ON THE MECHANISM OF INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS BY DEOXYADENOSINE IN VITRO*

BY K. OVERGAARD-HANSEN AND H. KLENOW

THE BIOCHEMICAL DIVISION OF THE FIBIGER LABORATORY, COPENHAGEN†

Communicated by H. M. Kalckar, March 8, 1961

It has previously been shown that the incorporation of both C14-formate into DNA\(^1\) thymine and P\(^{32}\)-orthophosphate into DNA phosphate of the hypotetraploid strain of Ehrlich ascites tumor cells in vitro is strongly inhibited by deoxyadenosine.\(^2\) It was, furthermore, shown that this inhibition is not due to a prevention of the de novo synthesis of acid-soluble thymine compounds. The incorporation of P\(^{32}\)-orthophosphate into RNA was not significantly inhibited by deoxyadenosine. These observations led to the postulate that deoxyadenosine in a direct or an indirect manner inhibits some specific step in the DNA synthesis of Ehrlich ascites tumor cells in vitro.

Prusoff\(^3\) has independently found a similar inhibiting effect of deoxyadenosine on the utilization of formate and of thymidine for the biosynthesis of DNA thymine in Ehrlich ascites tumor cells in vitro. Maley and Maley\(^4\) have concluded from recent experiments with labeled pyrimidine nucleosides that deoxyadenosine inhibits DNA synthesis in chick embryos in vitro, and Lark\(^5\) has found that the addition of deoxyadenosine to the growth medium of Alcaligenes fecalis LB blocks the synthesis of DNA also in these cells.

Among a number of ribosides and deoxyribosides only the synthetically prepared deoxyadenosine 1-N-oxide has been found to have an effect on the DNA synthesis in Ehrlich ascites cells similar to that of deoxyadenosine.\(^6\) \(^7\) It appears, however, that the inhibiting effect of deoxyadenosine is completely absent if deoxyguanosine is added simultaneously to tumor cell suspensions.\(^6\) When only deoxyguanosine is added, the incorporation of P\(^{32}\)-orthophosphate may be stimulated up to twofold, and it is possible, therefore, by addition of deoxyadenosine or deoxyguanosine to suspensions of Ehrlich ascites cells, to regulate the rate of DNA synthesis from an 85 to 90 per cent inhibition to a twofold stimulation, respectively. These findings have led to the assumption that deoxyadenosine inhibits DNA synthesis by interfering with the formation of deoxyguanosine or a closely related precursor of DNA.\(^6\)

The metabolism of deoxyadenosine in suspensions of Ehrlich ascites cells has now been investigated. The results have strongly suggested that the effect of this compound is partially or exclusively an indirect one and that a small part of the added deoxyadenosine in the cells is converted to a compound which is the actual inhibitor and which does not penetrate the cell membrane.

Since the cells of the hyperdiploid\(^8\) strain of the Ehrlich ascites tumor may be obtained without contaminating erythrocyes, these have been used in the present experiments in preference to the cells of the hypotetraploid\(^8\) strain previously used.

Materials.—Deoxynucleosides were obtained from the California Foundation for Biochemical Research, Los Angeles, California, and P\(^{32}\)-orthophosphate in sterilized isotonic solution from the Radiochemical Centre, Amersham, England.

Cells of the hyperdiploid strain of the Ehrlich ascites tumor were kindly furnished by Dr. G.
Klein, Karolinska Institutet, Stockholm, and they were maintained by transfers made by intra-peritoneal injections of ascites fluid into mice of strain DBA. The cells were used 4–5 days after transplantation.

Adenosine deaminase was a highly purified preparation from intestinal mucosa. One unit of enzyme was defined as the amount of enzyme which deaminates 1 μmole of deoxyadenosine per minute at pH 7.5. Xanthine oxidase was prepared as described by Klenow and Emberland. Nucleoside phosphorylase was prepared by the method of Price et al.

Methods.—The method for isolation of DNA and the determination of the specific activity of DNA phosphate was essentially as previously described.

Hyoxanthine, deoxyinosine, and deoxyadenosine were determined by enzymatic differential spectrophotometry using the enzymes xanthine oxidase, nucleoside phosphorylase, and adenosine deaminase according to the principle of Kalkar. Samples of the cell suspension were deproteinized with one volume of ice-cold 5 per cent perchloric acid and centrifuged in the cold. A fraction of the supernatant was neutralized with 0.5 N KOH, and when KClO₃ had precipitated, a known amount of the supernatant was transferred to a quartz cuvette containing 3.0 ml of 0.05 M phosphate buffer, pH 7.5. The determination of hypoxanthine, deoxyinosine, and deoxyadenosine was now carried out by successive addition of xanthine oxidase, nucleoside phosphorylase, and adenosine deaminase to the cuvette. By recording the increase in extinction at 292.5 mμ after addition of each of the enzymes, the amount of the corresponding compounds was calculated.

