Mechanism of caffeine-induced inhibition of DNA synthesis in *Escherichia coli*

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Caffeine inhibited DNA synthesis in toluene-treated *Escherichia coli* K12 strains to the same extent as in intact cells using the incorporation of [^{3}H]thymidine as a measure of DNA synthesis. The inhibition was found to be competitive with ATP, and it was not influenced by the concentrations of deoxynucleoside triphosphates to any extent. When caffeine was added together with other DNA synthesis inhibitors such as novobiocin, nalidixic acid or actinomycin D, the inhibition in all cases was non-additive. It is suggested that caffeine inhibits one of the ATP-requiring enzymes in the DNA replication machinery, possibly DNA polymerase III or one of the DNA helicases.

Caffeine *DNA synthesis* *Toluene-treated* E. coli *DNA enzymes*

1. INTRODUCTION

Methylated xanthine derivatives such as caffeine, theobromine and theophylline are widely distributed in nature and are used for many purposes both in nutrition and in biological research. The mode of action, particularly of caffeine has been extensively investigated, but still many aspects of its mechanism of action remain unclear [1]. With regard to interaction with the genetic material DNA, it has been shown that caffeine will bind to DNA, particularly single-stranded DNA [2]. Caffeine has also been shown to affect DNA repair [3,4], to increase mutation caused by other agents [5] and to interfere with purine requiring enzymes [6–8].

Work in this laboratory has shown that caffeine inhibits incorporation of thymidine into DNA in *Escherichia coli* and, moreover, caffeine also induces filamentous growth of the same strains [9]. Caffeine was also shown to inhibit the enzyme thymidine kinase [8]. However, studies of nucleotide pool sizes revealed that this inhibition was not the cause of the inhibition of DNA synthesis [10]. In order to gain more information about the mechanism of the caffeine effect on DNA synthesis, we have employed *E. coli* cells permeabilized by toluene-treatment and studied DNA synthesis in the presence and absence of caffeine in these cells. Permeable cells have the advantage that one can employ defined substrates and control their concentrations in the assay mixture and thereby change the conditions for the replication reaction [11]. Such manipulations may afford some clue as to the mechanism of action of caffeine. The results obtained clearly show that caffeine interferes with an ATP-requiring step in the replication process.

2. MATERIALS AND METHODS

2.1 *Chemicals*

Spermidine, actinomycin D, NAD, nalidixic acid, novobiocin and unlabelled nucleosides and nucleotides were from Sigma. Caffeine was a product of Koch Light Labs. [^{3}H]Thymidine and [^{3}H]dTTP were obtained from the Radiochemical Centre (Amersham).

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Abbreviation: dNMP, any of the 4 deoxynucleoside monophosphates

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2.2. *Bacterial strains and growth conditions*

E. coli K12, KMBL 1787 (F^-, thyA 301, argA 103, bio-87, pheA 97, endA 101, polA 1), *E. coli* K12, KMBL 1788 (F^-, thyA 301, argA 103, bio-87, pheA 97, endA 101) were originally obtained from Dr H.L. Heijneker and Dr B.W. Glickman, Laboratory of Molecular Genetics, Leiden State University.

The bacterial nucleoid was isolated from *E. coli* K12 KMBL 1788 grown in the minimal medium of Clark and Maaløe [12] supplemented with 20 μg/ml thymine, 20 μg/ml arginine, 20 μg/ml phenylalanine and 0.2 μg/ml biotin.

Before toluene treatment the bacteria were grown with aeration at 30°C in Tryptone medium, supplemented with 20 μg/ml thymine and 0.2 μg/ml biotin. Growth was followed at 450 nm or 650 nm in a Zeiss PMQ II spectrophotometer.

2.3. *Toluene treatment and assay of DNA synthesis*

A sample of 20 ml of exponentially growing bacteria at A_{650} of 0.5 was harvested by centrifugation, resuspended in toluenization buffer and treated with toluene as in [13]. The toluene-treated cells were used immediately after this treatment. The DNA synthesis reaction was started by adding a portion of 25 μl of the toluene-treated cells to the reaction mixture consisting of 50 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 130 mM KCl, 1.4 mM ATP, 1.4 mM 2-mercaptoethanol and 33 μM each of dCTP, dATP, dGTP and dTTP. The specific activity of dTTP was 0.4 Ci/mmol, and the reaction volume was 150 μl. The reaction was stopped by the addition of 2 ml 5% trichloroacetic acid and 0.02 M sodium pyrophosphate. The acid insoluble fractions were collected on glass-fibre filters, washed with trichloroacetic acid and ethanol, dried and counted in a toluene-based scintillation liquid.

