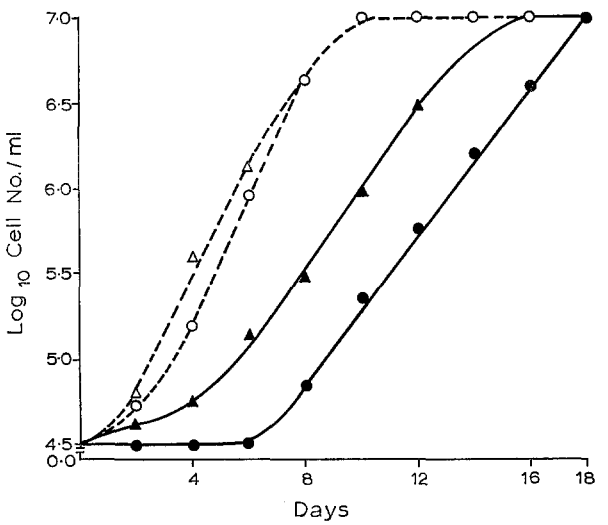

Fig.4

Effect of direct supplementation into the culture medium of 10 μg hydroxyurea plus 1.0 μg chloramphenicol/ml on growth of *A. variabilis*. o----o no hydroxyurea, no chloramphenicol; Δ----Δ hydroxyurea 10 μg/ml, no chloramphenicol; •——• no hydroxyurea, chloramphenicol 1.0 μg/ml; ▲——▲ hydroxyurea 10 μg/ml plus chloramphenicol 1.0 μg/ml

Post-Treatment and the Lag

When grown in basal medium, the hydroxyurea pretreated cells exhibit a shorter lag than basal grown cells (Fig.3). In the presence of 0.1 μg chloramphenicol/ml also the hydroxyurea pretreated alga shows a shorter lag than basal grown alga though the difference is smaller than in the previous case. In the presence of 0.5, 1.0 and 1.5 μg chloramphenicol/ml, hydroxyurea-pretreated cells show longer lag than untreated cells and the difference increases progressively in higher concentrations.

No growth was observed in 2.0 µg chloramphenicol/ml up to the 18th day following inoculation.

Direct Supplementation

The growth inhibition by chloramphenicol is lesser in the presence of hydroxyurea (Fig. 4) than in its absence. The lag in culture medium containing both chloramphenicol and hydroxyurea is less (about 3.5 days) than in one containing only chloramphenicol (6 days).

Discussion

In a previous paper (SINGH and KUMAR, 1968) we reported that the addition of hydroxyurea to basal culture medium stimulated the growth of *A. variabilis* and that the degree of growth stimulation increased with increasing concentrations of hydroxyurea up to a certain limit beyond which it exerted a lethal effect. The obvious conclusion to be drawn from such results is that the effect of hydroxyurea on this alga is essentially that of a metabolite or growth factor.

The growth curves of chloramphenicol-pretreated cells and of untreated cells in hydroxyurea supplemented and unsupplemented basal medium indicate that chloramphenicol pretreatment increases the duration of the lag-phase and that hydroxyurea supplementation abolishes the chloramphenicol-induced lag. Hydroxyurea also exerts similar effects on untreated controls (Fig. 1). These observations raise the following questions: 1. What sort of metabolite is hydroxyurea, and 2. How does it help the cells to overcome the chloramphenicol-induced lag?

The basal medium used in this study contains nitrate as the nitrogen source and the alga growing in it must therefore possess the enzyme system needed for reducing nitrate to the level of ammonium. For blue-green algae it has been observed that the enzyme systems converting nitrate to ammonia are inducible or adaptive and the algae growing with nitrate nitrogen show a lag in growth whereas with ammonium nitrogen the growth is not preceded by a lag-phase (see FOGG and WOLFE, 1954).

Chloramphenicol is an inhibitor of protein synthesis and if, as seems likely, the nitrate reducing enzymes of *A. variabilis* are inducible then chloramphenicol pretreatment would be expected to inhibit their formation and the degree of such an inhibition will be reflected in the duration of the lag-phase. Under such conditions if ammonium nitrogen is available for growth, then the chloramphenicol pretreated cells will start growing without any lag. This is precisely what happens with

chloramphenicol-pretreated cells growing in hydroxyurea-supplemented basal medium.

Hydroxyurea contains two amino groups and one hydroxyl group and on the basis of the results obtained with hydroxyurea in chloramphenicol-pretreatment experiments it can reasonably be assumed that the growth stimulatory effect of hydroxyurea is mainly due to its serving as a source of ammonium nitrogen. It seems that hydroxyurea decomposes to liberate ammonia but whether this decomposition takes place outside or inside the cells is somewhat difficult to decide. The proposed mechanism of hydroxyurea action in chloramphenicol-pretreated cells seems to be equally applicable to the results of chloramphenicol post-treatment and of chloramphenicol and hydroxyurea direct supplementation experiments.

In chloramphenicol post-treatment experiments, hydroxyurea grown cells are more sensitive to chloramphenicol than basal grown cells (Fig. 2). The presence of ammonium nitrogen in a medium containing nitrate nitrogen represses the formation of nitrate reducing enzymes and consequently hydroxyurea grown cells should lack such enzymes. Accordingly the inoculation of hydroxyurea pretreated cells, as compared to basal grown cells, into medium containing chloramphenicol, should show longer lag. The observed response of *A. variabilis* to post-treatment with chloramphenicol is in agreement with such an explanation. A similar explanation can be advanced to account for the data presented in Fig. 4.

Base analogues are inhibitors of nucleic acid synthesis and according to the generally accepted Central Dogma of the genetical control of inducible and repressible enzyme synthesis (viz., DNA → mRNA → protein), the results of the effects of pre- and post-treatments with base analogues can easily be explained provided one assumes that the analogues also affect the formation of nitrate reducing systems of *A. variabilis*. However, the conclusions arrived at with base analogues are at present purely tentative and further experiments to substantiate this view are in progress.

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