Results.—Evidence for intracellular formation of an inhibitor from deoxyadenosine: The concentration of deoxyadenosine has been determined at time intervals in suspensions of ascites cells incubated with this compound. In the experiment recorded in Figure 1B, 50 per cent of the deoxyadenosine had disappeared after about 50 minutes, and after about 3 hours no significant amount was present. Both deoxyinosine and hypoxanthine were formed in the cell suspension. Deoxyinosine was in the beginning formed at a faster rate than hypoxanthine, and deoxyadenosine is, therefore, probably first deaminated to ammonia and deoxyinosine, which is further converted to hypoxanthine and deoxyribose 1-phosphate by a phosphorolytic cleavage. It is, furthermore, seen from this figure that the sum of deoxyadenosine, deoxyinosine, and hypoxanthine decreases slowly until it has reached a level of about 80 per cent of the starting value. It appears from Figure 1A that, although almost no deoxyadenosine is present in the cell suspension after about 3 hours of incubation, the inhibition of P₃₂ incorporation into DNA is not released even after 5 hours. In another experiment (see Fig. 2) it was found that the inhibiting effect of deoxyadenosine is almost completely abolished when the compound has been preincubated with adenosine deaminase. This further supports a previous finding which suggested that the effect of deoxyadenosine is due to this compound per se and not to a contaminating impurity present in the preparations used.

The finding that the inhibition of P₃₂ incorporation into DNA is not released even after two hours after deoxyadenosine has disappeared from the cell suspension has led to the postulate that presence of deoxyadenosine in the cells gives rise to the formation of a compound which inhibits DNA synthesis.

This compound would be different from ammonia, deoxyinosine, and conversion products of deoxyinosine, and its inhibiting effect would not be abolished by the addition of adenosine deaminase to the cell suspension. The amount of inhibitor formed from deoxyadenosine would, furthermore, correspond to 20 per cent or less of the added deoxyadenosine. It is, however, not possible from the experiments to exclude the possibility that unconverted deoxyadenosine has an inhibiting effect on DNA synthesis.
It was previously observed⁸ that the inhibition of DNA synthesis may be abolished when deoxyguanosine is added to the hypotetraploid cells simultaneously with deoxyadenosine. This may now be explained in at least two ways: (1) Deoxyguanosine may prevent the formation of the inhibitor from deoxyadenosine. (2) It may counteract both the effect of the inhibitor formed from deoxyadenosine and a possible inhibiting effect of deoxyadenosine per se. In the first case it would not be possible with deoxyguanosine to release the inhibition of cells after preincubation for some time with deoxyadenosine. In the second case, however, it would be possible with deoxyguanosine to overcome the inhibition also after preincubation with deoxyadenosine, provided that the blockage of DNA synthesis in the period of preincubation has not caused other and irreversible changes.
The experiment in Figure 3 indicates that also for the hyperdiploid strain of the cells addition of deoxyguanosine at the beginning of the experiment has some stimulating effect on P32 incorporation into DNA and that this is even more pronounced in the presence of both deoxyguanosine and deoxyadenosine. The figure shows, furthermore, that the addition of adenosine deaminase to a sample of a cell suspension incubated for 160 minutes with deoxyadenosine does not release the inhibition of P32 incorporation into DNA although the concentration of deoxyadenosine rapidly fell to zero. Further addition of deoxyguanosine to such a sample did, however, immediately increase the rate of incorporation to that of the control cells treated in a similar way.
Figure 4 shows again a stimulating effect of deoxyguanosine when added to samples of cells preincubated both for 90 and 150 minutes with deoxyadenosine. In this case, deoxyadenosine was not removed from the samples by addition of adenosine deaminase before the addition of deoxyguanosine.

These findings may suggest, therefore, that the influence of deoxyguanosine is to counteract the effect of the inhibitor formed from deoxyadenosine, rather than to prevent the formation of the inhibitor.

Impermeability of the cell membrane to the postulated inhibitor formed from deoxyadenosine: Experiments have shown that no inhibitor is transferred to control cells with the extracellular fluid of cells that have been incubated with deoxyadenosine and after 100 minutes treated with adenosine deaminase. In another experiment, the acid-soluble fraction was isolated from cells that had been incubated for 180 minutes both under control conditions and in the presence of deoxyadenosine. Figure 5 shows that the incorporation of P32-orthophosphate was inhibited to about the same extent by the two extracts. These findings suggest, therefore, that the inhibitor which is postulated to be formed from deoxyadenosine in the cells does not pass through the cell membrane.

Discussion.—The experiments indicate that the inhibition of DNA synthesis of Ehrlich ascites tumor cells, by deoxyadenosine, in vitro, is partially or exclusively an indirect one. The inhibition of DNA synthesis persists also when deoxyadenosine, after incubation with the cells for about three hours, is removed by deamination. Since deoxyinosine has no effect on DNA synthesis it is postulated that a small part of the added deoxyadenosine in the cells is converted to an inhibitor which is not any more inactivated by adenosine deaminase. It has previously been shown that the inhibiting effect of deoxyadenosine may be prevented by simultaneous addition of deoxyguanosine. Now it has also been found that the effect of the postulated inhibitor may be reversed by deoxyguanosine. The inhibitor appears not to pass through the cell membrane.