2.4. *Preparation of envelope-free nucleoid*

A sample of 5 ml of an exponentially growing culture at A_{450} of 0.4 was mixed with 3 ml of a solution containing 0.1 M NaN_3 and 0.85 M NaCl. The cells were harvested by centrifugation and the envelope-free nucleoid isolated as in [14].

3. RESULTS

3.1. *Effect of caffeine on the rate of incorporation of dNMP*

The influence of increasing concentrations of caffeine on the incorporation of dNMP into DNA in *E. coli* K12 KMBL 1788 (*pol*A^+) was investigated and the results are shown in fig.1. Small concentrations, up to 3 mM, caused an increase in the incorporation followed by a linear decrease with higher concentrations. Thus in the presence of 10 mM caffeine a 40% decrease in incorporation was noted. The incorporation in these experiments was linear for at least 60 min (not shown). Similar studies were also carried out with a toluene-treated *pol*A^- strain, namely *E. coli* K12 KMBL 1787. Caffeine produced the same inhibition in this strain (not shown), suggesting that the synthesis and inhibition observed is not related to DNA repair synthesis carried out by DNA polymerase I. The DNA synthesis in toluene-treated *E. coli* cells has been shown to be semiconservative and to require ATP [11]. This was also shown to be the case using the present strain of *E. coli* K12 (not shown). No DNA synthesis was detected in the absence of ATP. GTP could not substitute ATP. Addition of NAD, ribonucleoside triphosphates or spermidine had little effect on the incorporation of dNMP or on the inhibition caused by 10 mM caffeine.

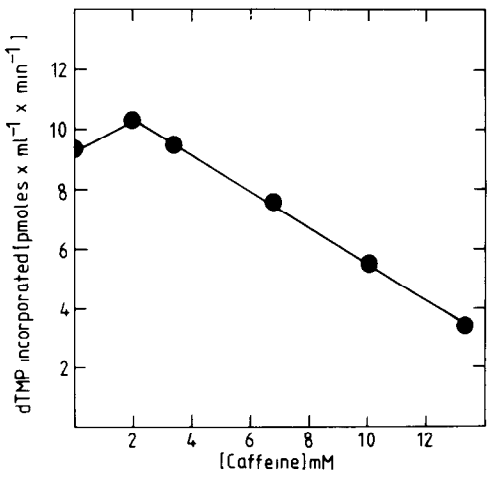

Fig.1. Rate of DNA synthesis measured in the presence of various concentrations of caffeine.

3.2. *Influence of dNTP, ATP and dATP concentrations on the caffeine induced inhibition*

Caffeine, being a purine analogue, might compete with dNTP or ATP for binding to the respective proteins in the DNA replication complex. We therefore investigated in more detail the effect of different concentrations of nucleoside triphosphates in the reaction mixture. Varying the dNTP concentration had no effect on the caffeine-induced inhibition, fig.2. In the presence of increasing concentrations of ATP, however, the inhibition decreased. When the data were plotted in a Lineweaver-Burk manner, $1/v$ *vs* $1/s$, the two lines obtained intersected at the $1/v$-axis indicating that caffeine is a competitive inhibitor with ATP, fig.3. The apparent K_i was 5.7 mM.

Similar experiments were also carried out by varying the concentration of dATP. In this case the two lines intersected at the $1/s$-axis, fig.4, suggesting that caffeine is a non-competitive inhibitor with dATP.

3.3. *Experiments with other DNA synthesis inhibitors*

The results observed indicated that caffeine might interfere with an ATP requiring step in the DNA replication process. Several enzymes involved in DNA synthesis are known which require ATP, namely DNA gyrase [15], DNA polymerase III [16] and the DNA helicases [18,19]. In the case of DNA gyrase two specific types of inhibitors are known, represented by novobiocin and nalidixic acid. Novobiocin, and the structural analogue coumermycin, interacts with the ATP site on the B

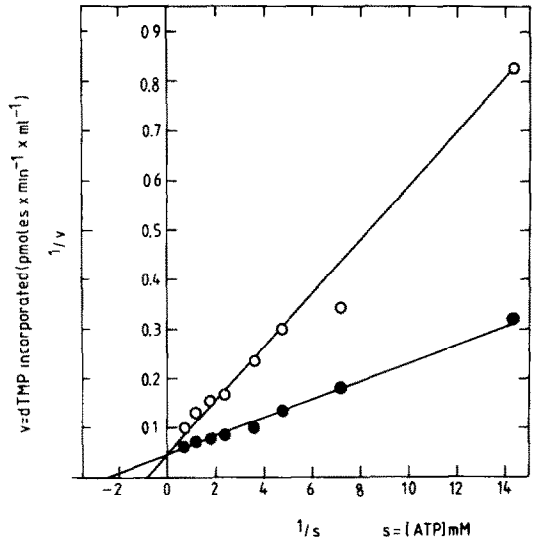

Fig.3. Effect of ATP concentration on the rate of DNA synthesis in the presence (○—○), and absence (●—●) of 10 mM caffeine in toluene-treated *E. coli* K12 KMBL 1788.

