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NEW PURINE ANALOGUES AS INHIBITORS OF PURINE METABOLISM

Thesis presented in fulfilment of the
requirements for the degree of
Doctor of Philosophy at the
University of the Witwatersrand

by

DHIRAJLAL BHAGWAN KASAN RAMA

Johannesburg
August, 1982

I, the undersigned herewith declare that this thesis is entirely my own work and has not been presented for any degree at another University.

DHIRAJLAL BHAGWAN KASAN RAMA

Johannesburg
August, 1982

To my parents, without whose support and encouragement this study would never have been undertaken.

PUBLICATIONS AND PRESENTIONS

The following were subjects of materials presented from this thesis:

Inhibition of hypoxanthine guanine phosphoribosyl transferase in hepatoma cell cultures S.A.Cancer Bulletin (1980) 24, 68 - 69.

Inhibition of hypoxanthine guanine phosphoribosyl transferase from human erythrocytes: Fourth congress of the S.A. Biochemical Society (1980).

Influence of phleomycin amplifiers on the excision repair mechanism of UV-irradiated E.coli : Fifth congress of the S.A. Biochemical Society (1981).

ABSTRACT

Phleomycin, a copper containing protein, has a broad range of antibiotic (3,4,6), antitumour (7,9), and anti-viral (5) activity. At low concentrations it induces DNA breakdown and death in growing cultures of E.coli B, but has little effect on stationary phase bacteria (10). However, its action can be strongly potentiated on stationary phase cells by the addition of certain compounds. This phenomenon is termed "amplification". The mechanism of amplification of phleomycin is not well understood. In this study, the effects of various purine and pyrimidine analogues reported as amplifiers of phleomycin (2) were investigated on several aspects of nucleotide metabolism.

Fistly, the effect of these amplifiers on the purine salvage enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT.EC.2.4.2.8) was investigated. No specific interaction of these amplifiers with the enzyme could be demonstrated and only three of the amplifiers were found to inhibit HPRT activity. Furthermore, all three amplifiers were found to inhibit HPRT activity in vivo in hepatoma cell cultures. Further analogues of one of the inhibitors, the benzoxazole derivative, were synthesised in an attempt to establish a structure function relationship. None of the analogues synthesised proved to be inhibitors of HPRT.

Secondly, the effect of the amplifiers on the excision repair mechanism of UV-irradiated E.coli was studied. All the amplifiers tested were found to inhibit the excision repair mechanism. This was determined by measuring the liquid holding recovery of UV-irradiated E.coli. However, there was no ordered pattern between phleomycin amplification activity and the ability to inhibit the excision repair mechanism.

Thirdly, direct interaction of the amplifiers with DNA was monitored by measuring:

i) The change in helix-coil transition of DNA in the presence of the amplifiers, as determined by the change in absorbance at 260 nm.
ii) The change in the optical activity of DNA as measured by circular dichroism.
iii) Viscosity measurements and,
iv) Electron microscopy.

Only circular dichroism measurements revealed direct interaction.

From the results reported in this thesis, it may be concluded that:

i) The mechanism of phleomycin amplification is not a result of the analogues being phosphoribosylated to their nucleotide forms and subsequently influencing the nucleotide pool, since most amplifiers tested do not affect HPRT activity, and
ii) The analogues may interact directly with DNA and alter the conformation of DNA so as to make it sensitive to the endonuclease suggested by Grigg (26).

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ABBREVIATIONS AND SYMBOLS.

APRT	:	adenine phosphoribosyl transferase
A - T	:	adenine - thymine base pair
ATP	:	adenosine triphosphate
BAC	:	benzyldimethylalkylammonium chloride
CD	:	circular dichroism
Ci	:	currie
cpm	:	counts per minute
CTP	:	cytidine triphosphate
DAB	:	4 - dimethylaminoazobenzene
DNA	:	deoxyribonucleic acid
ds	:	double stranded
E.coli	:	Escherichia coli
G - C	:	guanine - cytosine base pair
GTP	:	guanosine triphosphate
HPRT	:	hypoxanthine guanine phosphoribosyl transferase
LHR	:	liquid holding recovery
min	:	minutes
ml	:	millilitre
PEI	:	polyethyleneimine impregnated
poly dAT	:	poly - deoxyadenylic acid.poly-deoxythymidylic acid pair
POPOP	:	1,4-bis-2-(4-Methyl- -Phenyloxazylyl)-benzene
PPO	:	2,5-diphenyloxazole
PRPP	:	5-phosphoribosyl-1-pyrophosphate
Tm	:	helix-coil transition temperature
Tris	:	tris (hydroxymethyl) aminomethane
UTP	:	uridine triphosphate
UV	:	ultra-violet

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CHAPTER ONE

INTRODUCTION

Phleomycin, a copper containing protein isolated from Streptomyces verticillis (1,2) has a broad range of antibiotic (3-6), antitumour (7-9) and anti-viral (5) activity. The structure of phleomycin D_1 is shown in figure 1 (115). At low concentrations it induces DNA breakdown and death in growing cultures of E.coli B but has little effect on stationary phase bacteria (10). At higher concentrations this difference disappears.

Phleomycin influences the thermal melting of DNA and poly dAT. Phleomycin binds at the adenine-thymine rich regions in DNA, probably to the thymine bases (11,12), and distorts its secondary structure, inducing localized denaturation (13). These changes are thought to sensitize the DNA in vivo to enzymic attack, resulting in DNA breakdown and death (14).

In vitro, the DNA-dependent synthesis of DNA by DNA polymerase I from E.coli is inhibited by phleomycin, while the DNA dependent RNA polymerase is inhibited to a much smaller extent. Furthermore, the degradation of DNA by exonuclease 1 is affected by phleomycin, whereas the DNA degradation by endonuclease 1 is not. The inhibitory effect of phleomycin on DNA-dependent synthesis was found to be dependent on the concentration of the primer and on its content of adenine-thymine, and independent on the concentration of the enzyme (15).

At low concentrations ($\leq 2\mu g/ml$), phleomycin has no effect on stationary phase cells (10), but its action can be strongly potentiated by the addition of certain compounds (16,17,18,19,20). This phenomenon is termed "amplification" (21), and the compounds which effect phleomycin activity are known as amplifiers of phleomycin.

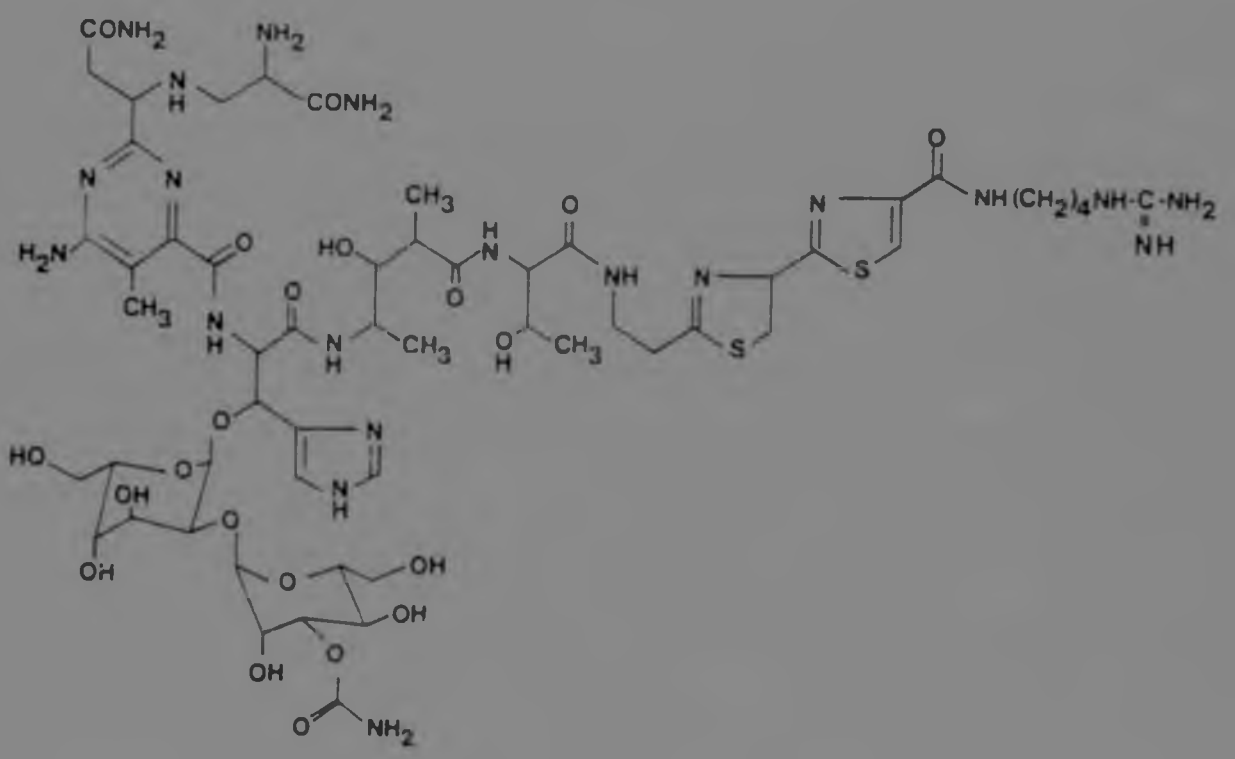


Fig.1 Structure of pnleomycin D_1

Although phleomycin has antibacterial and antitumour activity, the difference between its effective dose and toxic dose is very small (22,23). Hence, amplifiers could be used to enhance the effectiveness of phleomycin at lower dose levels (16,17,18,24).

Many purine and pyrimidine analogues have been synthesised by D.J.Brown and co-workers (25) and tested for phleomycin amplification activity. Many of these analogues have been found to possess high amplification activity *in vitro* and low toxicity with good aqueous solubility and high metabolic stability.

The mechanism of amplification of phleomycin by these analogues is not well understood. One model postulated to illustrate amplification by caffeine is decribed below.

Grigg *et al.* (26) have suggested that a single strand specific endonuclease attacks the phleomycin cleaved DNA where a number of adjacent bulky phleomycin-thymine complexes would cause local denaturation. DNA breakdown due to a combination of caffeine and phleomycin could also be produced by this endonuclease. A localized weakening of hydrogen bonds between complementary strands of the DNA by the presence of a thymine-phleomycin complex would allow binding of caffeine, which binds selectively to single-strand DNA. This would produce an effect similar to that of forming multiple thymine-phleomycin complexes on a number of neighbouring sites on the DNA (Fig.2).

The effect of these purine analogues on several aspects of nucleotide metabolism was investigated in an attempt to elucidate their mode of action.

An important pathway in this respect is the one catalysed by the purine salvage enzyme, hypoxanthine guanine

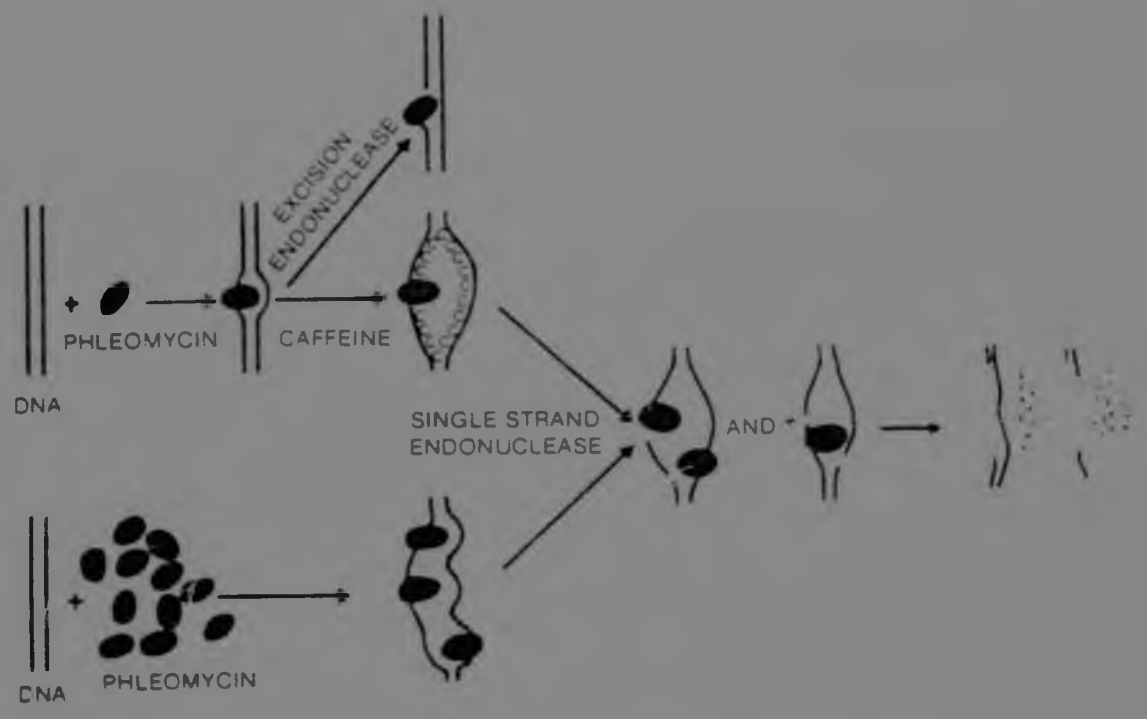


Fig. 2 An illustration of the hypothesis to explain the ability of caffeine to amplify the action of phleomycin in initiating DNA strand breakage and subsequent DNA breakdown and cell death. Phleomycin molecules bind to DNA and by so doing distort its secondary structure. This distortion may be recognized by an excision endonuclease which breaks a diester bond close by. Grigg *et al* (26) suggest that, if caffeine is present, it binds to the DNA where normal hydrogen bonding has been weakened by the distortion and thereby causes local cooperative denaturation. The denatured region is exposed to attack by single-strand specific endonuclease(s) which may introduce a break in one strand or in both. The latter would be expected to constitute a lethal event. High concentrations of phleomycin produce the same effect by binding to a number of thymine bases over a small restricted region of the DNA thereby disturbing its normal double-stranded structure and rendering it sensitive to single-strand endonuclease(s). These in turn produce breaks in one or both strands at the locally denatured spots as before, followed by DNA breakdown and by cell death.

From Grigg et al (26)

phosphoribosyl transferase (HPRT,EC.2.4.2.8). The nucleoside analogues formed may be incorporated into DNA following the action of the ribonucleotide reductase (EC.1.17.4.1). The interaction of these analogues with DNA was also studied by measuring

(i) The change of helix-coil transition temperature;
(ii) The change of the optical activity of DNA;
(iii) Viscosity;
(iv) Electron microscopy

Some amplifiers have been reported to be inhibitors of the excision repair mechanism of UV-irradiated E.coli (19,27). Hence, the effect of these analogues on the excision repair mechanism has been investigated.

HPRT (EC.2.4.2.8) catalyses the transfer of the 5-phosphoribosyl moiety from 5-phosphoribosyl-1-pyrophosphate (α-D-ribofuranose-1-pyrophosphate 5-phosphate; PRPP) to hypoxanthine (or guanine) to form the nucleotide inosinate (or guanylate) thereby returning the purine bases to the purine nucleotide pool. Cleavage of the pyrophosphate moiety of PRPP is accompanied by the anomeric inversion of the ribofuranose ring resulting in a N-β-riboside monophosphate. The reaction is summarised in Fig.3. In mammals, activity with both guanine and hypoxanthine resides in a single enzyme (28).

Another enzyme, adenine phosphoribosyl transferase (APRT, EC.2.4.2.7) converts adenine to adenylate by a similar reaction (28).

HPRT and APRT are often referred to as purine salvage enzymes (29).

Purine salvage enzymes are of special significance in the purine metabolism of cells and organisms in which purine biosynthesis de novo is deficient or inhibited

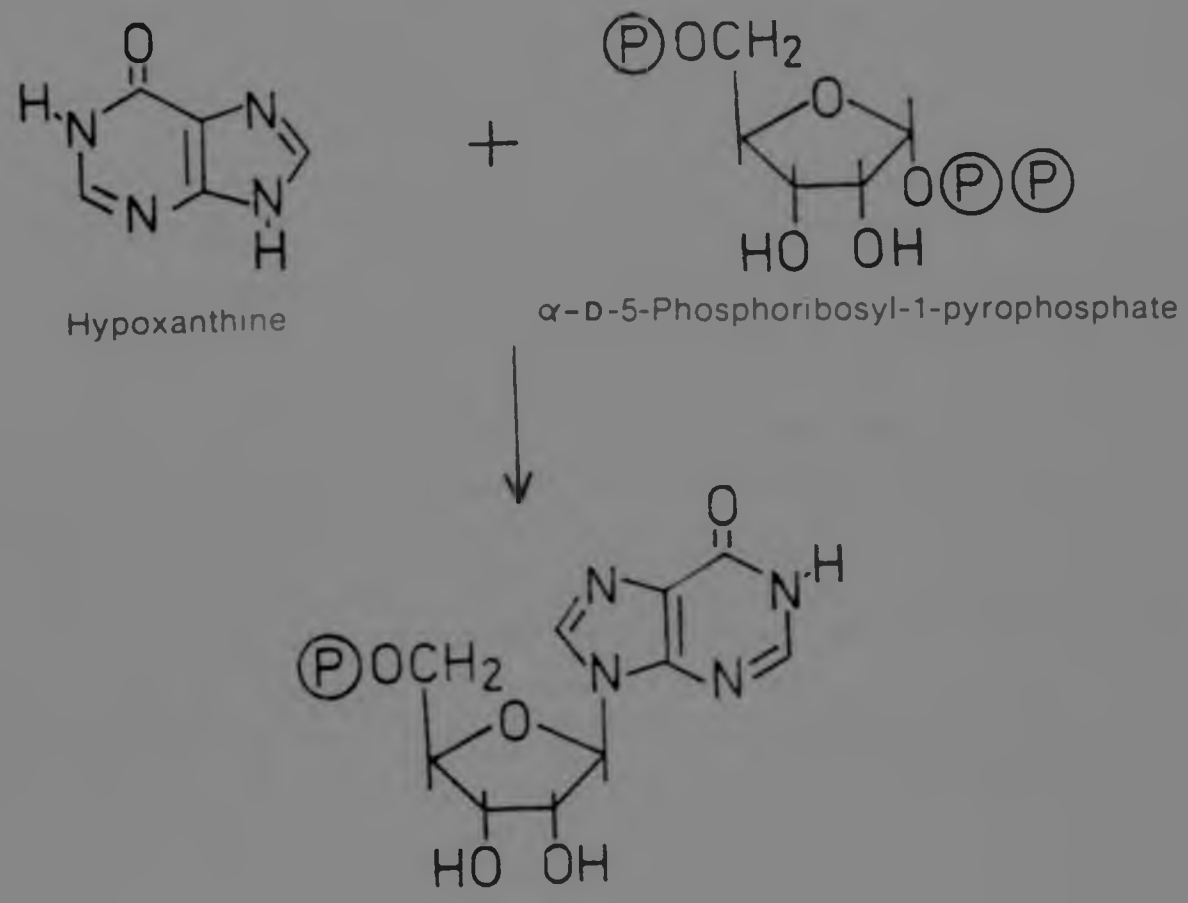


Fig. 3 Reaction catalysed by hypoxanthine-guanine phosphoribosyl transferase.

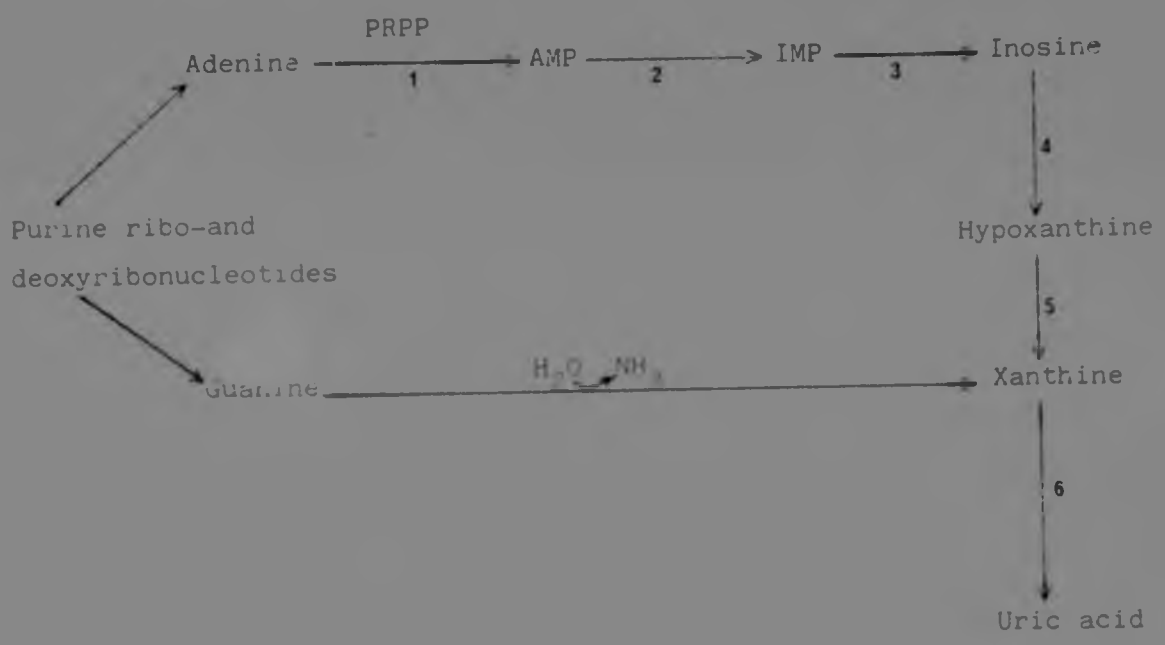
(e.g.erythrocytes and certain brain cells have a reduced capacity to synthesise purines de novo).