It has recently been shown by Munch-Petersen14 that appreciable amounts of deoxyATP accumulate in the cells incubated with deoxyadenosine. It is possible, therefore, that the inhibitor shown here...
to be formed in the cells from deoxyadenosine is identical with deoxyATP. The conclusion that deoxyguanosine presumably counteracts the effect of the inhibitor rather than preventing its formation would be in agreement with this concept since deoxyATP was found\textsuperscript{14} to accumulate also when cells were incubated with both deoxyadenosine and deoxyguanosine. Recent experiments in this laboratory have further supported the view that deoxyATP or a closely related compound is the actual inhibitor. It has been shown\textsuperscript{15} that addition of adenosine together with deoxyadenosine to a cell suspension not only prevents inhibition of DNA synthesis but also prevents accumulation of detectable amounts of deoxy-ATP. Furthermore, studies of the kinetics of formation and disappearance of deoxyATP in cells incubated with deoxyadenosine have shown\textsuperscript{16} a close correlation between inhibition of DNA synthesis and concentration of deoxyATP.

DNA synthesis in intact cells appears to be inhibited when the deoxyATP concentration increases over a certain level. Formation of deoxyATP in cells incubated with deoxyadenosine may, therefore, interfere with a very efficient mechanism for regulation of the rate of DNA synthesis.

Reichard and his co-workers\textsuperscript{17, 18} have recently found that extracts of chick embryo catalyze the reduction of CMP to deoxyCMP and of GMP to deoxyGMP. Both of these reactions appeared\textsuperscript{19} to be inhibited by extremely low concentrations of deoxyGTP and of deoxyATP. In the system studied here, deoxyATP or a closely related compound formed in the cells incubated with deoxyadenosine may in a similar way inhibit reduction of GMP to deoxyGMP. The antagonistic effect of deoxyguanosine may be due to the formation of deoxyGMP by direct phosphorylation. In this way the reduction of GMP to deoxyGMP would be bypassed and the level of deoxyguanosine phosphates may be restored to allow normal rate of DNA synthesis.

Summary.—The inhibition of DNA synthesis in the hyperdiploid strain of the Ehrlich ascites tumor cells, by deoxyadenosine, in vitro, persists also when deoxyadenosine is removed from the cell suspension by deamination. After the deamination of deoxyadenosine most of the compound can be accounted for as deoxyinosine and hypoxanthine which are both without effect on the DNA synthesis. It has been postulated, therefore, that a small part of the deoxyadenosine added to cell suspensions is converted to an inhibitor of DNA synthesis. The effect of the postulated inhibitor which appears not to pass through the cell membrane may be reversed by added deoxyguanosine. The findings are discussed in relation to recent observations of accumulation of deoxyadenosine triphosphate in cells incubated with deoxyadenosine and to the recently reported formation of deoxyribonucleoside phosphates in cell-free systems.

Valuable technical assistance was given by Miss Hanne Christensen.

\* This investigation was supported by a grant from the Danish State Research Foundation and by "Hestehandler af R‐sne, M. Jensens mindelegat."
\dagger Address: Frederik d. V's Vej 11, Copenhagen Ø, Denmark.
\textsuperscript{1} The abbreviations used are: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; deoxyATP, deoxyadenosine triphosphate; CMP, cytidine monophosphate; deoxyCMP, deoxycytidine monophosphate; GMP, guanosine monophosphate; deoxyGMP, deoxyguanosine monophosphate; and deoxyGTP, deoxyguanosine triphosphate.
RELATION BETWEEN RNA, DNA, AND PROTEIN SYNTHESSES IN THE REPLICATING NUCLEUS OF EUPLOTES

BY D. M. PRESCOTT AND R. F. KIMBALL

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Communicated by Alexander Hollaender, March 9, 1961

As a repository of genetic specificity DNA must perform two functions, the dissemination of information to the surrounding metabolic system and its own self-replication during growth. At the macromolecular level these two functions possibly proceed by mechanisms that are independent and separate from one another, and as a consequence, they may occur with mutual exclusiveness. Possibly only double-helix DNA can transmit information to the cell and only single-strand DNA is capable of self-replication. 1, 2

The proposition that information dissemination is carried out through DNA-directed synthesis of RNA is strongly supported by experiment (for review, see Prescott3). The most convincing evidence that DNA replication does not involve RNA or RNA synthesis is given by the in vitro deoxyribonucleotide polymerase system. 4 Some evidence has been presented, however, that can be interpreted to mean that there may be an intermediate carrier of genetic information between old DNA and the synthesis of new DNA. 5, 6

The macronucleus of the ciliate protozoan Euplotes has some unique features in the organization of its replication that make it possible to examine visually by autoradiography relations between RNA, DNA, and protein syntheses during nuclear replication. The macronucleus of this cell has the shape of a long narrow ribbon or rod (approximately 140 µ by 7 µ) through which two short-banded structures, the reorganization bands, pass during the last several hours of interphase. Ordinarily, the two bands originate, one at each end of the nucleus, travel through