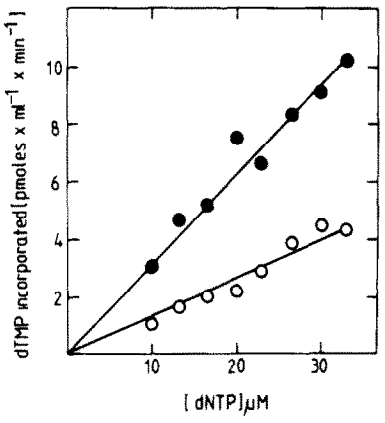

Fig.2. Rate of DNA synthesis measured at different concentrations of dNTP in the presence (○—○), and absence (●—●) of 10 mM caffeine in toluene-treated *E. coli* K12 KMBL 1788.

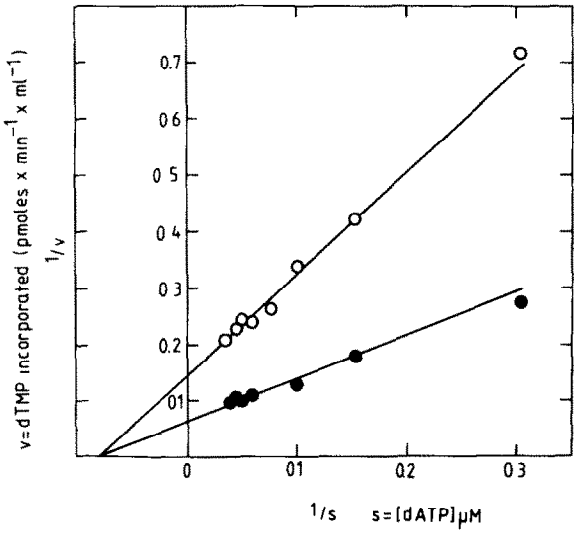

Fig.4. Effect of dATP concentration on the rate of DNA synthesis in the presence (○—○), and absence (●—●) of 10 mM caffeine.

subunit of the enzyme which is the product of the *cou* gene, thereby preventing ATP from binding to the DNA-gyrase complex. Nalidixic acid binds to the A subunit, the product of the *nal* gene, and appears to trap the covalent gyrase-DNA intermediate [20]. Experiments were carried out with both inhibitors in order to elucidate in more detail the mechanism of action of caffeine, and the results for novobiocin are given in fig.5. At a concentration of novobiocin of 3 μg/ml a 40% inhibition of incorporation of [^{3}H]dTMP into DNA in toluene-treated *E. coli* was observed. Increasing concentrations of caffeine in addition to novobiocin in the reaction mixture caused a further slow decrease in incorporation, but the effects of the two inhibitors were clearly not additive. Thus, at a concentration of caffeine of 13 mM the amount of [^{3}H]dTMP incorporation was the same, whether novobiocin was present or not. Similar results were also obtained with the other DNA gyrase inhibitor nalidixic acid (not shown).

The effect of caffeine on actinomycin D inhibition was also investigated. Actinomycin D causes inhibition of DNA synthesis by intercalation into the DNA, thereby preventing chain growth of RNA and DNA [20]. The inhibitory effects of actinomycin D and caffeine were clearly not additive, as shown in fig.5. The results were similar to those obtained for novobiocin.

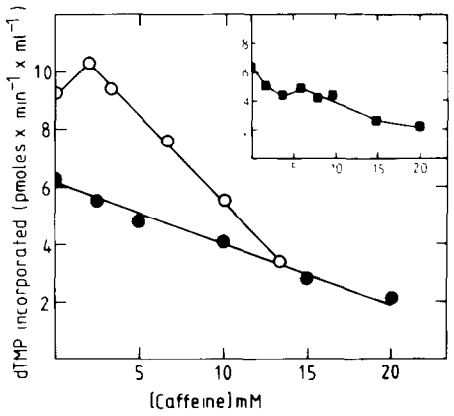

Fig.5. Rate of DNA synthesis measured in the presence of various concentrations of caffeine (○—○) in addition to 3 μg/ml novobiocin (●—●) or 10 μg/ml actinomycin D (■—■).