HPRT may play a major role in purine salvage in humans since circulating levels of adenosine and adenine are low (10^{-7}M or less), and the ubiquitous adenosine deaminase and purine nucleoside phosphorylase provide a continual supply of hypoxanthine (Fig 4).

The catabolism of purine ribo - and deoxyribonucleotides yields the free purine bases, adenine, guanine, hypoxanthine and xanthine through the concerted actions of a number of enzymes. Each of these bases can then be either returned to the nucleotide pool via the action of the phosphoribosyl transferases (HPRT/APRT) and nucleoside phosphorylase and kinase or oxidised to uric acid for excretion (30).

The conversion of the purine bases to the nucleotide form is subject to metabolic control and hence, the amount of uric acid excreted is regulated. The composition of the nucleotide pools (31) in animal tissues is determined by

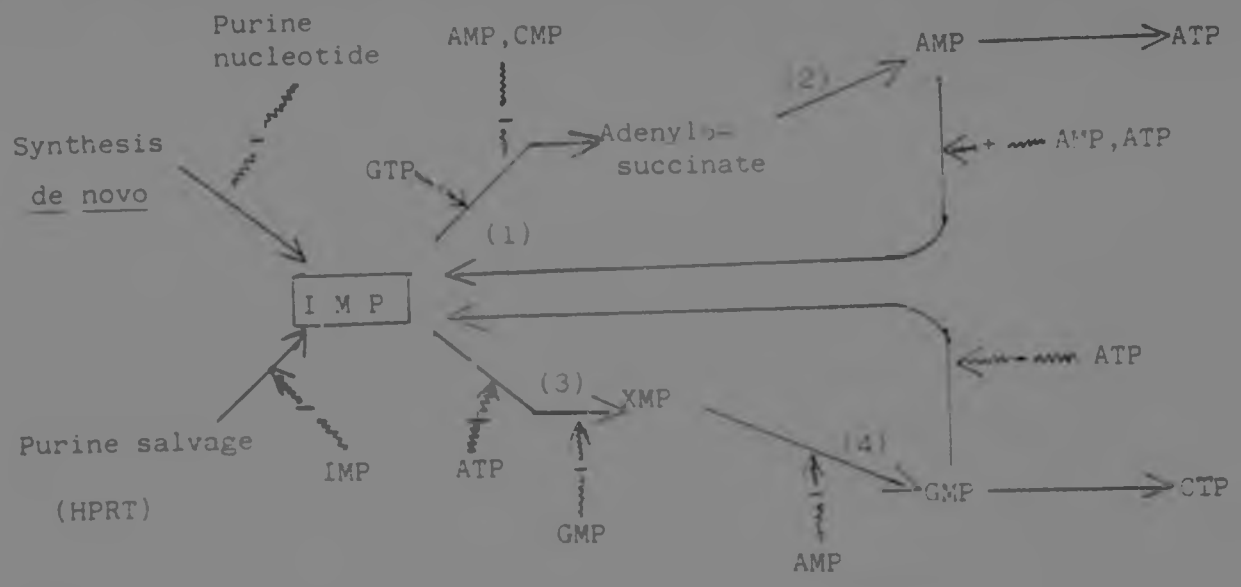
(a) Input processes - including synthesis de novo, recovery (or salvage) reactions of endogenously formed bases or nucleosides; and exogenous supply.
(b) Interconversions of the purine nucleotides.
(c) Output flow - which involves dephosphorylation, deamination and oxidation of purines and their derivatives, eventually leading to the end product of purine catabolism, uric acid.



(1) APRT (Adenine phosphoribosyl transferase) (EC.2.4.2.7)
(2) AMP deaminase (*)
(3) Phosphatase
(4) Purine nucleoside phosphorylase (*)
(5) Xanthine oxidase
(6) Guanine deaminase (*) : ubiquitous in humans

Fig 4 Enzymes involved in the production of uric acid

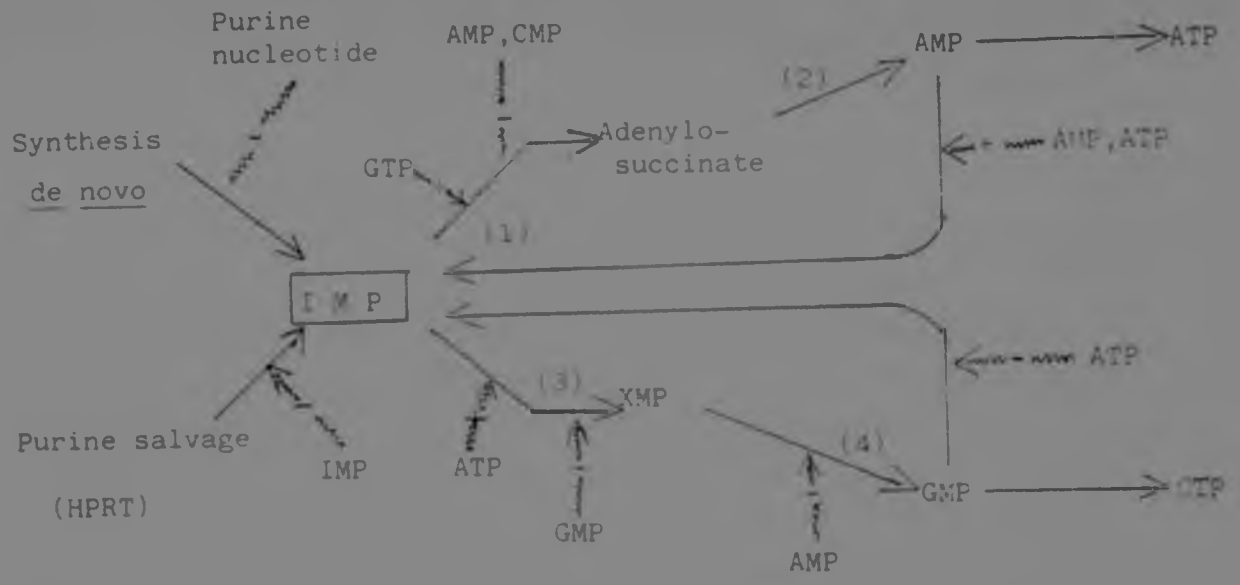
Inosinic acid is centrally located in the metabolic interrelationships of purines. Both of the primary nucleic acid purines, adenine and guanine, are derived in nucleotide form directly from IMP by amination reactions. A complex pattern of control loops regulate the relative concentrations of purine nucleotides in the metabolic pool (31,32,33). Coordination of the rates of synthesis of adenine and guanine nucleotides from IMP involves both positive and negative factors (Fig.5).



(1) Adenylosuccinate synthetase
(2) Adenylsuccinate lyase
(3) IMP dehydrogenase (inhibited by 6-mercaptopurine ribonucleotide)
(4) XMP aminase

Fig 5 Metabolic control of the purine nuclectide pool

(+) Indicates a stimulatory effect
(-) Indicates inhibition



(1) Adenylosuccinate synthetase
(2) Adenylsuccinate lyase
(3) IMP dehydrogenase (inhibited by 6-mercaptopurine ribonucleotide)
(4) XMP aminase

Fig 5 Metabolic control of the purine nucleotide pool

(+) Indicates a stimulatory effect
(-) Indicates inhibition

From the above figure (Fig.5) it is apparent that synthesis and degradation in nucleotide metabolism may not be considered as separate entities. The operation of cyclic paths linking nucleotides and their degradation products is of importance in regulating cellular concentrations of essential components (31).

Inosine can arise from adenine nucleotides by two paths as shown in Fig. 6. The level of ATP regulates both pathways (31). ATP inhibits the adenosine route by its action on 5'-nucleotidase, and stimulates the path through IMP as an effector of adenylate deaminase. The route through IMP is normally the major one.

Inosine is then catabolised to uric acid via the purine bases hypoxanthine and xanthine respectively.

The salvage of these purine bases (see (a) above) is significant and this is based on indirect or negative evidence (29). The total quantity of purine salvaged can be estimated. On a purine free diet normal adult males excrete approximately 400mg of uric acid per 24 hours in the urine (i.e.6mg/day/kg body weight) (34,35). Hyperuricemic children who lack HPRT excrete an average of 660mg of uric acid per day (47mg/day/kg body weight) (35,36). The average 24 hour uric acid excretion by control children of similar age and body weight as the hyperuricemic children was about 10mg/day /kg body weight (36). If lack of the enzyme which salvages hypoxanthine is the only biochemical lesion in these children, then the difference is an estimate of the amount of purines normally salvaged by this enzyme. Hence the overproduction of uric acid by these patients unable to utilize hypoxanthine indicates significance of the salvage of this compound.

A genetic deficiency of HPRT results in a condition known as the Lesch-Nyhan syndrome, a bizarre neurological disorder characterised by compulsive self-destructive behaviour (37,38).

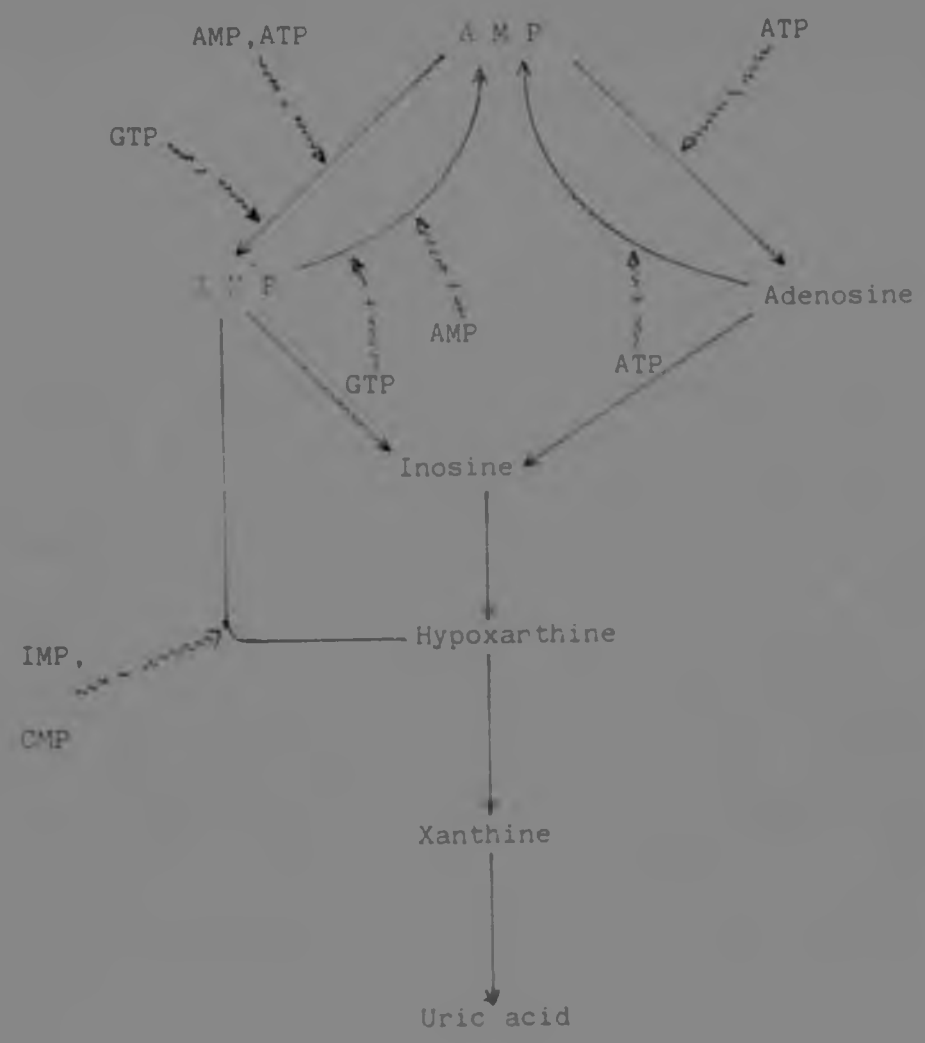


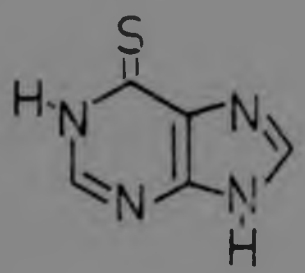
Fig 6 Cyclic reactions of the purine pool

(+) Indicates stimulatory effect

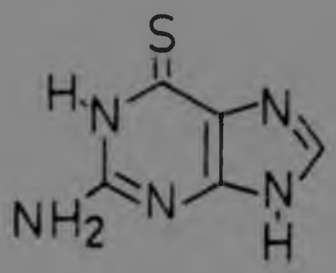
(-) Indicates inhibition

Thus, this salvage pathway evidently serves a critical role which is not yet clearly understood.

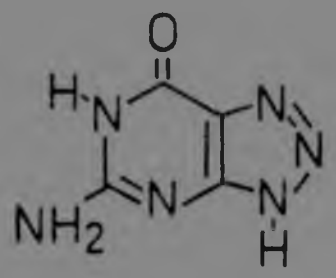
HPRT is of pharmacological importance by virtue of its ability to "activate" certain drugs used in cancer chemotherapy (i.e. for the cytotoxic effects of purine analogues. For example, 6-mercaptopurine (6-thiohypoxanthine), 6-thioguanine (the sulphur analogue of the normal substrate, 8-azaguanine and 8-azahypoxanthine are competitive inhibitors of this enzyme, and are phosphoribosylated to the corresponding nucleotice analogues - these being the biologically active forms of the drugs (37 39,40). The structures of these analogues are shown in Fig.7.



6-Mercaptopurine



6-Thioguanine



8-Azaguanine

Fig. 7 Structures of some of the known inhibitors of HPRT.

If the purine and pyrimidine analogues found to be inhibitors of HPRT are phosphoribosylated to the nucleoside forms, they would presumably be incorporated into the ribonucleotide pool. These could then be converted to the deoxyribonucleotide forms by ribonucleotide reductase, and become available for DNA synthesis.

Ribonucleotides are converted to deoxyribonucleotides without cleavage of the glycosidic bonds in reactions catalysed by ribonucleotide reductase (nucleoside diphosphate reductase ; EC.1.17.4.1) (13).

Two related characteristics (41) of the ribonucleotide reductases of various species are of particular importance :

(i) The enzyme activity is regulated by ATP and 2'-deoxyribonucleoside 5'-triphosphate levels; and

(ii) In at least some mammalian tissues it appears likely that the enzyme catalyses the rate-limiting step in the synthesis of deoxyribonucleotides for DNA synthesis.

Reichard and co-workers (42) have found that under several conditions deoxyribonucleoside triphosphate pools in E.coli change in the manner predicted from the pattern of inhibition and activation effects found for the pure enzymes. These effects seem to provide physiological control of deoxyribonucleotide synthesis. It is not yet clear as to how the known regulation of the reductase activity would provide physiological control. Furthermore, these deoxyribonucleotide analogues may regulate ribonucleotide reductase activity.

The effect of these analogues on UV-irradiated E.coli has also been examined.

The colony forming ability of bacterial cells is reduced after irradiation with ultra-violet light. However, irradiated cells of E.coli B show increased survival if [illegible] for several hours in a non-nutrient liquid prior [illegible]plating (43,47). This effect, termed "liquid-holding recovery" overlaps completely with photoprotection. Jagger et al. (44) suggested that liquid holding increases UV survival by delaying cellular growth, thus allowing more time for repair process to occur. The bhleomycin amplifier, caffeine, has been reported to inhibit liquid-holding recovery if present during the liquid holding treatment (27).

Pyrimidine dimers are the principal photoproducts formed in DNA of bacterial cells after UV-irradiation (45,46). Subsequent removal of the dimers is crucial to the survival of the irradiated cells. The discovery that the cells can recover from radiation induced damage has not only provided a major stimulus to the general field of radiation biology, but has also made a major impact on the field of mutagenesis (47), carcinogenesis (48) and ageing (49).

Three modes of DNA repair (50) have been documented:

(i) Photoreactivation: The damaged part of the molecule is restored to its functional state in situ. This may result from the spontaneous 'decay' of the damage to an innocuous form, for example dehydration of pyrimidine photohydrates or the recombination of radicals to yield a restored molecule; or it may be accomplished by some enzymatic mechanism (photoreactivating enzyme). In the dark, the enzyme combines with cyclobutyl pyrimidine dimers in UV-irradiated DNA to form an enzyme-substrate complex. The absorption of light between 320-410 nm activates the complex to convert the cyclobutyl pyrimidine

dimers to monomeric pyrimidines. This is followed by the release of the enzyme.

(ii) Excision repair: Excision repair of pyrimidine dimers involves principally four enzymatic steps:

(a) UV-specific endonuclease recognises the dimers and makes an incision break at the 5' ends of the dimers;
(b) DNA polymerase I resynthesizes the DNA through the opposite intact template;
(c) The damaged section of the DNA is excised, and,
(d) Ligase seals off the resynthesized DNA.

(iii) Postreplication repair: The damage itself is not repaired, but rather it is bypassed during replication, leaving gaps in the daughter strand DNA. The missing genetic information is subsequently supplied by redundant information within the cell.

Liquid holding recovery was first described for bacteria, using E.coli B (51), but is also observed in rec A strains of E.coli K-12 (52). Mutations at uvrA, uvrB or uvrC block liquid holding recovery (53), hence indicating that the molecular basis of liquid holding recovery is excision repair. This suggested another means by which the analogues may function and hence the analogues were tested as inhibitors of excision repair by measuring liquid holding recovery.

Furthermore, it has been shown that fully-denatured DNA interacts with caffeine whereas native DNA does not (54). Since UV induced photoproducts are thought to cause partial

denaturation of the DNA (55), Domon (56) showed that UV-irradiation of DNA stimulates caffeine binding. Thus, one model for the action of caffeine on UV-irradiated cells is as follows: (i) caffeine binds to the locally-denatured regions in the DNA which are induced by UV-irradiation (56) and (ii) during subsequent DNA-synthesis, abnormalities are induced in the DNA which lead to cell death (57).

Many workers (54,56,57) have reported that caffeine, amongst many other purine analogues, interacts more strongly with the single-stranded, random coil form of DNA than with the double helical form.

Hence, various physical methods have been employed to study the interaction of DNA with the phleomycin amplifiers in an attempt to elucidate their mode of action:

(i) Optical density - thermal denaturation and reassociation of DNA in the presence of amplifiers was measured by monitoring the change in absorbance at 260nm.

(ii) Circular dichroism - circular dichroism measurements were carried out to detect any changes in optical activity of DNA treated with the analogues.

(iii) Electron microscopy - DNA treated with phleomycin or a mixture of phleomycin and amplifier were visualized directly to detect any changes in the contour of DNA. and

(iv) Viscosity - the viscosity of treated and untreated DNA was measured by means of an Ostwald viscometer.

CHAPTER TWO

INHIBITION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE FROM HUMAN ERYTHROCYTES

2.1 INTRODUCTION

Kinetic studies of HPRT from human erythrocytes have been undertaken. In most of the work described previously, the enzyme was assayed by incubating the [^{14}C]-labelled purine base with PRPP, Mg^{++} ions, buffer and the enzyme preparation (58). Separation of the product was achieved by paper chromatography (60), thin layer chromatography, high-voltage electrophoresis (38, 64), column chromatography (58,61,63), lanthanum salt precipitation (61) or by electrophoresis on cellulose acetate membranes (62). These methods are complex and time consuming, and one of them requires a specific apparatus for high voltage electrophoresis.

In this study, a simple and rapid radiochemical method based on that described by Wohlheuter (65) was employed in an attempt to determine the influence of various purine and pyrimidine analogues on the kinetics of HPRT.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Human blood was donated by the South African Blood Transfusion Service. Sephadex G-100, CM-Sephadex G-50 and sodium-5-phosphoribose-1-pyrophosphate were obtained from Sigma Chemical Company. 8-[^{14}C]-Hypoxanthine (59m Ci/mmole) was purchased from the Radiochemical Centre Ltd., Amersham, and diluted with non-radioactive hypoxanthine (Sigma Chemical Co.) to about 7dpm per pmole.

Thin-layer chromatography sheets of polyethylenimine-impregnated cellulose ("Polygram Cel 300 PEI") were obtained from Brinkmann Instruments.

All other chemicals used were of standard grade.

METHODS

2.2.2 PURIFICATION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE FROM HUMAN BLOOD

The method of Krenitsky *et al.* was adopted (67). All procedures were carried out at 3°C unless otherwise stated.

Step I: Preparation of Erythrocyte Hemolysate

500ml of stored human blood were centrifuged at 8000 g for 30 min in a Sorvall GSA rotor. The supernatant was discarded and the cell pellet resuspended in 200 ml of 0.9% NaCl. The suspension was centrifuged at 8000 g for 45 min in a Sorvall SS 34 rotor. The resulting cell pellet was taken up in 150 ml of distilled water.

Step II: Stroma precipitation

The pH of the lysed suspension was adjusted to 5.2 by the slow addition of 0.24M HCl with mechanical stirring. The precipitated stroma was removed by centrifugation at 5000 g for 30 min. The pH of the resulting supernatant was brought to 6 by the slow addition of 1M KOH. This fraction was termed the "pH 5.2 lysate" (so called because it is the fraction remaining after precipitation of proteins at pH 5.2).

Step III: CM-Sephadex batch filtration

This step removes most of the haemoglobin. 100 ml of a gel which was previously prepared by swelling 10 g CM-Sephadex C-50 in 300 ml of a 0.01 M tris-0.0025 M magnesium sulphate - 0.016 M acetic acid buffer, pH 6.0 were added to the fraction designated "pH 5.2 lysate". After mechanical stirring for 15 min, the suspension was centrifuged at 1500 g for 30 min in a Sorvall SS 34 rotor. The pellet was discarded, and the resulting supernatant, termed "first CM-Sephadex fraction", was treated as above with 150 ml of the gel, and this supernatant fluid termed "second CM-Sephadex" fraction.

Step IV: Precipitation by heating

The second CM-Sephadex fraction was placed in a 1l- Erlenmeyer flask and immersed in a water bath at 75°C. After agitation for 10 minutes the temperature of the fluid had reached 73°C. The flask was then immersed in an ice-bath. When cool, the suspension was filtered by gravity through Whatman No.1 filter paper. The filtrate designated the "heat-treated" fraction was shell-frozen and lyophilized.

Step V: Gel filtration chromatography

The lyophilized powder was dissolved in 3 ml of 0.02 M tris-HCl buffer, pH 7.7at 24°C, containing 0.2 mM $MgSO_4$, and applied to a Sephadex G-100 column, equilibrated and eluted with the same buffer. Fractions of 2 ml were collected and

assayed for enzyme activity. Those containing the highest activity were pooled and frozen at - 10°C.

2.2.3 ASSAY OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE

The method of Wolheuter (65) was adopted and modified as decribed:-

2.2.3 a Principle

The assay is based on the separation of the radioactively labelled product [^{14}C] IMP, from the radioactively labelled substrate, [^{14}C]-hypoxanthine, by binding the former on an anion-exchanger. PEI-cellulose plates (Polyethylene imine-impregnated cellulose) has been used as the ion-excahanger.

2.2.3 b Reagents

(i) Assay buffer: 2 M Tris-HCl, 5CM $MgCl_2$ pH=7.5
(ii) Substrate : [^{14}C]-Hypoxanthine, 0.812 mM. (7.3dpm per pmole).
(iii) Na_4PRPP : 13 mM (i.e. Phosphoribosylpyrophosphate tetrasoduim salt).

2.2.3 c Procedure

The following amounts were added to disposable plastic test tubes using a Hamilton microsyringe:

10 µℓ assay buffer
25 µℓ [^{14}C]-hypoxanthine
20 µℓ enzyme extract
35 µℓ distilled water

The mixture, less PRPP, was brought to 30°C and the reaction started by addition of 10µℓ

Na_4 PRPP. Hence, the reaction mixture of 100µℓ contained 200 mM Tris HCℓ buffer/pH 7.8, 5 mM $MgC\ell_2$, 0.203 mM [^{14}C]-hypoxanthine, enzyme and 1.3 mM PRPP. Thirty minutes later, 20µℓ aliquots were removed with a Hamilton syringe, spotted on a PEI-cellulose plate and the plates dried with a hot air-blower. The appearance of PEI-bound radioactivity is a measure of nucleotide formed and hence enzyme activity.

In practice, a 10 x 20 cm sheet of PEI-cellulose was cut into 2.5 x 2.5 cm squares. The spotted PEI-cellulose pieces were accumulated until all reactions were complete. Unreacted hypoxanthine was washed off the plates by swirling them in three washes of distilled water for 5 minutes each and finally in methanol.

After drying in air, the PEI-cellulose plates were placed upright in scintillation vials containing 10 ml of scintillation fluid (3.0 g 2,5-Diphenyloxazole (PPO) and 0.2g 1,4-bis-2-(5-phenyloxazyl)-benzene (POPOP) per litre of toluene. Scintillation counting was done in a Beckmann liquid scintillation counter (model number LS 255).

2.2.4 DEFINITION OF UNIT AND SPECIFIC ACTIVITY

One unit is defined as the amount of enzyme that catalyzes the phosphoribosylation of 1 n mole of hypoxanthine to form 1 n mole IMP per minute from 0.203 mM hypoxanthine and 1.3 mM PRPP, under the assay conditions described above.

Specific activity is expressed in terms of units per mg of protein.

2.2.5 PROTEIN DETERMINATION

The protein content was determined by the method of Lowry et al.(66), using bovine serum albumin as standard.

2.3 RESULTS

2.3.1 ASSAY OF HPRT ACTIVITY

The method of assay is simple and rapid and capable of measuring HPRT activity of samples of low protein content. [^{14}C]-Hypoxanthine was quantitatively removed by the washing procedure, and like the results of Wohlhueter, backgrounds of 200 counts per minute of a total of 22000 counts per minute were left behind. The counting efficiency of the plates was found to be 84% as determined by spotting known concentrations of [^{14}C] hypoxanthine.

2.3.2 ENZYME PURIFICATION

The results of the purification procedure are summarised in Table I. The haemaglobin was removed effectively by the CM-Sephadex-C 50, and the heat treatment removed APRT. The other proteins were removed by Sephadex G100, resulting in an overall yield of HPRT of 13.6%.

TABLE I PURIFICATION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE FROM HUMAN RED BLOOD CELLS

Fraction	Volume (ml)	Protein content (mg)	Total activity (units)	Specific activity (units/mg protein)	% Yield	Purification fold
pH 5.2 lysate	175	420	1722	0.4	100	0
First CM-Sephadex	170	255	1581	6.2	91	15
Second CM-Sephadex	175	166	1282	7.7	74	18
Heat treated	150	135	705	5.2	40	12
Sephadex G100 eluate	12	4	23	48.8	13	118

TABLE I PURIFICATION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE FROM HUMAN RED BLOOD CELLS

Fraction	Volume (ml)	Protein content (mg)	Total activity (units)	Specific activity (units/mg protein)	% Yield	Purification fold
pH 5.2 lysate	175	4200	1722	0.4	100	0
First CM-Sephadex	170	255	1581	6.2	91	15
Second CM-Sephadex	175	166	1282	7.7	74	18
Heat treated	150	135	705	5.2	40	12
Sephadex G100 eluate	12	4	234	48.8	13	118

2.3.3 KINETICS OF HPRT

Under the assay conditions used, the reaction rate was found to be linear up to 40 minutes. As shown in Table II and Fig 8 the correlation between IMP formation and the amount of enzyme used is linear.

Initial velocities (V) and substrate concentrations (s) from the kinetic experiments were fitted by an IBM 37158 computer to the equations relating to the Lineweaver-Burk, Haynes, Eadie-Hofstee and the modified Eadie-Hofstee plots. The fortran program developed by Manchester was used. From this procedure, values for V and K_m were obtained, where V is maximum velocity and K_m the Michaelis constant.

The apparent K_m (Michaelis constant) of HPRT for the substrate hypoxanthine was found to be 67μM (Table III, Fig. 10) at 0.203mM hypoxanthine These values compare favourably with literature values (119). The lineweaver Burk plots for the results from the kinetic experiment are illustrated in this study.

Various purine analogues (Table V and Fig.12) were tested as inhibitors of HPRT for the substrate, hypoxanthine. Three in particular were found to be active inhibitors of the enzyme. Detailed kinetic studies of these inhibitors are indicated in figures 13,14 and 15 respectively. The type of inhibition is summarised in Table VI.

NUCLEOTIDE FORMATION AS A FUNCTION OF ENZYME CONCENTRATION

TABLE II NUCLEOTIDE FORMATION AT VARYING ENZYME CONCENTATIONS AT 0.203 mM HYPOXANTHINE AND 1.3 mM PRPP

Enzyme (µg)	Velocity IMP formed nmoles/min
20	0.89
30	1.31
40	1.80
60	2.66
80	3.80

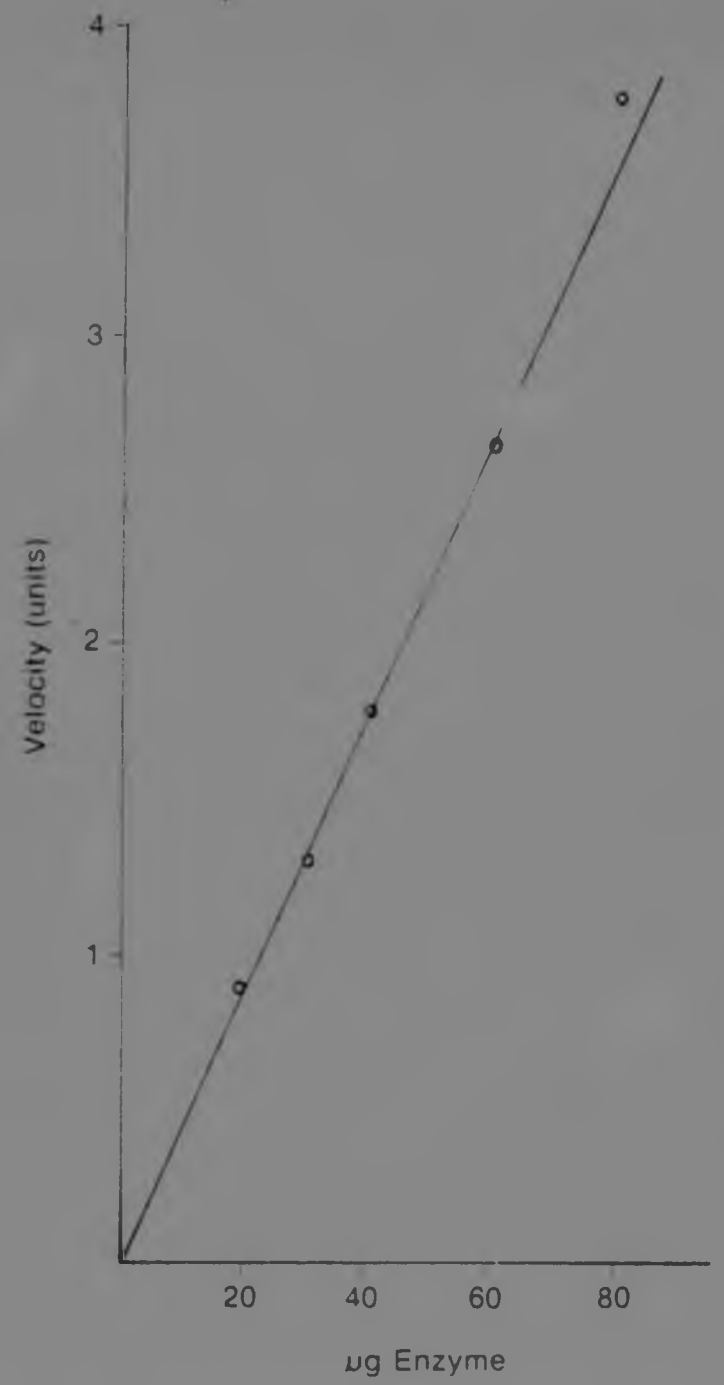


Fig. 8
Nucleotide formation at varying enzyme concentrations at 0.203mM Hypoxanthine and 0.13mM PRPP.

DETERMINATION OF THE MICHAELIS CONSTANT (K_m) OF HPRT FOR HYPOXANTHINE AT CONSTANT PRPP CONCENTRATION

TABLE III NUCLEOTIDE FORMATION AT VARYING HYPOXANTHINE CONCENTRATIONS AND 1.3 mM PRPP

[^{14}C]Hypoxanthine (mM) (59 mCi/mmol)	Velocity (units)	Hypoxanthine^{-1} (mM^{-1})	Velocity^{-1} ($units^{-1}$)
0.040	0.40	4.94	2.45
0.080	0.59	12.46	1.69
1.120	0.67	8.31	1.47
0.217	0.85	4.61	1.17
0.281	0.87	3.56	1.14
0.321	0.91	3.11	1.09
0.361	0.90	2.77	1.10

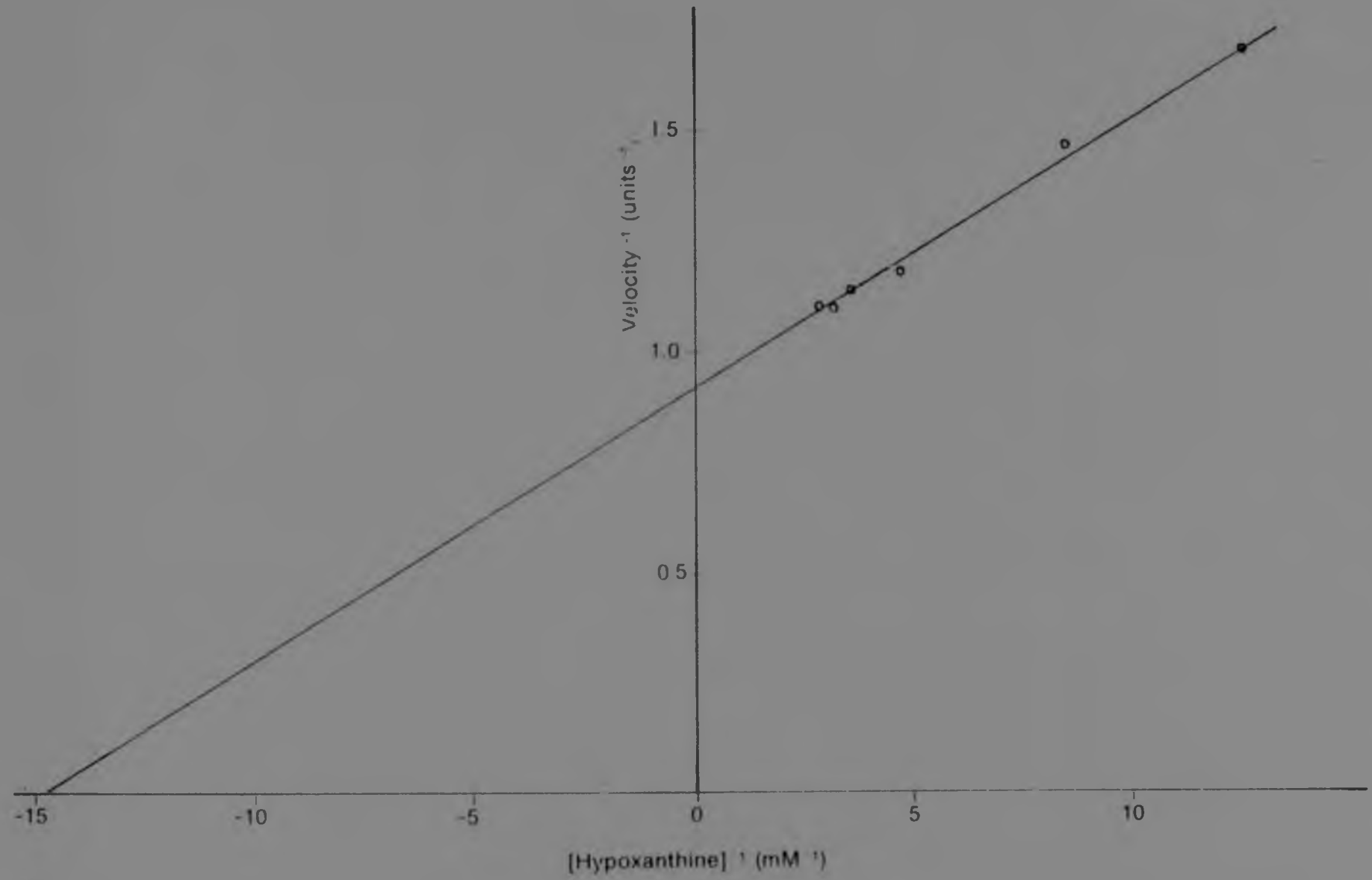


Fig. 9
Lineweaver-Burk plot for Hypoxanthine

DETERMINATION OF THE MICHAELIS CONSTANT (K_m) OF HPRT FOR PRPP (5-PHOSPHORIBOSYL-1-PYROPHOSPHATE)

TABLE IV NUCLEOTIDE FORMATION AT VARYING PRPP CONCENTRATIONS AND 0.2 mM HYPOXANTHINE

PRPP (mM)	Velocity (units)	$PRPP^{-1}$ (mM^{-1})	$Velocity^{-1}$ ($units^{-1}$)
0.3	1.84	3.3	0.54
0.4	2.18	2.5	0.45
0.5	2.50	2.0	0.39
0.8	3.09	1.2	0.32
1.0	3.14	1.0	0.31
1.5	3.57	0.66	0.28

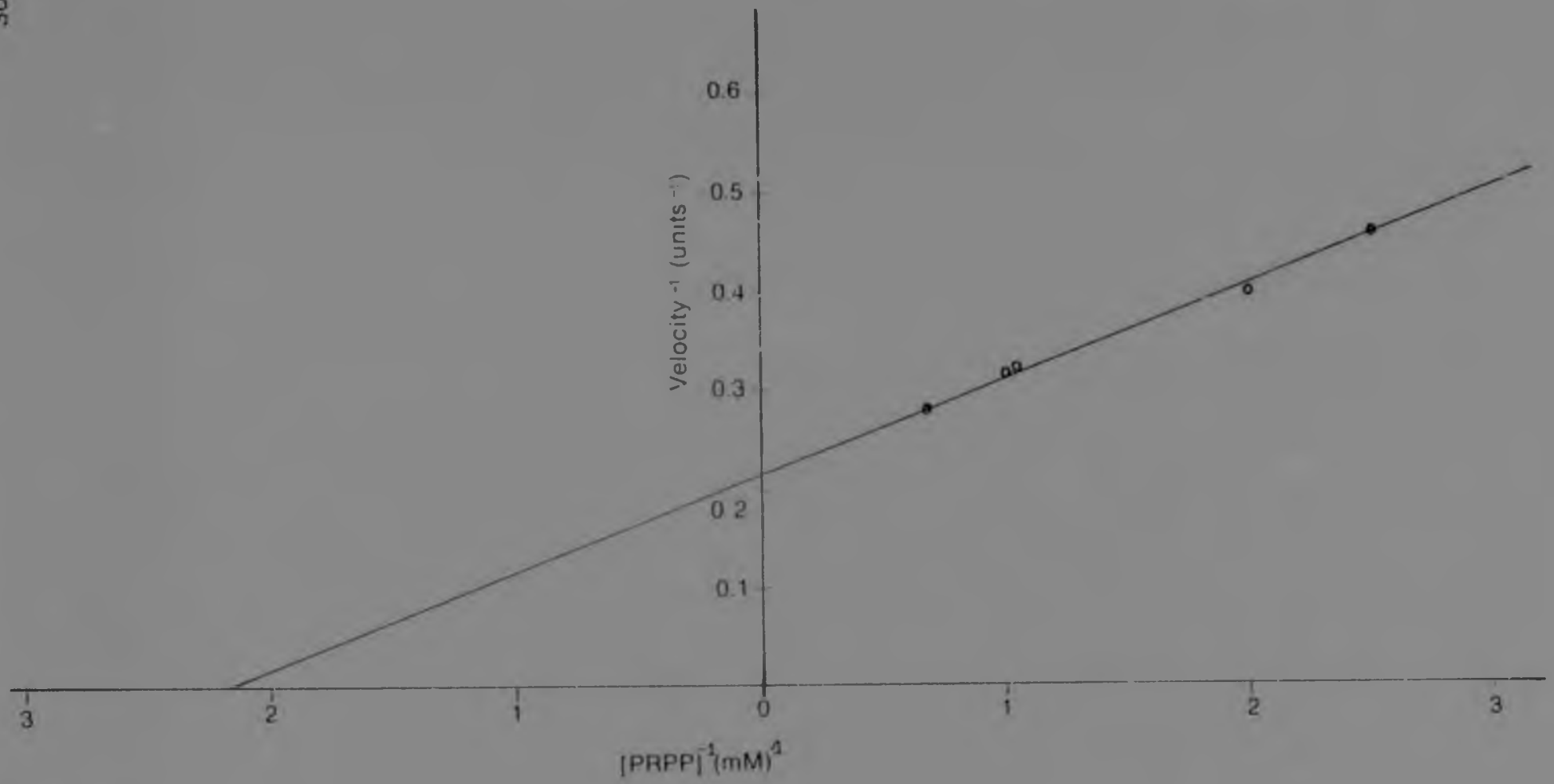
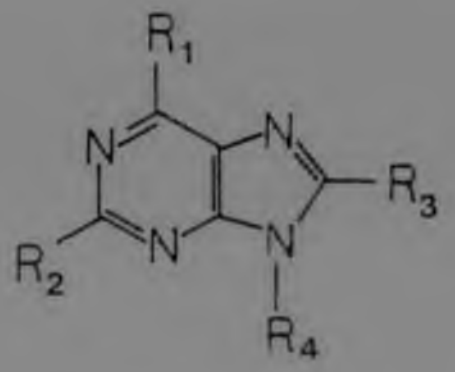
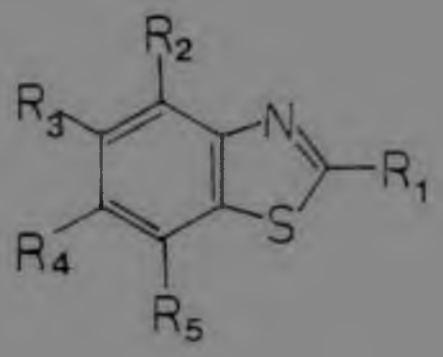


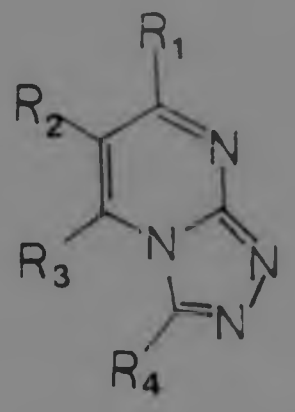
Fig. 10
Lineweaver-Burk plot for PRPP.