4. DISCUSSION

The present work deals with the effect of caffeine on DNA synthesis in toluene-treated strains of *E. coli* K12. Caffeine in small concentrations caused stimulation of the incorporation of dNMP into DNA. At large concentrations, however, caffeine inhibited the DNA synthesis reaction, and the magnitude of this inhibition was similar to that observed by measuring thymidine incorporation in untreated cells [9]. The fact that the inhibition by caffeine was competitive with ATP strongly suggests that caffeine interferes with one or several of the energy-requiring steps in the replication process. Moreover, since the inhibition could not be overcome by increasing the dATP concentration or that of the other deoxynucleoside triphosphates, it seems likely that caffeine itself does not directly inhibit the binding of the deoxynucleoside triphosphates to the polymerase. Several studies have clearly shown that in toluene-treated *E. coli* semiconservative DNA synthesis proceeds along preexisting replication forks [13,22]. Thus, caffeine inhibits the elongation process in the growing forks and not the initiation of replication at the chromosomal origin. This study also confirmed that the synthesis observed was semiconservative and not DNA repair synthesis catalyzed by DNA polymerase I. Movement of the replication fork along the chromosome can in general be divided into 3 main events:

(a) Unwinding of the DNA; (b) chain growth; (c) introduction of supercoils in the replicated DNA.

All three processes involve the action of several proteins, and they also require energy in the form of ATP. The respective ATP-dependent enzymes are the DNA helicases [18,19], DNA polymerase III [16,17] and DNA gyrase [15]. Caffeine may affect any of these enzymes. However, the fact that the inhibition caused by 13 mM caffeine was the same whether novobiocin, nalidixic acid or actinomycin D were present or not, may suggest that caffeine acts at a site preceding the gyrase and chain elongation step. With regard to the effect of caffeine on supercoiling, studies carried out in this laboratory support the view that caffeine does not affect DNA gyrase. If caffeine inhibited DNA gyrase, one would expect the nucleoid to be partial-

ly relaxed and have a smaller sedimentation coefficient than that found in untreated cells as is seen when *E. coli* cells are treated with coumermycin [23]. It should also be possible to observe such a relaxation with a phase contrast microscope. The sedimentation coefficient of the nucleoid, however, remained essentially unchanged after the cells had grown for up to 2 h in the presence of 10 mM caffeine. In this short time no bacterial filaments were formed. Furthermore, the size and shape of the nucleoid as viewed by phase contrast microscopy were not altered significantly by the presence of caffeine (not shown).

A possible target for the caffeine effect is DNA polymerase III. The holoenzyme of DNA polymerase III consists of several subunits, and ATP is required for binding of the holoenzyme to the 3′-terminus of the template. When the holoenzyme moves along the template and attaches deoxynucleoside triphosphate residues to the 3′-end, ATP is hydrolyzed to ADP and P_i [16]. Caffeine might interfere with the latter step. However, a detailed investigation will be required to elucidate the possible mechanism of action of caffeine on this multisubunit enzyme [17]. In the case of DNA polymerase I earlier studies have shown that caffeine inhibited both the 5′ → 3′ and 3′ → 5′ nuclease activities, but had no effect on the polymerase activity [24].

Concerning the unwinding enzymes at least 3 ATP-dependent DNA helicases have been isolated from *E. coli*, and these are DNA helicase I [25], DNA helicase II [18] and Rep protein [19]. The function of DNA helicase I in DNA replication is not known. In the case of DNA helicase II this enzyme catalyzes the unwinding of DNA molecules which initiate DNA replication as closed circles [18]. The Rep gene product also participates in the unwinding of DNA at the replication forks, since the rate of the fork movement is reduced in Rep mutants compared to the wild type [26]. It is possible that DNA helicase II and Rep protein operate in different directions in the growing forks [18]. Since caffeine is known to bind preferentially to single-stranded DNA [2], small concentrations of caffeine might help the unwinding process by stabilizing the single-stranded regions of the growing forks and thereby increase fork movement. A small increase in DNA synthesis was in fact observed at low concentrations of caffeine. However, the inhibition observed at high concentrations is more likely due to protein–caffeine than to DNA–caffeine interaction, since the caffeine-induced inhibition was competitive with ATP. More information about the structure of the helicases and their substrate requirements and role in DNA replication are needed in order to make a more definite assessment of the caffeine effect.

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