(i) Purine

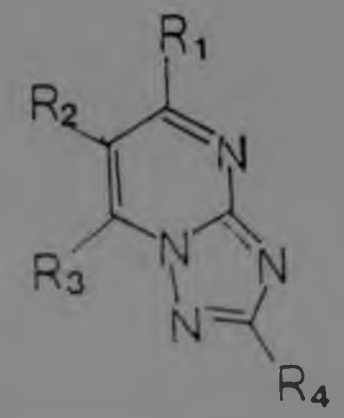


(ii) 2-Benzothiazole

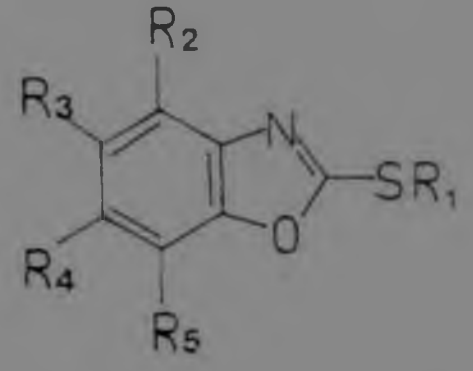


(iii) s-Triazolo-[4,3-a]-pyrimidine

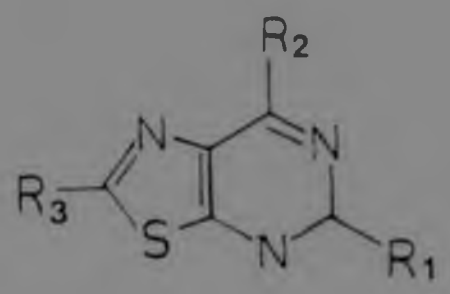
Fig. 12 Parent structures of the phleomycin amplifiers investigated.



(iv) s-Triazolo-[1,5-a]-pyrimidine

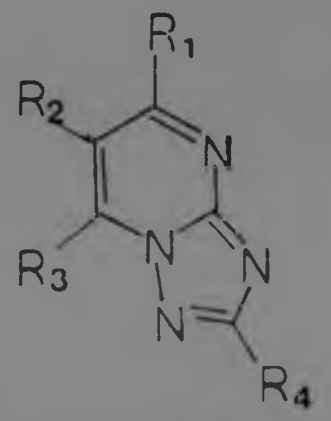


(v) 2-Thiobenzoxazole

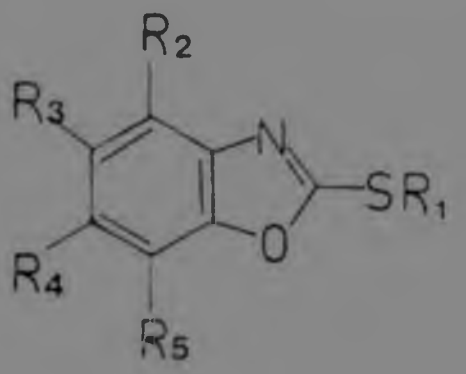


(vi) Thiazolo-[5,4-d]-pyrimidine

Fig. 12 (Continued)



(iv) s-Triazolo-[1,5-a]-pyrimidine



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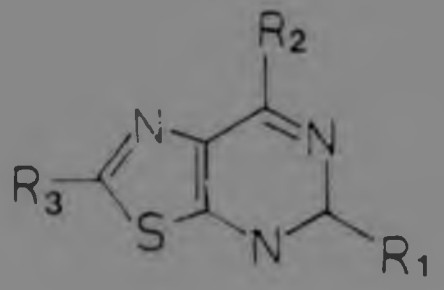
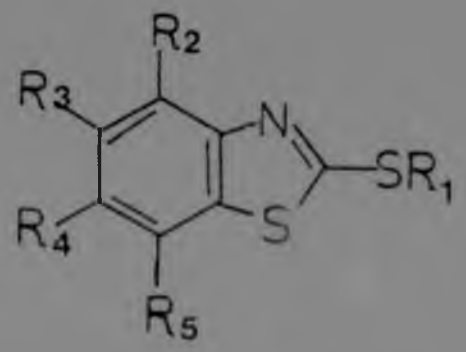


(vi) Thiazolo-[5,4-d]-pyrimidine

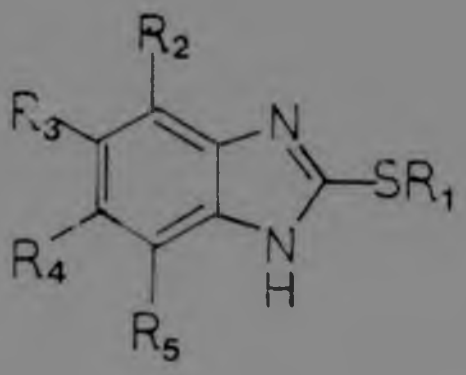
Fig. 12 (Continued)



(vii) 2-Thiobenzothiazole

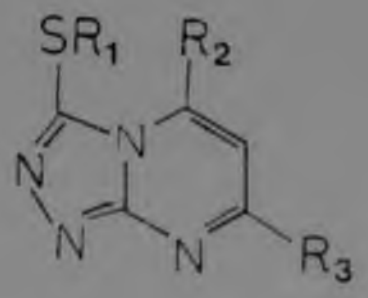


(viii) *s*-Triazolo-[4,3-a]-pyrimidine

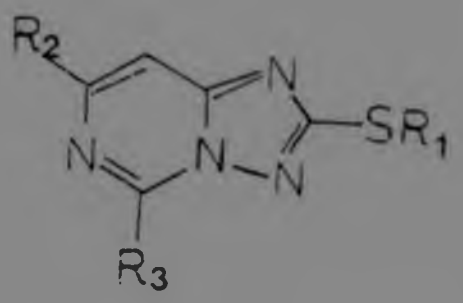


(ix) Thiobenzimidazole

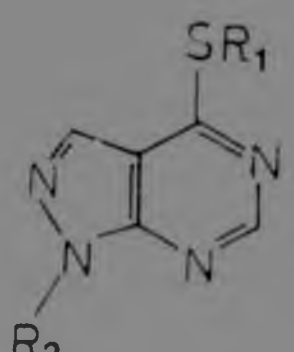
Fig. 12 (Continued)



(x) *s*-Triazolo-[4,3-a]-pyrimidine



(xi) *s*-Triazolo-[1,5-c]-pyrimidine



(xii) Pyrazolo-[3,4-d]-pyrimidine

Fig. 12 (Continued)

TABLE V STRUCTURAL FEATURES OF THE PHLEOMYCIN AMPLIFIERS INVESTIGATED

Analogue	Parent structure (see figure)	Substituents
2-α-Carbamoylmethylthio-6,9-dimethylpurine	Purine (i)	R_1: $-CH_3$ R_2: $-SCH_2CONH_2$ R_3: $-H$ R_4: $-CH_3$
2-Carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine	Purine (i)	R_1: $-CH_3$ R_2: $-SCH_2CONH_2$ R_3: $-CH_2OH$ R_4: $-CH_3$
2-Carbamoylmethylthio-6,8,9-trimethylpurine	Purine (i)	R_1: $-CH_3$ R_2: $-SCH_2CONH_2$ R_3: $-CH_3$ R_4: $-CH_3$
2-α-Carbamoylethylthio-benzothiazole	Benzothiazole (ii)	R_1: $-SCHCONH_2$ (CH_3) R_2: $-H$ R_3: $-H$ R_4: $-H$

TABLE V (cont.)

Analogue	Parent structure (see figure)	Substituents
3-α-Carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine	s-Triazolo-[1,5-a]-pyrimidine (iv)	R_1: -H R_2: -H R_3: -H R_4: $-SCHCONH_2$ CH_3
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	s-triazolo-[4,3-a]-pyrimidine (iii)	R_1: -H R_2: -H R_3: -H R_4: $-SCHCONH_2$ CH_3
2-α-Carbamoylethylthio-benzoxazole	Benzoxazole (v)	R_1: $-SCHCONH_2$ CH_3 R_2: -H R_3: -H R_4: -H R_5: -H
3-Carbamoylmethylthio-7-methyl-s-triazolo-[4,3-a]-pyrimidine	Thiazolo-[5,4-d]-pyrimidine (vi)	R_1: SCH_2CONH_2 R_2: CH_3 R_3: SCH_2CONH_2

TABLE V (CONT.)

Analogue	Parent structure (see figure)	Substituents
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	s-triazolo-[4,3-a]-pyrimidine (viii)	R_1: -SCHCONH$_2$ (CH$_3$) R_2: -H R_3: -H R_4: -H R_5: -H
2-α-Carbamoylethylthio benzimidazole	Thiobenzimidazole (ix)	R_1: -CHCONH$_2$ R_2: -H R_3: -H R_4: -H
3-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[4,3-c]-pyrimidine	s-triazolo-[4,3-c]-pyrimidine (x)	R_1: -CH$_2$CONH$_2$ R_2: -CH$_3$ R_3: -CH$_3$
2-Carbamoylmethylthio 5,7-dimethyl-s-triazolo-[1,5-c]-pyrimidine	s-triazolo-[1,5-c]-pyrimidine (xi)	R_1: SCH$_2$CONH$_2$ R_2: -CH$_3$ R_3: -CH$_3$
4-Carbamoylmethylthio-1-methyl-1H-pyrazolo-[3,4-d]-pyrimidine	pyrazolo-[3,4-d]-pyrimidine (xii)	R_1: -CH$_2$CONH$_2$ R_2: -CH$_3$

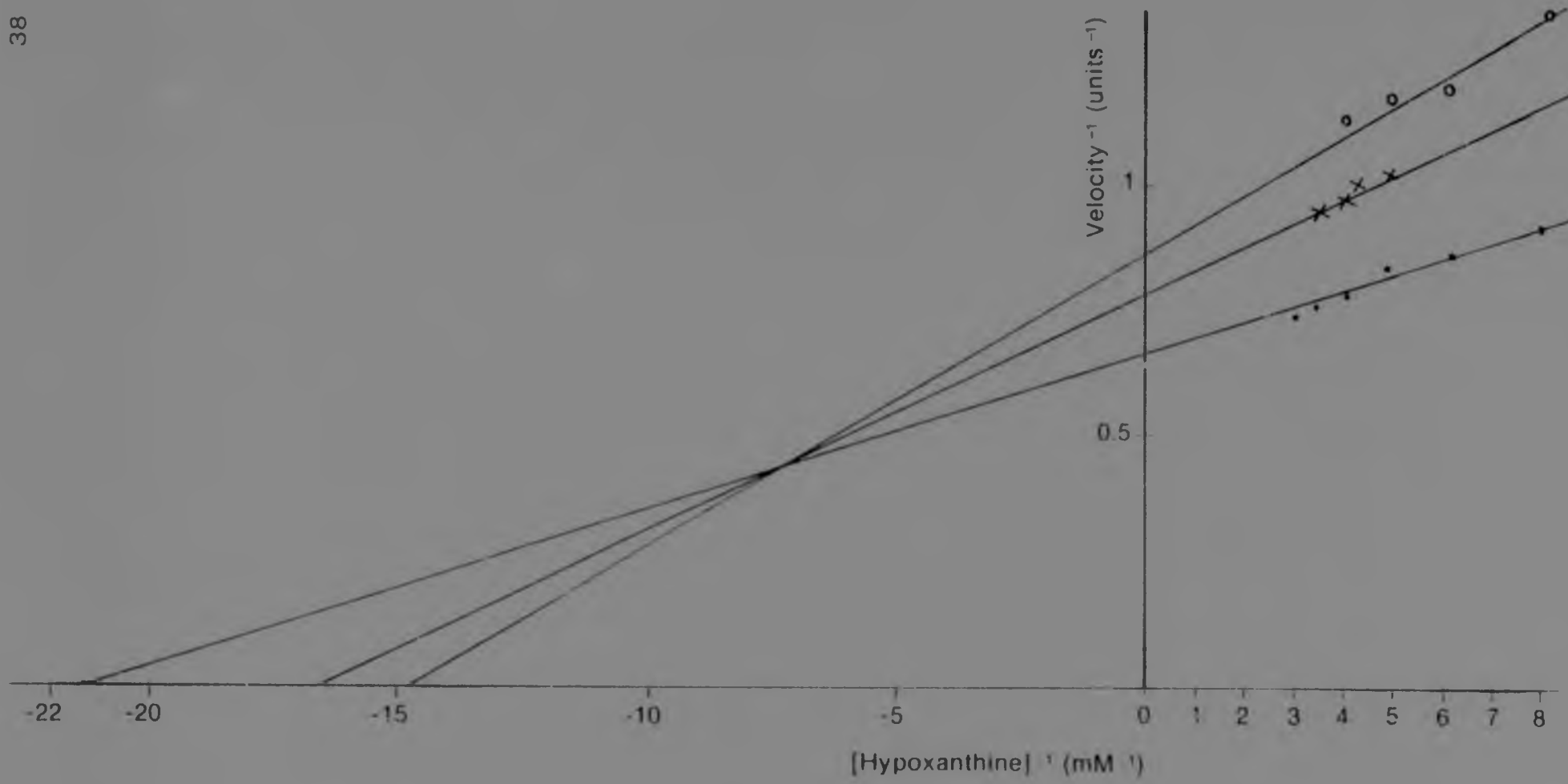


Fig. 13
Double reciprocal plots of the inhibition by 2-∝-carbamoylethylthiobenzoxazole on HPRT activity for the substrate Hypoxanthine.

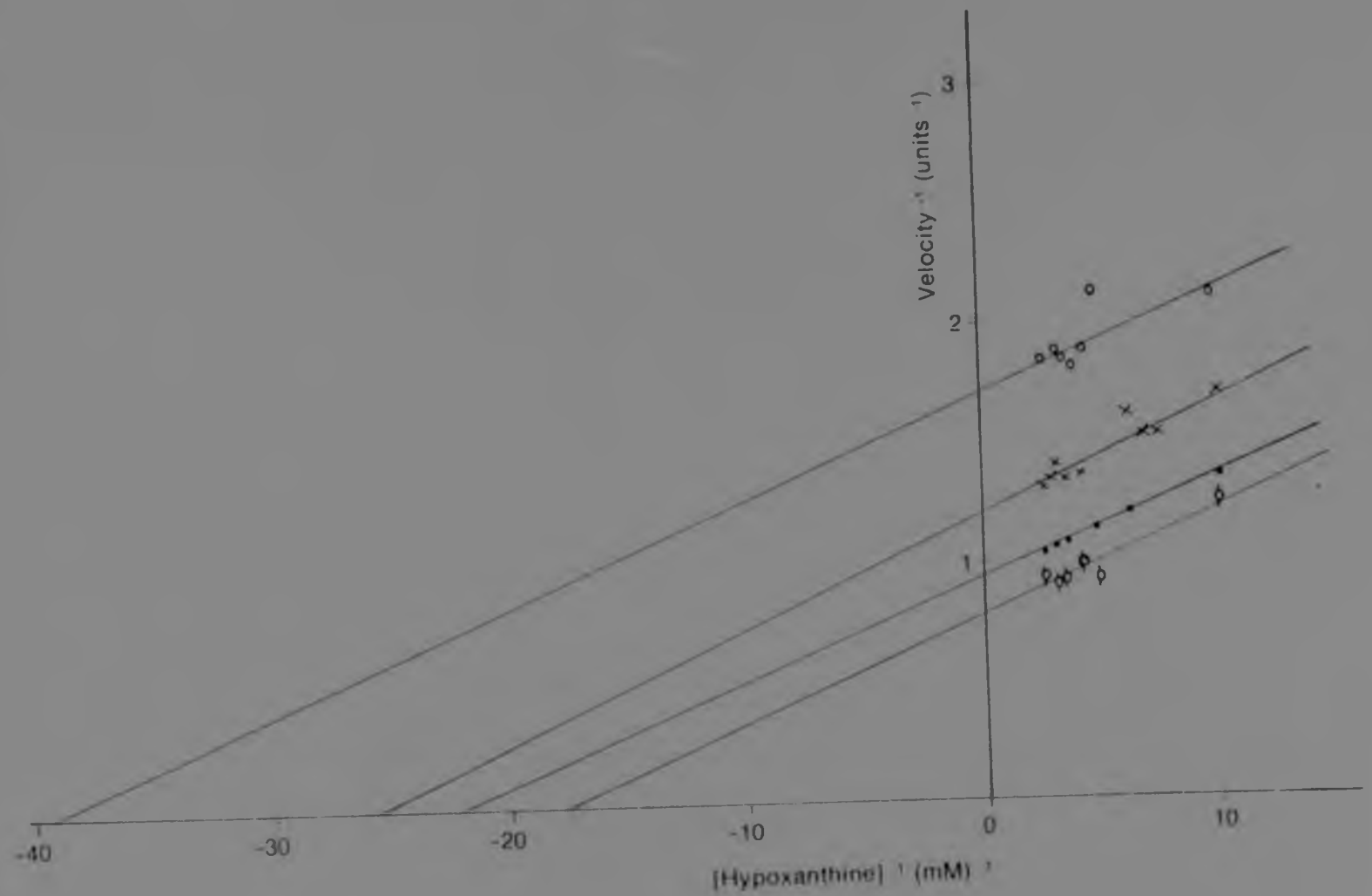


Fig. 14
Double reciprocal plots of the inhibition by 2-∝-carbamoylethylthio -s-triazolo- [1,5-a]-pyrimidine on HPRT activity for the substrate Hypoxanthine.

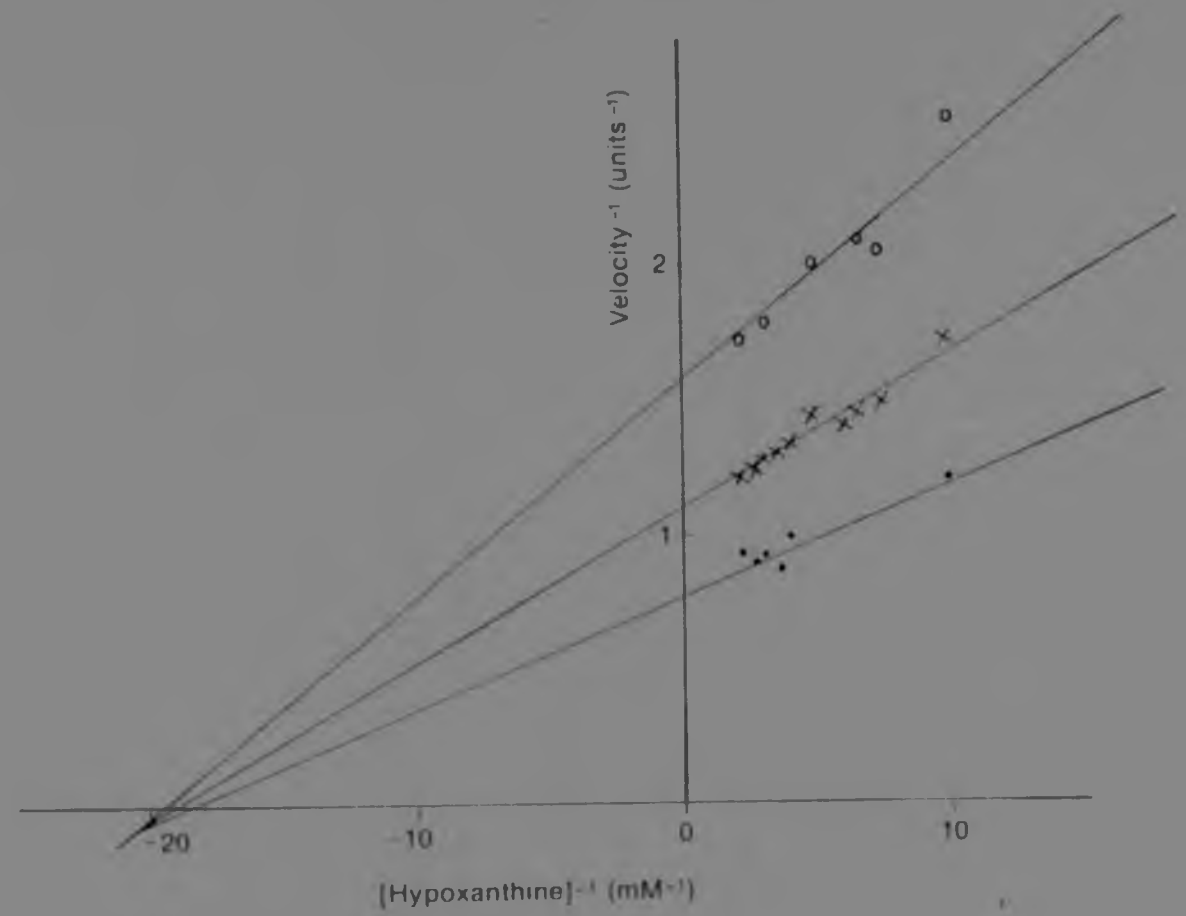


Fig. 15
Double reciprocal plots of the inhibition by 2,5-biscarbamoyl-methylthio-7-methylthiazolo-[5,4-d]-pyrimidine for the substrate Hypoxanthine.

TABLE VI INHIBITORS OF HUMAN ERYTHROCYTE HPRT

Inhibitor	Type of inhibition
2-α-Carbamoylethylthiobenzoxazole	Non competitive
2-α- Carbamoylethylthio-s-triazole [1,5-a]-pyrimidine	Uncompetitive
2,5-Biscarbamoylmethylthi -7-methylthiazolo-[5,4-d]-pyrimidine	Non competitive

2.4 DISCUSSION

Krenitsky *et al.* (68) have shown that the apparent K_m values of PRPP is dependent on the concentration of the tris buffer and K_m values for PRPP of 0.24 mM to 0.63 mM has been reported. A K_m value of 0.47 mM for PRPP has been obtained in this study which compares favourably (68). In this study a K_m of 67 µM for hypoxantnine was obtained. This value compares favourably with some values reported (119). However, K_m values as low as 5µM for hypoxanthine have been reported by other workers (67,68). These differences may be either attributed to the different enzyme assay methods used or the nature of the enzyme form.

Many studies explain the nature of the differences between the various normal forms of HPRT. Der Kaloustian *et al.* (69) have reported the presence of three active forms of the enzyme and have suspected the presence of a fourth from results obtained by isoelectric focusing in a polyacrylamide gel. Results of Arnold and Kelley (70) may also suggest the presence of a fourth component. Later Gulumian and Wabic (71) isolated four separate components of normal HPRT by the technique of isoelectric focusing in a sucrose gradient.

Hence a complete knowledge of the different forms of HPRT is essential to elucidate the enzyme structure, stability and catalytic or regulatory properties. Arnold and Kelley (70) have proposed a hypothesis to explain the different forms of HPRT: since the genetic coding for HPRT resides in a single locus, the existence of different forms could not be due to *de novo* synthesis of

different proteins,thus suggesting a post-transcriptional alteration in the enzyme. Heterogeneity of the native human erythrocyte HPRT has been observed by several other investigators (72,73,74).

Krenitsky et al. have shown that several purine and pyrimidine analogues also serve as substrates for the enzyme (68). The enzyme has a high affinity for purine analogues with 6-oxo or thio with or without 2-amino groups. However, guanine and hypoxanthine bind most tightly to the enzyme. 6-Mercaptopurine and 6-thioguanine were found to bind slightly less effectively than the corresponding N-methyl derivatives of guanine and hypoxanthine.

Further derivatives with alkyl, hydroxyl or amino groups bound even more poorly. Furthermore, they suggested that the imidazole ring was important for binding since other condensed pyrimidine systems such as the pyrazolo-[3,4-d]-pyrimidines and the triazolo-[4,5-d]-pyrimidines bound relatively poorly. However the imidazole portion itself is not sufficient for the enzyme binding as indicated by the high K_i values they obtained for 5-aminoimidazole-4-carboxamide and its formyl derivative.

In the present study three compounds were found to be inhibitors of HPRT from a series of analogues tested. The type of inhibition varied and the structure-inhibition relationship could not be concluded. Many derivatives containing an imidazole portion did not inhibit HPRT and two of the inhibitors reported were not imidazole derivatives (Table VI).

CHAPTER THREE

INHIBITION OF HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE FROM HEPATOMA CELL CULTURES

3.1 INTRODUCTION

The three purine-pyrimidine analogues (Fig 16) which proved to be active inhibitors of HPRT from human erythrocytes:

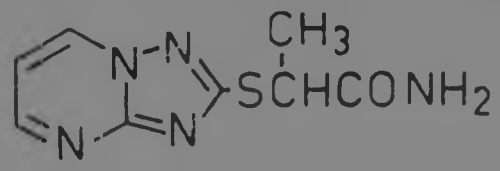
(i) 2-α-carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine

(ii) 2,5-biscarbamoylmethylthio-7-methylthiazolo [5,4]-pyrimidine

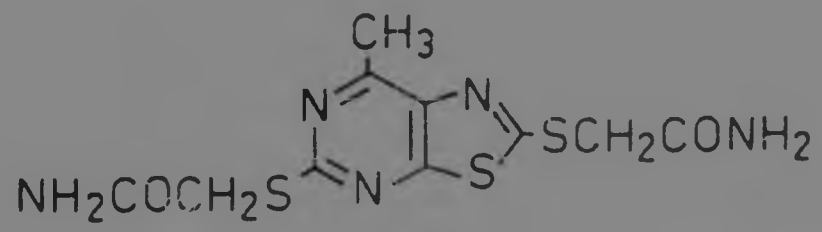
(iii) 2-α-carbomoylethylthiobenzoxazole,

were tested in hepatoma cell cultures to investigate their effects *in vivo*. Their effects were tested in vivo since many purine analogues, for example, 6-mercaptopurine are reported to possess pharmacological activity. 6-Mercaptopurine was reported to possess significant antitumour activity and is used in the treatment of acute lymphocytic leukemia in children (30). Furthermore, antifolic acid drugs such as aminopterin and amethopterin which contain a modification in the pyrimidine (or triazine) moeity of the folic acid molecule possess substantial antitumour activity (30). Amethopterin, for example, is employed in the treatment of many forms of neoplasia. The purine bases, guanine and hypoxanthine, and several purine analogues are converted to the ribonucleotide derivatives by hypoxanthine phosphoribosyl transferase. For guanine and hypoxanthine, this represents a salvage pathway for nucleotide production. The effectiveness of the purine analogues, 6-mercaptopurine and 6-thioguanine, in inhibiting cell growth is dependent on their transformation to their ribonucleotide forms by this enzyme.

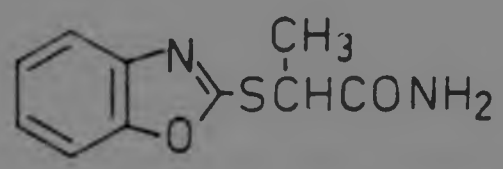
6-Methylthiopurine ribonucleoside is converted by adenosine kinase (ATP: adenosine 5'phosphotransferase EC.2.7.1.20) to the nucleotide which exerts a potent inhibition of the de novo purine pathway. Thus by blocking de novo purine nucleotide biosynthesis, it lowers the ATP content of the cell to such as extent that production of nucleic acids is reduced. Thus, the inhibition of both de novo and salvage-pathway production of GMP and IMP may be related to their toxic effects on tumours (30). Hence, the purine and pyrimidine analogues reported as inhibitors of human erythrocyte HPRT were tested in hepatoma cell cultures.



(i) 2-[1-carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine



(ii) 2,5-bis-carbamoylmethylthio-7-methylthiazolo-[5,4-d]-pyrimidine



(iii) 2-[1-carbamoylethylthiobenzoxazole

Fig. 16 Phleomycin amplifiers detected as inhibitors of human erythrocyte HPRT

3.2 MATERIALS AND METHODS

All reagents used were of analytical grade.

3.2.1 HEPATOMA CELL CULTURE

Rat hepatoma (75) cells, originally derived by treatment of rats with 3-methyl-4-dimethylaminoazobenzene were grown as described by Grimm (76) and Gulumian (77). They were grown in Eagle's minimal essential medium with glutamine which was supplemented with 5 mg% ascorbic acid, 5% foetal calf serum, 10.5 mg/ℓ L-serine, 7.5 mg/ℓ glycine, 50 units/ml of both penicillin G and streptomycin. and 750mg/ℓ sodium bicarbonate. The cells were grown in glass or plastic bottles. They were kept at 37°C in an atmosphere of 95% air, 5% CO_2.

3.2.2 SUBCULTURE

The old medium was decanted. The cells were rinsed with 3.0 ml warmed trypsin solution. To detach the cells from the substrate, 5.0 ml trypsin was added and the culture was allowed to stand at room temperature for 3 minutes. The trypsinization process was completed when the cell sheet appeared to be loosened from the glass or plastic surface. The cells were then aspirated with a 10 ml syringe and transferred into centrifuge tube. After 3 minute centrifugation at low speed, the trypsin solution was decanted and 2.0 ml medium were added. The cells were then dispersed before distributing into new culture vessels containing 5.0 ml medium. The number of cells in these subcultures was always kept in the region of five million per culture. They were then incubated at 37°Cand the medium was

changed after [illegible] days.

[illegible] contained:

-[illegible]0g	NaCl
-[illegible]4g	KCl
-1[illegible]	D-(+)-glucose
-[illegible].8g	$NaHCO_3$
-2.0g	EDTA
-5.0g	Trypsin (Difco 1:250)
-10.0ml	(0.2% solution) phenol red
-10.0ml	Penicillin G (10^6 units)
-5.0ml	H_2O containing 1g streptomycin

20mg phenol red and 5.8g $NaHCO_3$ were dissolved in 100ml deionised water. The mixture was added to the other ingredients and made up to one litre and stored at -20°C. For subcultures, this solution was diluted 5 times and sterilized by passing through millipore filters with positive pressure.

3.2.3 CELL COUNTS

To determine cell numbers, cells were scraped off the culture dish into isotonic saline and counted in a Coulter counter Model D. The counting is based on a difference in conductivity between cells and suspending medium. The sensing device is a tube containing a small cylindrical aperture (140µm in diameter) which is immersed in a beaker containg cells to be enumerated and suspended in isotonic saline. A small DC voltage is applied across this gate. When a metered volume of suspension (0.5ml) is drawn through the opening by vacuum, the passage of an individual cell causes a voltage drop due to increased aperture impedance. The corresponding pulse can be visualised by means of an oscilloscope and is recorded

by the instrument counting circuits. The dilutions are chosen to give an estimated machine count of 10000 or less per 0.5ml to minimize coincidence which can hence be ignored.

It is important in making instrument counts to choose settings which register all pulses above background noise. Therefore, the aperture current and the amplitude controls were adjusted to give an oscilloscope pattern in which the impulses were one-half to two-thirds of the screen height.

3.2.4 HPRT ACTIVITY IN HEPATOMA CELL CULTURES

3.2.4 a ASSAY METHOD

The assay method described for human blood HPRT (chapter 2, this thesis) was employed.

3.2.4 b ENZYME EXTRACT

The cells were scraped off the culture dish and suspended in the buffer of the following composition: 0.02mM Tris-HCℓ, 0.2mM $MgSO_4$, pH 7.8. These cells were lysed by freeze-thawing in a dry-ice-acetone mixture. This lysate was then centrifuged and the supernatant used to assay HPRT.

3.2.4 c KINETICS OF HPRT

The Michaelis constants (Km) for hypoxanthine was calculated by the method described for human erythrocyte HPRT.

3.2.4 d INHIBITION OF HPRT

(i) In vitro: Lysed cells were assayed for HPRT in the presence of analogues to detect inhibition.

(ii) In vivo: Cells in culture were incubated with the analogues (at 10μM) for one hour. The cells were washed free of analogue with buffer, scraped off the culture dish and assayed for HPRT. Furthermore, cells were reincubated for varying time intervals in analogue - free medium to detect any change in HPRT activity.

3.3 RESULTS AND DISCUSSION

Kinetic studies showed that this enzyme is active in hepatoma cell cultures, and this activity is directly proportional to the number of hepatoma cells present. The results are indicated in Table VIII and Fig 17.

Further kinetic studies showed that HPRT activity in hepatoma cells has a K_m of 86μM for hypoxanthine at 1.3mM PRPP (Table IX and Fig 18).

Also, the average HPRT activity in hepatoma cells was calculated to be 25.8 n moles/min/10^6 cells.

A complete loss of HPRT activity resulted when cells in culture were incubated with these analogues for one hour. Removing the medium, and reincubating the cells for 2 hours in analogue free medium did not restore HPRT activity. Presumably during the 2 hours, the analogue (or its metabolite) is still present in sufficient concentration for inhibition. Furthermore, the analogues had no effect on cell number and HPRT activity was detected within 6 hours.

Enzyme extracts from hepatoma cell cultures incubated in analogue -free medium were then assayed in the presence of these three analogues. The following results were obtained (Table X):

Analogues (ii) and (iii) resulted in complete loss of HPRT activity at the concentrations used.

However, with analogue (i) there was decreasing activity with increasing analogue concentration.

The dependence of activity on analogue (i) is shown in Table XI.

Table VIII Nucleotide formation at varying enzyme concentration

Enzyme extract (µℓ)	n moles product/ mℓ from 0.203mM Hypoxanthine	n moles product/ mℓ from 0.1624mM Hypoxanthine
5	1.75	1.26
10	3.69	3.38
15	5.74	5.19
20	7.16	6.96
25	9.38	8.78
30	10.83	9.63
35	12.42	11.08
40	13.64	12.13

Cell number = 14 million cells/mℓ

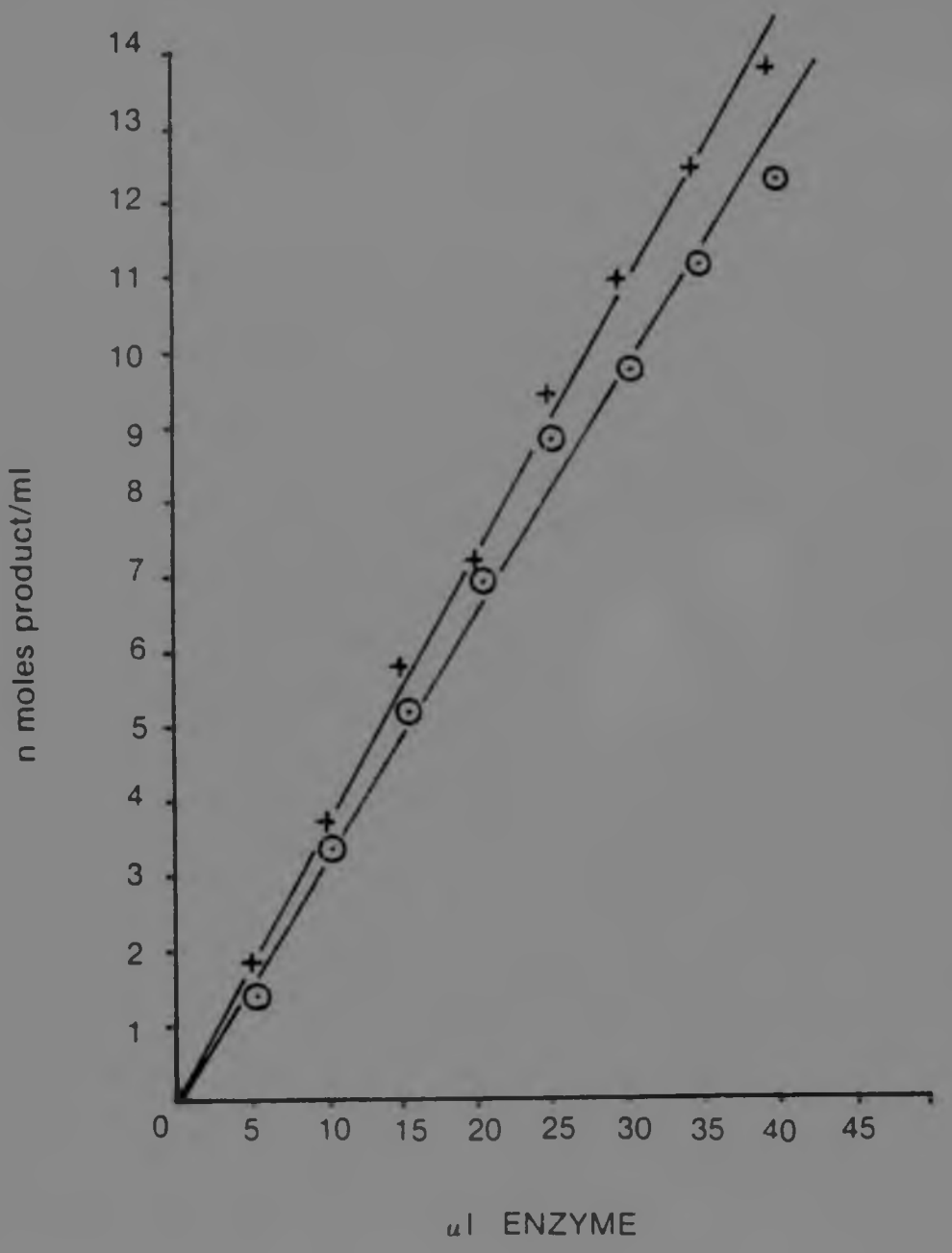


Fig. 17 Velocity of the HPRT reaction as a function of amount of Enzyme extract at
(i) 0.203 mM Hypoxanthine (-+-)
(ii) 0 162 mM Hypoxanthine (o-o)

Table IX Nucleotide formation at varying hypoxanthine concentrations

Hypoxanthine (mM)	Hypoxanthine^{-1}	VELOCITY units IMP formed (n moles/mℓ/ 10^6 cells)	Velocity^{-1}
0.0609	16.42	15.37	0.0650
0.0812	12.315	17.67	0.0565
0.1015	9.85	19.79	0.0505
0.1218	8.21	22.01	0.0454
0.1624	6.158	25.03	0.0399
0.203	4.926	26.13	0.0382
0.2842	3.519	27.51	0.0363

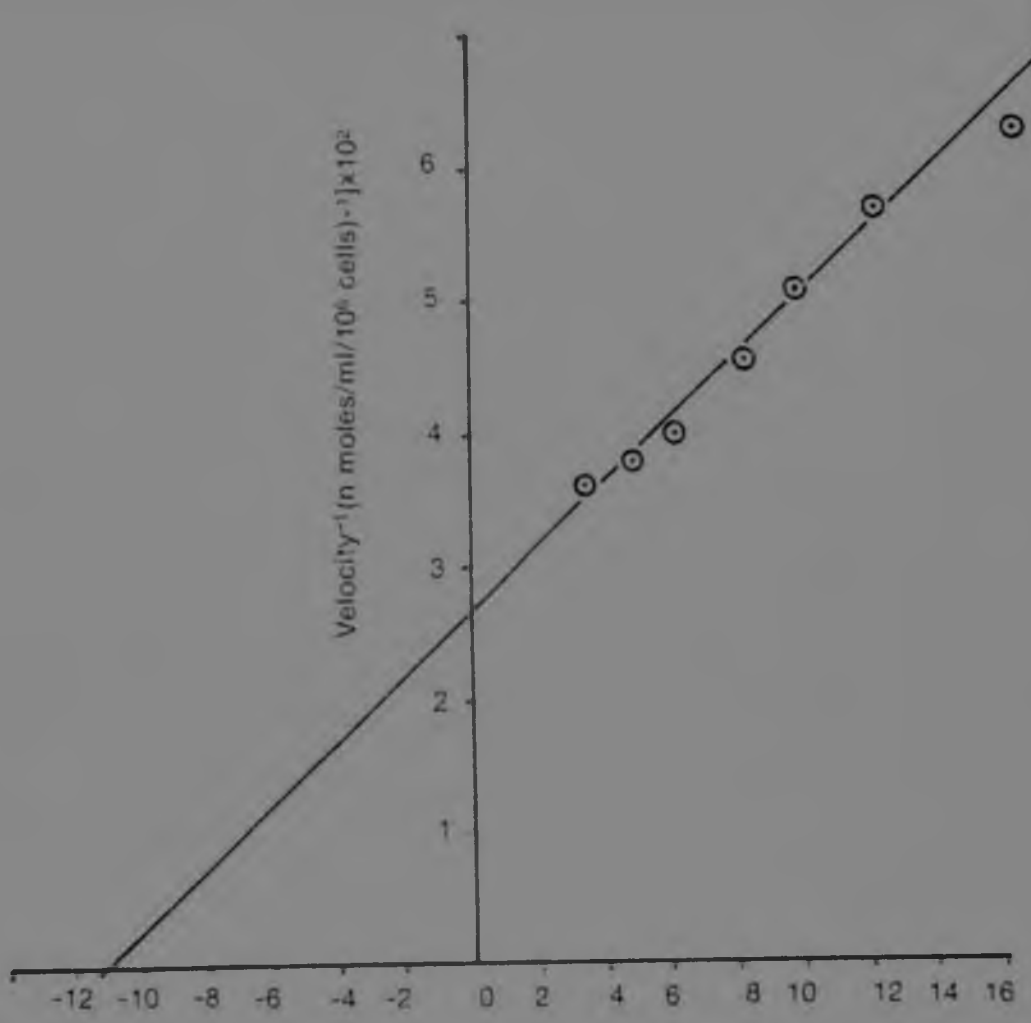


Fig. 18 Determination of Km of hypoxanthine of HPRT from Hepatoma cell cultures.

Table X Inhibition of HPRT of hepatoma cells

Enzyme concentration (µℓ enzyme extract)	Inhibitor concentration (mM)	Analogues (i)	(ii)	(iii)
		(n moles/ml/10^6 cells)		
40	0	23.4	23.4	23.4
	0.05	20.0	-	-
	0.10	18.7	-	-
	0.15	15.6	-	-
30	0	27.8	27.8	27.8
	0.10	20.4	-	-
	0.20	16.2	-	-
	0.25	13.8	-	-

Table XI Inhibition of HPRT of hepatoma cells by 2-α-Carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine

Inhibitor concentration (mM)	HPRT activity	
	UNITS	%
0	23.4	100
0.05	20.0	85
0.10	18.7	80
0.15	15.6	66
0.20	13.6	58
0.25	11.6	49

CHAPTER FOUR

INFLUENCE OF THE ANALOGUES ON THE EXCISION REPAIR MECHANISM OF UV-IRRADIATED E.coli

4.1 INTRODUCTION

An association between phleomycin induced cell death following DNA breakdown and enzymes of the dark-repair process has been implicated. Grigg (10) showed that excision defective mutants were neither killed by phleomycin ($\leq 2\mu g/ml$) nor their DNA broken down.

Phleomycin binds to thymine bases in the DNA (78) and induces breakage of DNA and corresponding cell death by two distinct mechanisms:-

(i) a non-enzymic mechanism, chiefly at low phleomycin concentrations, and
(ii) an enzymic mechanism

The non-enzymic DNA breakage is mediated by sulphydryl compounds. Sleigh and Grigg (78) have proposed a mechanism which involves metal ion/O_2 catalysed oxidation of the sulphydryl group to yield its free radical form, followed by reaction of the radical with the phleomycin bound DNA resulting in DNA breakage and regeneration of the sulphydryl.

The enzymic mechanism invclves the single strand-specific endonucleases. Phleomycin produces local denaturation in DNA which is then sensitive to these endonucleases.

Double - stranded nucleic acids exhibit a helix-coil transition (Tm) within a defined temperature

range, at a given ionic strength and pH. The premature denaturation of the DNA by the phleomycin amplifier caffeine, as indicated by the drop in Tm, suggests that it interacts more strongly with the single-stranded random coil form of the DNA than with the double helical form: hence, shifting the equilbrium in favour of the random coil form.

It has been suggested that substances which exhibit selectivity of binding for single-strand DNA may inhibit excision repair (19,56). As with caffeine the mode of action could be by denaturation of the DNA where hydrogen bonding between the complementary strands of DNA is weakened. Since the endonuclease which initiates excision repair is a double-strand specific enzyme, it presumably cannot recognise the substrate.

Hence, the phleomycin amplifiers reported were investigated as inhibitors of the excision repair mechanism. The excision repair mechanism was studied by measuring the liquid holding recovery of UV-irradiated E.coli B as described in the introduction (chapter 1. this thesis).

4.2 MATERIALS AND METHODS

The method of Harm (27) was adopted as follows:-

4.2.1 BACTERIAL STRAIN

E.coli B (supplied by the Department of Genetics, University of the Witwatersrand, Johannesburg).

4.2.2 GROWTH AND DILUTION OF BACTERIA

All cultures were freshly grown overnight in 0.8%

ent Broth in a 37°C incubator shaker. The suspension at stationary phase was then inoculated in fresh medium (0.8% nutrient broth) and grown to logarithmic phase (2 hours). The cells were immediately collected by centrifugation, washed and resuspended in phosphate buffer of the following composition: 19.5mM-Na_2HPO_4,22mM-KH_2PO_4, 85mM-NaCℓ and 2mM-$MgSO_4$. ($MgSO_4$ was autoclaved separately before making up the buffer to avoid precipitation).

4.2.3 ULTRAVIOLET RADIATION

Irradiation was carried out with an ultraviolet lamp at a dose rate of 0.10 ergs/mm^2/sec. (6.25J/m^2) at 254nm.

4.2.4 KILL CURVE

Cell samples were irradiated in sterile petri dishes with continuous agitation to ensure uniform irradiation. All irradiation was carried out in the dark while post-irradiation treatment was carried out under yellow light (545nm) to prevent photoreactivation. 100 µℓ aliquots were removed after various time intervals of irradiation to monitor cell survival.

4.2.5 LIQUID HOLDING

Bacteria were irradiated at the above mentioned dosage for 12.5 seconds to yield 20% cell survival as determined from the kill curve. Irradiated samples (or unirradiated control samples at the same concentration) were diluted 1:10 into phosphate buffer or phosphate buffer containing 1 mM purine analogue and kept at room temperature (24°C) for various periods of time.

4.2.6 CELL VIABILITY

Suspension of bacteria, suitably diluted in 0.9% saline , were spread on a nutrient agar medium and the petri dishes incubated at 37°C. All plating and dilutions were rapidly performed immediately the samples were withdrawn from the experimental cultures.

4.3 RESULTS

The kinetics of UV-inactivation of E.coli is shown in Fig . 19.

The results of the liquid holding in phosphate buffer (free of analogue) are shown in Figure 20 . As described by Harm (27 , most of the liquid holding recovery is found within 4 hours after irradiation, holding for still longer periods adds relatively little to the effects. Control suspensions of unirradiated cells, held in buffer for the same periods of time, showed very little change in cell viability. Furthermore, the analogues had no significant effect on the survival of the control suspensions of unirradiated cells held in analogue containing buffer (Table XII).

Table XIII shows the effects of the purine analogues on liquid holding recovery.

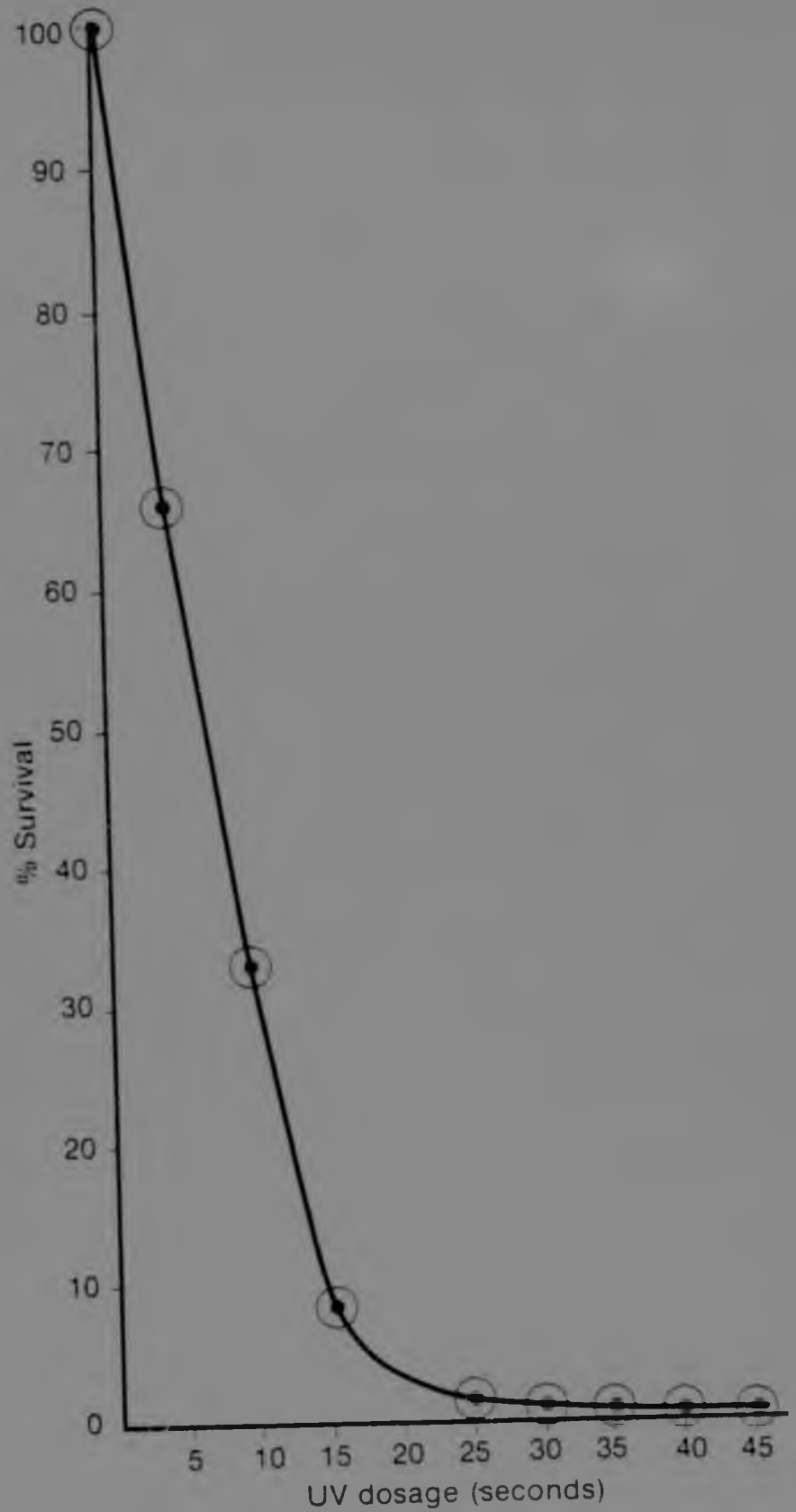


Fig. 19 The effect of time of irradiation on survival of E coli.

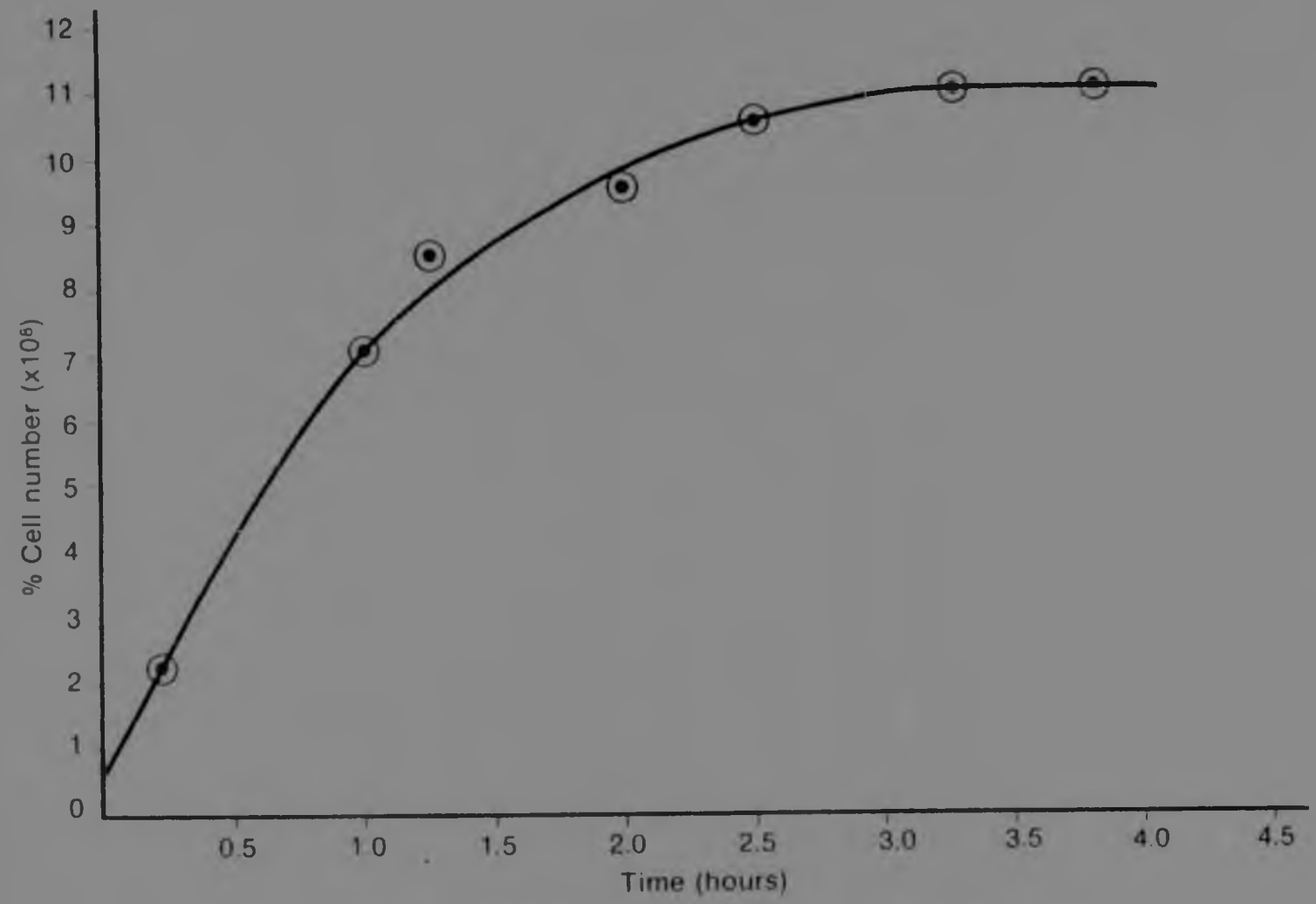


Fig. 20 UV survival of E coli B as a function of the time of liquid holding in buffer before plating.

TABLE XII INFLUENCE OF PHLEOMYCIN AMPLIFIERS ON THE SURVIVAL OF THE CONTROL SUSPENSIONS OF UNIRRADIATED CELLS HELD IN ANALOGUE CONTAINING BUFFER FOR 3 HOURS

ANALOGUE	% SURVIVAL
2-α-Carbamoylmethylthio-6,9-dimethylpurine	94
2-Carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine	91.5
2-Carbamoylmethylthio-6,8,9-trimethylpurine	93
2-α-Carbamoylethylthiobenzothiazole	29
2-α-Carbamoylethylthio-s-triazole-[1,5-a]-pyrimidine	96
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	98
2-α-Carbamoylethylthiobenzoxazole	93.5
2,5-Biscarbamoylmethylthio-7-methylthiazolo-[5,4-d]-pyrimidine	95
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	93.5
3-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[4,3-c]-pyrimidine	97
2-Carbamoylmethylthio 5,7-dimethyl-s-triazolo -[1,5-c]-pyrimidine	90
4-Carbamoylmethylthio-1-methyl-1H-pyrazolo-[3,4-d]-pyrimidine	94.5
1,3,7-Trimethylxanthine (caffeine)	25

TABLE XIII INFLUENCE OF PHLEOMYCIN AMPLIFIERS ON THE LIQUID HOLDING RECOVERY OF E coli B CELLS IRRADIATED WITH UV FOR 12.5 SECONDS

ANALOGUE	% SURVIVAL	% INHIBITION OF LHR
2-α-Carbamoylmethylthio-6,9-dimethylpurine	7	93
2-Carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine	9	91
2-Carbamoylmethylthio-6,8,9-trimethylpurine	8	92
2-α-Carbamoylethylthiobenzothiazole	9	91
2-α-Carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine	47	53
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	47	53
2-α-Carbamoylethylthiobenzoxazole	7	93
2,5-Biscarbamoylmethylthio-7-methylthiazolo-[5,4-d]-pyrimidine	12	88
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	86	14
3-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[4,3-c]-pyrimidine	40	60
2-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[1,5-c]-pyrimidine	33	67
4-Carbamoylmethylthio-1-methyl-1H-pyrazolo-[3,4-d]-pyrimidine	29	71
1,3,7-Trimethylxenthine (caffeine)	54	46

TABLE XIV PHLEOMYCIN AMPLIFICATION ACTIVITY IN RELATION TO THE ABILITY TO INHIBIT EXCISION REPAIR

ANALOGUE	% INHIBITION OF EXCISION REPAIR	AMPLIFICATION ACTIVITY (ADJUSTED TO CAFFEINE)
2-α-Carbamoylmethylthio-6,9-dimethylpurine	92	510
2-α-Carbamoylethylthio-benzoxazole	92	31/2mM 98/8mM
2-Carbameylmethylthio-6,8-9-trimethylpurine	91	130
2-α-Carbamoylethylthio-benzothiazole	90	125
2,5-Biscarbamoylmethylthio-7-methyl thiazolo-[5,4-d]-pyrimidine	88	650
4-Carbamoylmethylthio-1-methyl-1H-pyrazole-[3,4-d]-pyrimidine	71	7 /2mM 34/8mM
2-α-Carbamoylethylthio-s-triaxolo-[1,5-a]-pyrimidine	52	100/8mM 100/2mM
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	52	275/8mM:85/2mM
1,3,7-Trimethylxanthine (caffeine)	46	30
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	14	275/8mM 55/2mM

4.4 DISCUSSION

An association between phleomycin induced cell death and the inhibition of dark repair processes has been reported by Grigg (10). Furthermore, some phleomycin amplifiers tested by him (e.g. caffeine, coumarin) were found to inhibit the excision repair processes (19).

In this study a series of new phleomycin amplifiers were found to inhibit the excision repair mechanism of UV-irradiated E.coli.

Many studies (10,19) have reported the inhibition of excision repair by caffeine which binds specifically to single-stranded DNA (54). Domon et al. (56) concluded that this property is important in explaining inhibition of excision repair. The phleomycin amplifiers, coumarin, pyronin Y and 6,9-dimethyl-2-methylthiopurine reported by Grigg (19) to be inhibitors of excision repair share with caffeine the ability to bind specifically to single-stranded DNA (19,54,14). However, the order of ranking of the inhibitors of excision repair differed to that of their relative efficiencies as amplifying substances.

The results reported here also indicate no ordered pattern between phleomycin amplification activity and inhibition of excision repair. This is illustrated in Table XIV which lists a few of the analogues in decreasing order of their ability to inhibit excision repair.

CHAPTER FIVE

EFFECTS OF THE PHLEOMYCIN AMPLIFIERS AT THE MOLECULAR LEVEL

5.1 INTRODUCTION

Many workers have suggested that the activity of phleomycin occurs through a direct effect on DNA, though a precise mechanism has not been established. Stern et al. (79) have shown on sucrose density gradients the ability of phleomycin to act directly upon viral DNA resulting in single-strand breaks. Degradation of DNA in the presence of phleomycin has also been observed in bacteria (10). Phleomycin may act either directly in breaking DNA or, as suggested by Grigg (10), indirectly by activating an endogenous DNA endonuclease. Native DNA, treated in vitro with phleomycin and/or caffeine produced sites sensitive to the single-strand specific endonuclease purified from Neurospora crassa (13). Kato and Fraser (80) have shown ultra-violet irradiated DNA to be sensitive to this enzyme. They thus concluded that the enzyme recognised the DNA as single stranded where clusters of pyrimidine adducts occurred Phleomycin-thymine complexes could have similar effects on the secondary structure of DNA.

To obtain a stable interaction between any molecule and DNA, the free energy of the molecule-DNA complex must be lower than for the separated components. The decrease in entropy must be overcome. The attainment of favourable free energies of interaction between ligands and nucleic acids require the involvement of a number of potential intermolecular forces. These include:

(a) hydrogen bonding,
(b) charge-charge (electrostatic) interactions,
(c) hydrophobicity,
(d) aromatic ring stacking interactions, and
(e) van der Waals forces

Of the forces, charge-charge and stacking provide the greatest contribution to the free energy of binding (81).

Various techniques have been employed to study the interaction of DNA with phleomycin and/or phleomcin amplifiers in an attempt to elucidate their mode of action:

(i) optical density
(ii) circular dichroism
(iii) electron microscopy
(iv) viscosity

5.1.1 OPTICAL DENSITY

The complementary polynucleotide strands of DNA form the double helix by non-covalent bonds. The non-covalent bonds consist (81) mainly of

(i) hydrogen bonds between adenine (A) and thymine (T) or guanine (G) and cytosine (C), and
(ii) stacking forces between the purine and pyrimidine rings.

The free energy of hydrogen bonding is about -3kcal (G.C.) or -2kcal (A.T.) per mole of base pair while the stacking free energy corresponds to about -7kcal per mole of base pair. Under physiological conditions, the double helix is stable. However, drastic changes, such as large increases in temperature or changes of pH disrupt both interactions and hence destabilize the double helix causing DNA denaturation. According to Szybalski (82) denaturation consists principally of three overlapping steps:

(i) collapse of the hydrogen bonded double-helical structure;
(ii) collapse of base stacking; and
(iii) dissociation or complete separation of the complementary strands.

In the resulting single strands, hydrogen-bond donor and acceptor sites are immediately occupied by water molecules. The energy difference between the different states contributes to the stability of the double stranded conformation. Therefore, stacking interactions are the main stabilizing force (83). The denaturation is accompanied by marked changes in the physical and chemical properties of the DNA, which may be followed by means of various techniques (e.g. circular dichroism). The most frequently used technique in DNA denaturation studies is absorption spectrophotometry: the optical density at 260nm is measured as a function of temperature. A curve as shown in Fig.21 is obtained.

The increase of optical density in the region B-C is connected with the disturbance of interaction of electrons between bases, owing to the collapse of the double-helical structure. Conformational changes occuring in the region B-C are quickly reversed if the temperature is decreased. Changes in the region C-D are basically irreversible. However, if the DNA solution contains a relatively homogeneous population of molecules (bacterial DNA) these changes, under special conditions, can be slowly reversed to a great extent (renaturation).

It has also been shown that the base composition of DNA is related to its thermal denaturation temperature. The midpoint of the transition resulting from the increased temperature has been

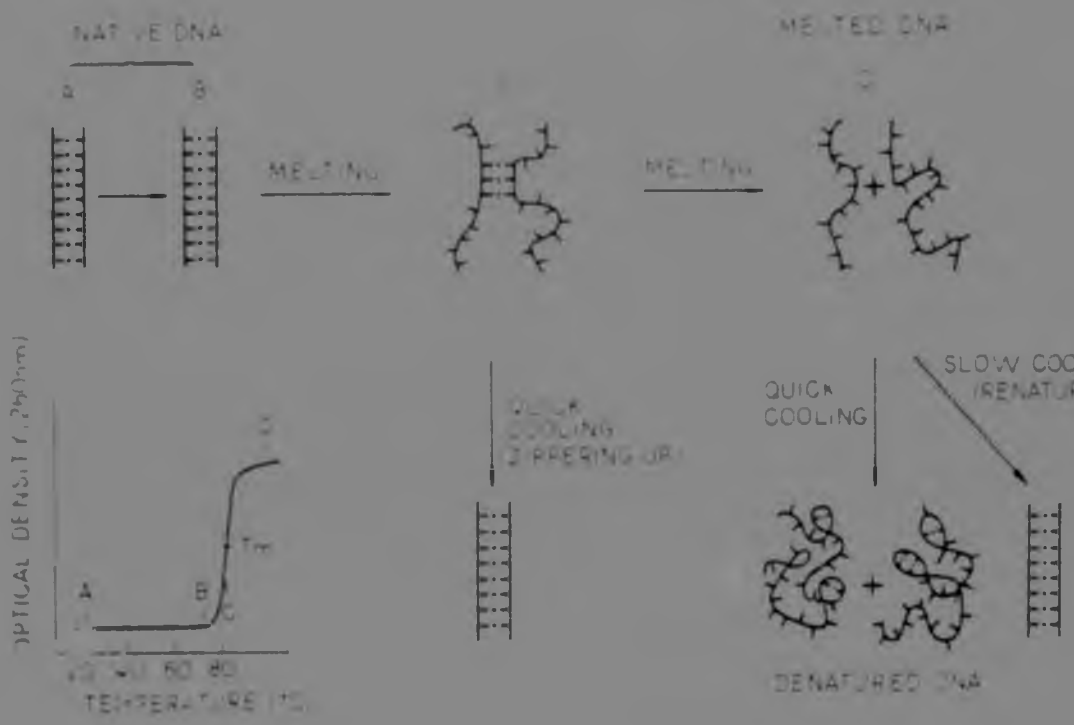


Fig. 21 Schematic presentation of DNA thermal helix-coil transition.

termed Tm (the helix-coil transition temperature). The Tm is linearly related to the average base composition - a higher guanine-cytosine (G-C) content confers a higher thermal stability. The mole per cent guanine-cytosine of polymerised DNA may be determined (under specified conditions, e.g. ionic strength) from the relationship:

$$Tm = 69.3 + 0.41\ (G-C)$$

Ts'O and Lu (54) have shown that the Tm is lowered by purines, pyrimidines, nucleosides, their analogues, and other derivatives. The order of their effectiveness indicated that hydrophobic and stacking interactions were important. Equilibrium dialysis experiments have shown that these compounds interact more strongly with the single-stranded coil form of nucleic acids than with the double-stranded helical form, thus shifting the equilibrium in favour of the coil form. They thus assumed that this difference of affinity provides the driving force for the compounds as denaturing agents.

In this study, the effect of various phleomycin amplifiers on the Tm of E.coli DNA has been investigated by measuring the optical density at 260 nm as a function of temperature. The influence of these compounds on the optical activity of DNA has also been measured by monitoring changes in circular dichroism. Circular dichroism measurements have the special feature that the added highly UV - absorbing substances which are optically inactive do not interfere with the measurements. The effect of these analogues on the gross structure of DNA was also investigated by viscosity measurements and electron microscopy.

5.1.2 CIRCULAR DICHROISM (CD)

5.1.2 a INTRODUCTION

Three classes of biopolymers, nucleic acids proteins and polysaccharides are optically active. The interest in the optical properties of these polymers is not in the rotations arising from the asymmetric configuration, but in correlating the optical activities with the conformations of these polymers (i.e.the secondary, tertiary and quarternary structures). The constituent pentoses of DNA and RNA, deoxyribose and ribose, are optically active. But it is the induced optical activity of the base chromophores attached to the pentoses that provide information concerning the overall molecular architecture of the nucleic acids.

5.1.2 b DEFINITION OF CIRCULAR DICHROISM

Polarized light is employed in the measurements of optical activity. Linearly polarized light may be regarded as the resultant of right and left circularly polarized light of the same frequency. The two components are of equal magnitude but rotate in opposite directions.

When linearly polarized light is passed through an optically active medium, the intensity of both circularly polarized light beams is reduced. If they are absorbed unequally, circular dichroism results, i.e. the difference in absorbance between the two circular polarizations.

5.1.2 c MATHEMATICAL RELATIONS FOR CIRCULAR DICHROISM

Circular dichroism (84,99) results from a differential absorption of left and right-circularly

polarized light by a sample that exhibits molecular assymmetry. This can be expressed as

$$A = A_L - A_R \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where A_L: absorbance of left-circularly polarized light

A_R: absorbance of right-circularly polarized light

Note: CD occurs only in a region of the spectrum where the sample absorbs whereas circular birefringence occurs in all wavelength regions of an optically active substance.

The circular dichroism (equation 1) is defined as the corresponding molar absorptivity (extinction co-efficient) difference, or by analogy with the definition of absorbance (optical density):

$$\varepsilon_l - \varepsilon_r = \Delta\varepsilon_\lambda = \frac{\Delta A_\lambda}{C\ell} \quad \ldots\ldots\ldots\ldots (2)$$

where C : molar concentration

ℓ : path length in cm

λ : wavelength in nm

The CD is sometimes expressed in plots of $\Delta\varepsilon$. Commonly, however, the ellipticity ($deg.cm^2$ $decimol^{-1}$) is the quantity plotted. For the ellipticity of plane polarized light after passage through the optically active medium is another measure of circular dichroism. The ellipticity, written $[\Theta]$ is related to circular dichroism by

$$[\Theta]_\lambda = 3300\Delta\varepsilon_\lambda$$

and emerges in the units of degrees cm^2/decimole.

5.1.2 d NUCLEIC ACIDS : DNA

Many workers (84) have reported the CD spectrum of DNA with one positive band at about 273 nm and one negative band at about 243 nm. The optical activity of the nucleic acid is the sum of the contributions from the monomeric units and the polymeric conformation. By comparing the circular dichroism of the native polymer to that of its monomeric units, the contribution from the interactions in the native polymer conformation may be calculated. This is illustrated for DNA and RNA in Fig.22. The dramatic difference between the solid and dashed curves demonstrates that the principal contribution to the CD of the nucleic acids arises in the conformaticnal structure of the polymer.

Loss of the double-stranded structure (due to thermal denaturation) decreases the amplitude of both bands. On the contrary,elevation of temperature in the premelting zone increases the positive CD band. The premelting behaviour is qualitatively the same for all DNAs examined (e.g.calf thymus, phage, bacterial, salmon sperm) (85,86-91). In contrast to double stranded (ds) DNA, denatured DNA shows a linear decrease of the positive CD band (85,86) with increase of temperature in the premelting zone.

The conformation of the DNA is thus important in describing the Cotton effects. The configurations of the different constitutents is described below in order to understand the known conformations of ds DNA.

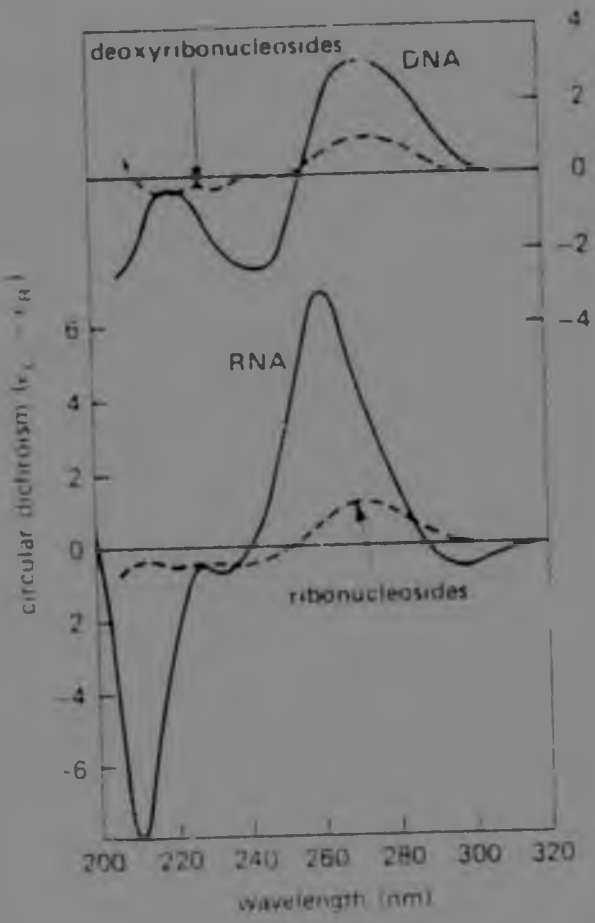


Fig. 22 The circular dichroism of double-stranded DNA and RNA compared with their component mononucleosides. *M. lysodeikticus* DNA data from Allen et al., *Biopolymers*, 11, 853 (1972). Rice dwarf virus RNA data from Samejima et al., *J. Mol. Biol.*, 34, 39 (1968). Nucleoside spectra calculated from the base composition (72% G + C for the DNA, 44% G + C for the RNA) and the C D data of Cantor et al., *Biopolymers*, 19, 1059, 1079 (1970). (from Bloomfield et al (84)).

5.1.2 d.i NUCLEOSIDES

DNA contains 2'deoxynucleosides, i.e. they are - D-2'deoxyriboncleosides.

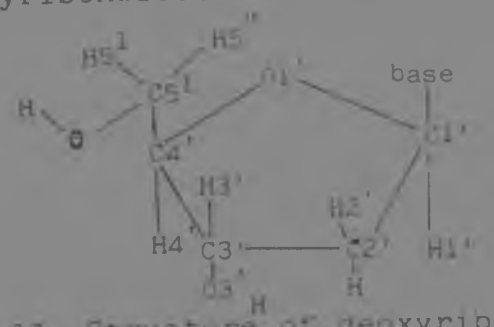


Fig.23. Structure of deoxyribonucleoside

The base is attached in the β- configuration, i.e. the base is on the same side of the sugar ring as the 5' carbon (Fig.23).

5.1.2 d.ii ORIENTATION OF THE RIBOSE MOEITY

X-ray crystal data show that four of the sugar ring atoms form a plane to within ± 0.04Å and the fifth atom is significantly displaced. The four nearly planar atoms are C1',C4',O1' and either C2' or C3'. If the out of plane ring atom is on the same side of the plane as the 5' carbon, the conformation is designated 'endo'. The conformation is 'exo' if the out-of-plane ring atom is on the opposite side from the 5' carbon. This is illustrated in Fig.24.

X-ray data for crystals and the NMR data for solutions indicate that either C2' or C3' will be out of plane. The energy differences between the various endo and exo forms and the barriers to interconversion in solution are not known.

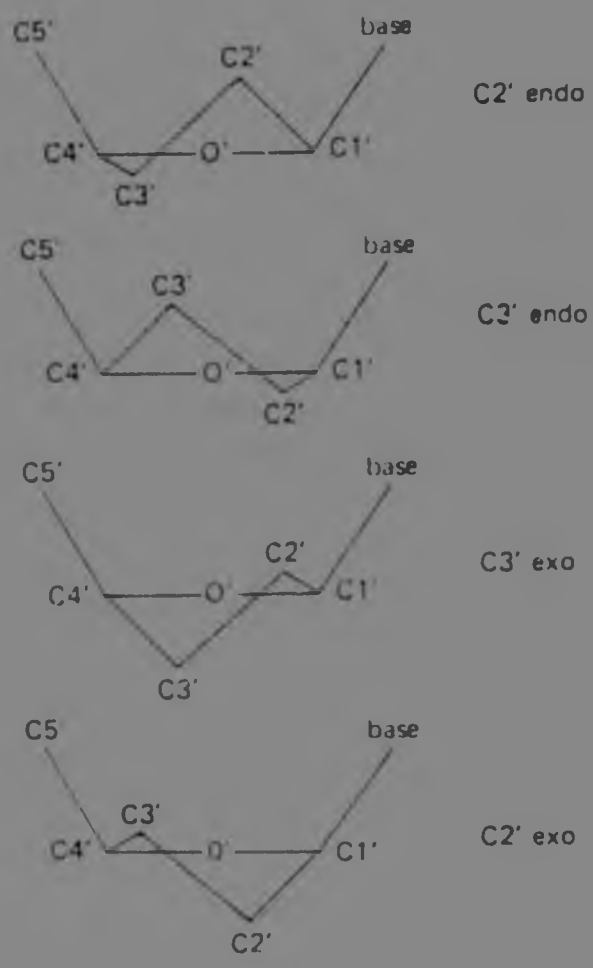


Fig. 24 The conformation of the sugar rings found in crystals of various nucleosides. Four atoms of the sugar ring are nearly in a plane: C1', C4', 01', and either C2', or C3'. The out-of-plane atom is designated *endo* or *exo* as illustrated. (from Bloomfield et al (84)).

5.1.2 d.iii ORIENTATION OF THE BASE RELATIVE TO THE SUGAR

It has been shown by X-ray analysis that the carbonyl oxygen (O2) of cytosine and uracil is away from the ribose. This conformation is called 'anti'. Purine nucleosides are mainly 'anti' with the six membered ring of the purine away from the ribose, but some are 'syn'.

Calculations by Haschemeyer and Rich (111) of the possible conformations on the basis of van der Waals contacts between atoms are in agreement with the published data for crystals. All the pyrimidine nucleosides are anti, but some purine nucleosides are syn. Pyrimidines are more hindered in the syn position because the O_2 carbonyl oxygen crowds the H2' and H3' of the sugar whereas the N3 of the purines is further away.

Miles et al. (112,113) have concluded that the sign of the CD band near 260 nm is a good measure of the conformation around the glycosidic bond. For pyrimidine nucleosides there is a positive CD band for the anti conformations and a negative CD band for syn. For purines, the sign of the CD is reversed.

5.1.2 d iv DNA CONFORMATION IN SOLUTION

The geometries of three classical DNA forms have been found experimentally in X-ray fibre studies (92,93). It should be noted that the sequence of nucleotides dictates the properties of DNA and, in some cases the conformation of the DNA (94).

1 A-Form of DNA

A-DNA is formed at low humidities with Na^+ or K^+ as counterion. A-DNA is characterized by furanose rings in the C3'-endo conformation with the bases tilted substantially (about 20°) from the axis of

the helix. The helix of the A-DNA has a larger diameter (i.e.greater distance between the helical axis and the paired bases) and the large groove is deeper and narrower. The sugar rings in the A-form are incorporated into the main helical chain as can be seen in Fig.25.

2 B-Form DNA

At high humidities and in the presence of excess salt, DNA assumes the B configuration which is characterized by the furanose rings in a standard C3'-exo conformation. The bases are nearly perpendicular to the axis and the sugar rings are situated radially from the helical axis (Fig.24).

3 C-Form DNA

At low humidities lithium-DNA assumes the C-form which is closely related to the B-form, (see Table XV).

4 Other forms of DNA

Many workers have suggested that DNA secondary structure does depend on the base composition and that the A,B and C forms are not the only ones in which DNA can exist.

5.1.2 e INTERACTION OF SMALL MOLECULES WITH DNA AS DETECTED BY CD

Polylysine distorts the DNA helix (95), decreasing the maximum and increasing the minimum amplitudes of the DNA CD spectra (96). This has been attributed to dehydration and charge neutralization of DNA leading to a DNA conformational change from B to a combination of B and C (96) forms.

Intercalation of aromatic planar molecules alters the conformation of single- and double-stranded DNA polymers (97). It has been suggested that intercalation would increase the distance between nucleotides and alter the base stacking. Tyramine

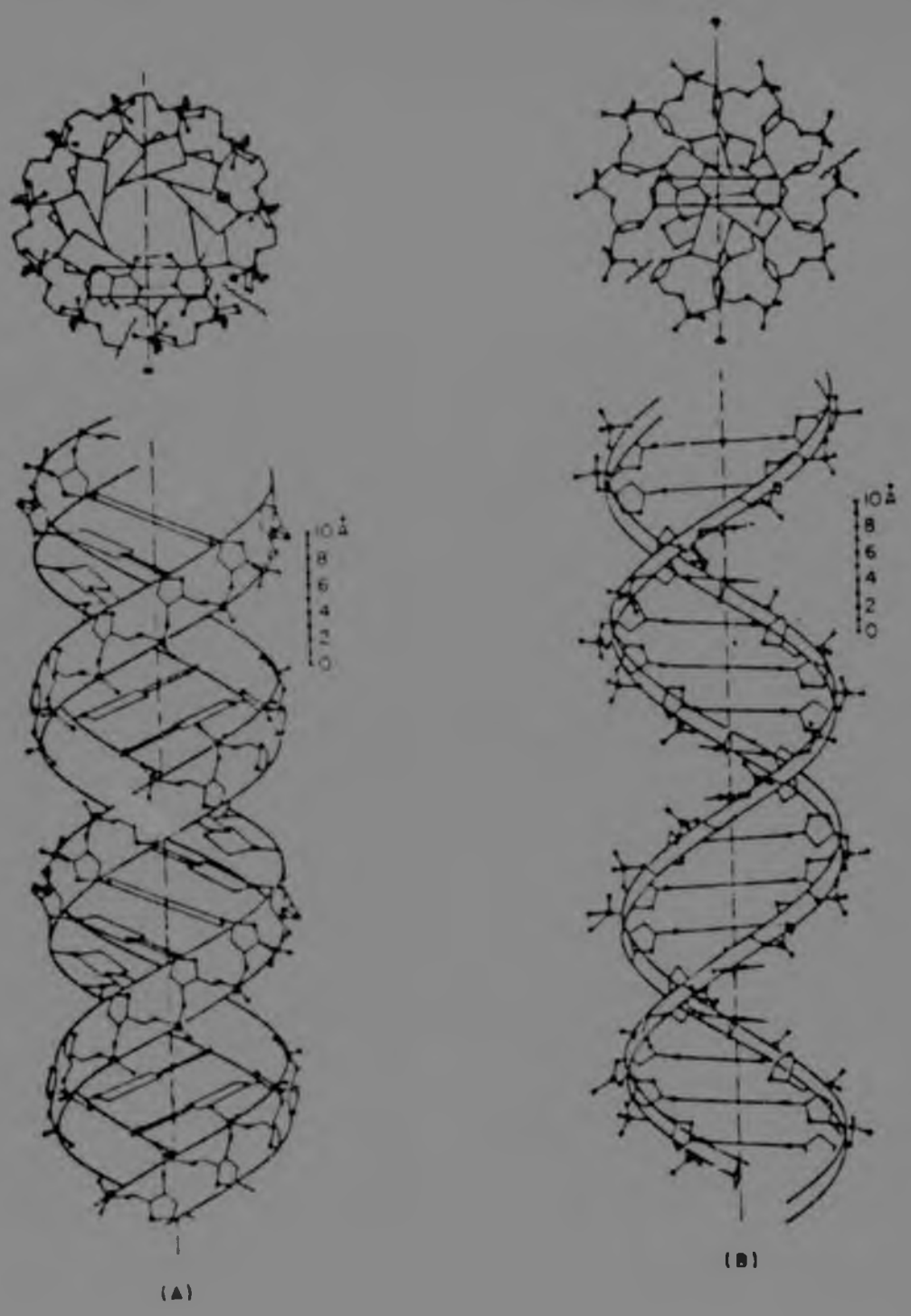


Fig. 25 Double stranded helical model of nucleic acid (A) A-Form of DNA; (B) B-form of DNA.

TABLE XV HELIX PARAMETERS OF THE CLASSICAL DNA CONFIGURATIONS

Helix type	Pitch(A)	Residues/ Turn	Rotation/residue (degrees)	Translation/ Residue (A)	Tilt (deg)	Twist (deg)
A-DNA	28.15	11	32.7	2.56	20	-8
B-DNA	33.7	10	26.0	3.37	-2	5
C-DNA	33.2	9.3	38.6	3.32	-6	5

TABLE XV HELIX PARAMETERS OF THE CLASSICAL DNA CONFIGURATIONS

Helix type	Pitch(A)	Residues/ Turn	Rotation/residue (degrees)	Translation/ Residue (A)	Tilt (deg)	Twist (deg)
A-DNA	28.15	11	32.7	2.56	20	-8
B-DNA	33.7	10	36.0	3.37	-2	5
C-DNA	33.2	9.3	38.6	3.32	-6	5

intercalation in calf thymus DNA decreases the amplitude of the maxima and minima of the CD spectra of the B-conformation. This has been interpreted as a decrease in base-stacking interactions (98).

VISCOMETRY

A number of hydrodynamic techniques; for example, ultracentrifugation, viscosity and molecular-sieve chromatography, may be employed for obtaining information on the size and shape of biopolymers (99). Viscosity measurements may be employed to detect small changes in the macromolecular shape when small molecules bind to nucleic acids. Many workers have suggested that the intercalating drugs increase the viscosity of DNA by producing either an increase in molecular length or an increase in chain stiffness, or both.

Viscosity measurements have a special feature as noted by its experimental simplicity. In most cases, the capillary viscometer suffices for accurate measurement of the viscosity of solutions. However, the disadvantage entails the shearing forces generated by the flow gradients in a typical capillary viscometer which are quite large. Much lower shear stresses can be obtained by using a rotating cylinder viscometer (100).

The intrinsic viscosity [η] is expressed as follows(101):

$$[\eta] = 2.5 (\bar{V}_2 - \delta_1 \bar{V}_1)$$

where $\bar{V}_2$: partial specific volume of solute

$\bar{V}_1$: partial specific volume of water

δ_1: hydration in grams solvent per grams solute.

The absence of any dependence on molecular weight is clear, and this independence has been confirmed experimentally. However, although there is no molecular weight dependence, viscosity is very sensitive to shape. For coiled molecules, it is a function of the dimensions of the coil and of the extent to which solvent can drain freely through the coil without becoming entrapped by hydrodynamic interactions. Hydrodynamic properties of chain molecules are sensitive to chain conformation. In particular, the intrinsic viscosity of random coils is related to the mean square and end-to-end distance, so that viscosity measurements provide a convenient way to evaluate chain dimensions of coils. Frictional and sedimentation coefficients are also sensitive to chain dimensions. Most important, the dependence of molecular weight of the intrinsic viscosity (or frictional or sedimentation coefficient) can be used to determine whether a chain behaves as a random coil or as another shape and character. However, some of the hydrodynamic parameters are interrelated through the Mandelkern-Flory-Scheraga constant in a way that is particularly useful for calculating molecular weights from a combination of viscosity and sedimentation measurements (100). In the case of double-stranded DNA, the molecular weight dependence of the intrinsic viscosity and of sedimentation coefficient has been studied. The results show that up to molecular weights of approximately 10^6 daltons, the molecule exhibits quasi rod behaviour and that, above this molecular weight, it is more like a coil. This suggests that

the chain is a stiff, wormlike coil. Such coils are conveniently treated in terms of an artificial model known as the Porod-Kratky chain (100). This model gives a useful description of DNA conformation and dimensions.

5.1.4 ELECTRON MICROSCOPY

Direct visualization of nucleic acids has proved to be an important technique to study a variety of problems. Some of the information yielded include the direct measurement of single-stranded DNA (102), the analysis of partially denatured DNA (103), the replication and recombination complexes (104) and the physical mapping of genes in nybrid molecules (105). In this study the interaction of phleomycin and/or phleomycin amplifiers with DNA was investigated.

5.2 MATERIALS AND METHODS

5.2.1 ISOLATION OF DNA FROM E.coli

Method: The method of Marmur (59) was adopted. E.coli was a gift from S.Goldstein (Department of Biochemitsty, University of the Witwatersrand), and all chemicals used were of standard grade.

Reagents: (Note: The procedure adopted for 100g wet packed cells is described).

(1) Saline-EDTA: 0.15 M NaCl + 0.1M EDTA, pH = 8
(2) 25% Sodium dodecyl sulphate.,
(3) 5M Sodium perchlorate
(4) $CHCl_3$: Isoamyl alcohol (3:1)
(5) EtOH : 96%
(6) Concentrated saline citrate:1.5M -NaCl + 0.15 M trisodium citrate.

(7) Saline citrate : 0.15M-NaCl + 0.015M Na_3citrate:
Dilute "concentrated saline citrate" one to ten time and adjust pH to 7.0 ± 0.2

(8) Dilute saline citrate : 0.015M -NaCl + 0.0015 M Na_3citrate:
Dilute "concentrated saline citrate" 100 times

(9) Ribonuclease : 0.2% - 0.15M NaCl, pH 5.0

(10) Acetate-EDTA 3M NaAc + 0.001M EDTA pH 7.0

(11) Isopropanol

6.2.1 b <u>ISOLATION PROCEDURE</u>

The volumes are only approximate unless otherwise stated. All operations were carried out at room temperature, except the RNAse treatment, which was carried out at 37°C.

Bacteria: tryA2/F' try A2 strain of <u>E.coli.</u>

Bacteria grown to the logarithmic phase of their growth cycle were harvested by centrifugation (8000rpm for 40 minutes in a Sorvall GSA rotor) and washed once with 2.5 volumes saline EDTA. After collecting by centrifugation, the cells were suspended in a total volume of 1250 ml of saline-EDTA. Lysis was effected by the addition of 100 ml SDS, heating the mixture in a 60°C water bath for 10 min, and then cooling to room temperature. (Lysis of the culture resulted in a dramatic increase in viscosity accompanying the release of the nucleic acid components and some clearing).

Perchlorate was added to a final concentration of

1M to the viscous. lysed suspension and the whole mixture shaken with an equal volume of $CHCl_3$: isoamyl alcohol in a 5ℓ flask for 30 minutes. The resulting emulsion was separated into 3 layers by a 5 min centrifugation at 8 000 rpm in a Sorvall GSA rotor.

The upper aqueous phase containing the nucleic acids was carefully pipetted into a 2ℓ flask by water pump suction and then transferred to a 5ℓ beaker. The nucleic acids were then precipitated by gently layering approximately 2 volumes of ethanol on the aqueous phase. When these layers were gently mixed with a stirring rod, the nucleic acids "spooled" on the rod as a threadlike precipitate. The precipitate was drained free of excess alcohol by pressing the spooled rod against the vessel. The precipitate was then transferred to 750 ml of dilute saline citrate and gently removed from the stirring rod by swirling it back and forth. The DNA was dissolved using a magnetic stirrer (slow speed). The solution was adjusted to the standard saline citrate concentration by adding concentrated saline citrate, shaken as before with an equal volume of $CHCl_3$: isoamyl alcohol for 15 min, centrifuged and the supernatant removed. It was then deproteinized repeatedly with chloroform-isoamyl alcohol, as described earlier until very little protein was observed at the interface, and three repeated extractions were required.

The supernatant obtained after the last of the series of deproteinizations was precipitated with EtOH and dispersed in saline citrate (0.5 to 0.75 the volume of supernatant, in this case 850 ml), in the manner already described (i.e. ± 350 ml). Ribonuclease was added to a final concentration of 50 µg/ml and the mixture incubated

at 37°C/30 min. The digest was again subjected to a series of deproteinizations until there was little or no denatured protein visible at the interface after centrifugation (twice). Ribonuclease was again added to a final concentration of 50 µg/ml and the mixture incubated at 37°C for 30 min. The digest was again subjected to a series of deproteinization steps. (Ribonuclease treatment need not be repeated but was done so as to ensure greater purity).

The supernatant, after the last treatment, was again precipitated with EtOH and the drained nucleic acid dissolved in 500 ml dilute saline citrate. Once dissolved, 50 ml acetate-EDTA was added and while the solution was rapidly stirred, 0.54 volumes isopropyl alcohol was added dropwise into the vortex (a burette was used for the isopropyl alcohol addition). The DNA threads wound around the glass propeller after about 0.5 volumes of isopropyl alcohol addition. It was necessary to use three rods in succession to ensure collection of all the DNA.

The final precipitate was washed free of acetate and salt by gently stirring the adhered precipitate in ethanol-water mixtures of progressively increasing ethanol concentrations (70% - 95%). The DNA was then dissolved in 195 ml of saline-citrate buffer at pH 7.0.

5.2.2 DETERMINATION OF THE T_m (THERMAL DENATURATION TEMPERATURE) OF E.coli DNA

5.2.2 a MATERIALS AND EQUIPMENT

(i) Spectrophotometer

A Gilford model 2000 UV-visible spectrophotometer

was used with 3 ml quartz cuvettes of 1 cm path length. Temperature control was effected using the thermostatted cuvette chamber assembly as shown in Fig 26. The assembly is fitted with dual thermo-spacer plates (a,a') as well as with insulating spacers (b,b') each of which is located next to the thermospacers distal to the cuvette chamber. The outer set of spacers (c,c') is cooled with a very slow flow of cold water (tap water) in order to protect the instrument. Temperature in the chamber was increased by circulating hot ethylene glycol through the inner pair of thermal spacer plates by the action of gravity (syphoning). The flow rate was adjusted intermittently to regulate temperature. Temperature measurements in the cuvette chamber were accomplished by sealing a thermo-couple into a cuvette. This thermo-couple wire was connected directly to a recorder.

(ii) DNA sample and solvent

The DNA purified from E coli was dissolved in the buffer to be employed for T_m determination. The buffer used was standard saline citrate (SSC) of the following composition: 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0. DNA was diluted with solvent to a concentration of approximately 20ug/ml. The value of $E^{\%}_{260}$ of native DNA was taken to be 200. The absorbance at 260 nm was therefore about 0.4.

This is a convenient region of the absorbance scale; it is an accurate portion of the scale and represents a concentration below that at which aggregation effects are noted.

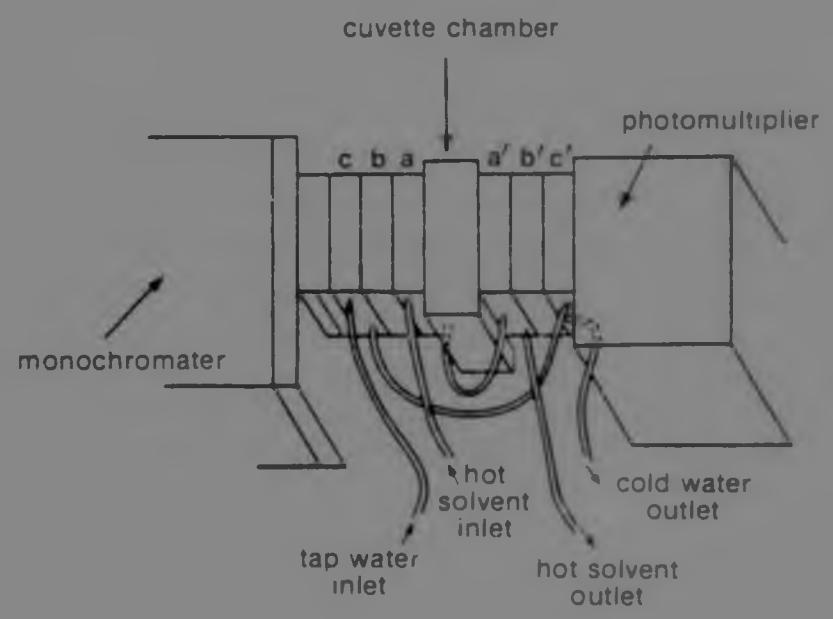


Fig. 26 An illustration of the thermostatted cuvette chamber assembly.

5.2.2 b MELTING TEMPERATURE DETERMINATION

The DNA was dissolved in standard saline citrate (59) in a 3 ml quartz cuvette with a 1 cm light path to give an absorbance of approximately 0.4-0.7 at 260 nm. The blank was standard saline citrate alone. The samples were placed in the Gilford spectrophotometer chamber and the temperature raised by approximately 1°C per minute. The absorbance at each temperature was divided by the value at 25°C and the ratio (relative absorbance) plotted against temperature. The temperature corresponding to half the sharp increase in absorbance when the double-stranded DNA denatures is designated the thermal denaturation temperature. Absorption spectra were recorded during heating (denaturation) and cooling (renaturation).

5.2.2 c MELTING TEMPERATURE DETERMINATION IN THE PRESENCE OF PURINE AND PYRIMIDINE ANALOGUES

The DNA was dissolved in standard saline citrate containing an analogue. Two blanks were required:
(i) saline citrate containing analogue at the same concentration as the standard, and
(ii) saline citrate containing DNA.

5.2.3 DETERMINATION OF THE OPTICAL ACTIVITY OF E.coli DNA: CIRCULAR DICHROISM MEASUREMENTS

5.2.3 a MATERIALS AND METHODS

(i) Materials

DNA purified from E coli by the method of Marmur and Doty was dissolved in 10 mM - Tris HCℓ, pH = 7.3 buffer (106). This buffer was also used as the blank and for dissolving the phleomycin amplifiers and the intercalating agents (see results).

(ii) Circular dichroism.

C.D. spectra were recorded at 25°C with a Jasco J-20 automatic recording spectro-polarimeter in a 1 cm light path cell. Concentration of the DNA samples were kept at 0.0294 mg/ml (approximately 0.6 absorbance units). The slit setting was on automatic mode, the time constant set at 4 seconds and the sensitivity was set on 0.002. The base line was recorded under the same conditions using the buffer.

The spectra were computed using a Hewlett Packard computer in terms of ellipticity (deg x cm^2 x $dmol^{-1}$) on the basis of DNA residue concentration, after correction for base line deviation. All spectra were recorded from 225 nm to 310 nm.

5.2.4 HYDRODYNAMIC PROPERTIES OF DNA: VISCOSITY MEASUREMENTS

5.2.4 a MATERIALS AND METHODS

Viscosities were determined with an Ostwald viscometer supplied by Poulton, Selfe and Lee Ltd. England.The viscometer was a Cannon Manning Semi-micro size and was calibrated in accordance with the standard method of test for kinematic viscosity as specified in ASTM D445-IP 71.

All measurements were carried out at 37°C and exactly one millilitre of sample was used for all experiments. At least five determinations were made to ensure that the results agreed to within 1%. Viscosity of the DNA was measured in the presence of 1 mM purine analogue.

5.2.5 ELECTRON MICROSCOPY

5.2.5 a MATERIALS AND METHODS

The protein-free method of Coetzee and Pretorius (107) involving benzyldimethylalkylammonium chloride (BAC) has been employed in investigating the interactions of various purine and pyrimidine analogues with DNA.

(i) Chemicals

The carbon rods, mica sheets and gold-palladium wire were purchased from Allan Agar Company, England. The carbon rods were spectrographically pure and the gold-palladium wire (60:40) was 0.2 mm in diameter. All chemicals were of analytical grade purchased from Merck. Uranyl acetate was obtained from Hopkin and Williams (Searle Co.). The DNA was purified from E.coli by the method of Marmur and Doty (59).

(ii) Solutions

Double glass-distilled water was used throughout. The following stock solutions were prepared:

A. 250 mM sodium acetate; 50 mM triethanolamine, pH 8, 30% aqueous formamide.
B. 0.2% (W/V) BAC in formamide.
C. 50 mM uranylacetate - 50 mM HCℓ.

The solutions were stored at 4°C.

(iii) Film preparation

Carbon films were prepared by evaporating carbon onto freshly cleaved mica sheets at $< 10^{-4}$ Torr. The films were then floated off onto redistilled water and deposited onto 300-mesh copper grids. Grids were stored on filter paper in petri dishes.

(iv) Nucleic acid preparation technique

To solution A (50 µℓ) containing DNA (0.2 µg/ml) was added 50 µℓ of solution A (or to investigate the interaction of phleomycin and/or purine analogues on DNA, 50 µℓ of solution A containing phleomycin and/or phleomycin amplifier was added), and 150 µℓ BAC (solution B freshly diluted 20 times in formamide).

Droplets of 50 µℓ were immediately formed on a parafilm sheet. The droplets were then covered with petri dishes to avoid contamination.

After 10 minutes, the droplets were briefly touched with individual grids. Each droplet was used only once. The specimen grids were exposed to double-distilled water by touching the collected droplet to the solution surface for 30 seconds. The grid were then totally submerged for 30 seconds in a 1:1500 dilution of solution C in 95% ethanol, and briefly rinsed in 90% ethanol. The grids were then air-dried on filter paper, carbon face up.

Specimens were rotary shadowed at an angle of 6-10° and 10 cm from the source.

Electron micrographs

Electron micrographs were taken on a JEM-100S microscope at 80 keV and magnification 8000 unless stated otherwise.

5.3 RESULTS

5.3.1 PURIFICATION OF E coli DNA

The yield of DNA was found to be 1.5 mg/g E coli and was of high purity as determined by the O.D. ratios. The UV spectrum of the purified DNA is

illustrated in Fig.27. The optical density ratios of the DNA purified at 260:230:280 nm was found to be 1.0:0.46:0.508. This compares favourably to reported values (57) for pure DNA.

3.3.8 THERMAL DENATURATION AND RE-ASSOCIATION OF DNA

The Tm of E.coli DNA was found to be 90- 0.2° and this value compares favourably with reported values (57). A typical curve illustrating denaturation and re-association is shown in Fig.28.

Thermal denaturation and re-association of DNA in the presence of the phleomycin amplifiers had no influence on the helix-coil transition of DNA. However the compounds could only be tested at a maximum concentration of 0.3 mM. Concentrations above 0.3 mM could not be used because of their high extinction at 260 nm. The T_m of DNA in the presence of the amplifiers is summarised in Table XVI. Caffeine, a weak amplifier of phleomycin lowered the Tm of DNA by 2° at 0.3 mM (Fig.29.)

The Tm of DNA in the presence of the amplifiers is summarised in Table XVI

3.3.9 THE OPTICAL ACTIVITY OF DNA

A typical CD spectrum of DNA recorded is shown in Fig.30 with one positive band at about 273 nm and one negative band at about 243 nm. Similar CD spectra have been reported by many workers (84). Furthermore elevation of temperature in the pre melting zone increased the positive CD band as shown in Fig. . This finding is also in agreement with reported spectra (84). Any phleomycin amplifier incubated with DNA induced significant changes in the circular dichroism spectra of native DNA.

DNA treated with 1 mM amplifier results in complete loss of the positive band and marked changes in the negative band. High concentrations of phleomycin (50µg/ml) results in complete loss of both positive and negative bands. Figure 32 illustrates the circular dichroism spectra of phleomycin and DNA. A typical spectrum obtained for DNA treated with 1 mM amplifier is shown in Fig.33. The CD spectra obtained for caffeine, and DNA treated with varying concentrations of caffeine, are also shown (Figures 34,35,36 and 37).None of the amplifiers tested proved to possess significant optical activity. Figures 38, 39, 40 and 41 illustrate the CD spectra of DNA treated with some of the phleomycin amplifiers. Furthermore, superimposing the CD spectra of DNA, amplifier and phleomycin does not contribute to a final spectrum in an additive fashion. New spectral peaks result which is a strong indication of interaction.

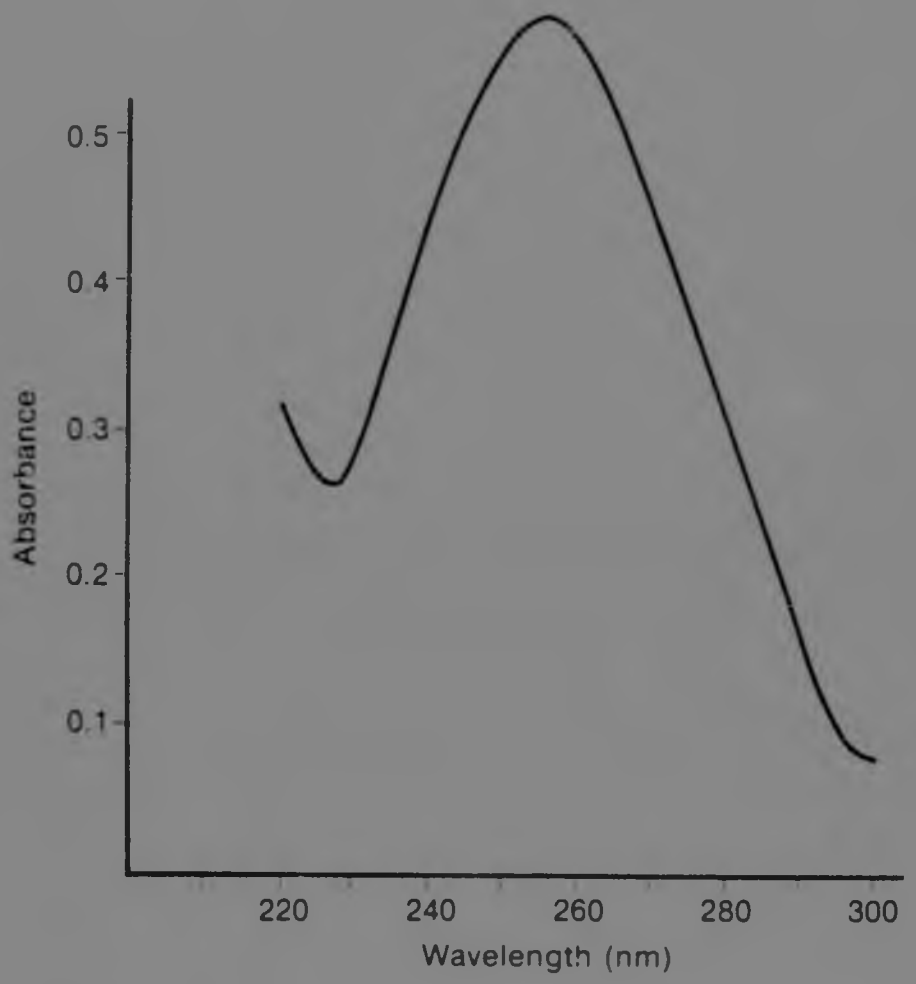


Fig. 27 UV spectrum of DNA purified from E coli

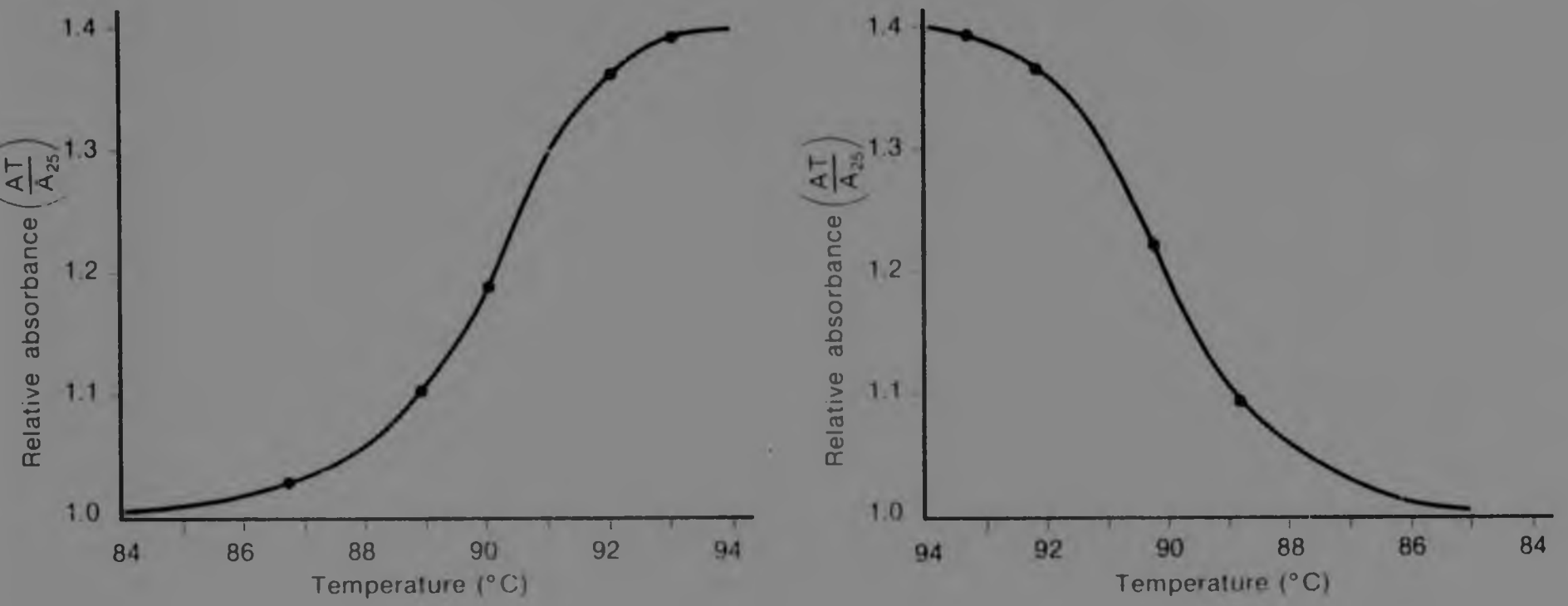


Fig. 28 Thermal denaturation and reassociation of DNA isolated from E coli.

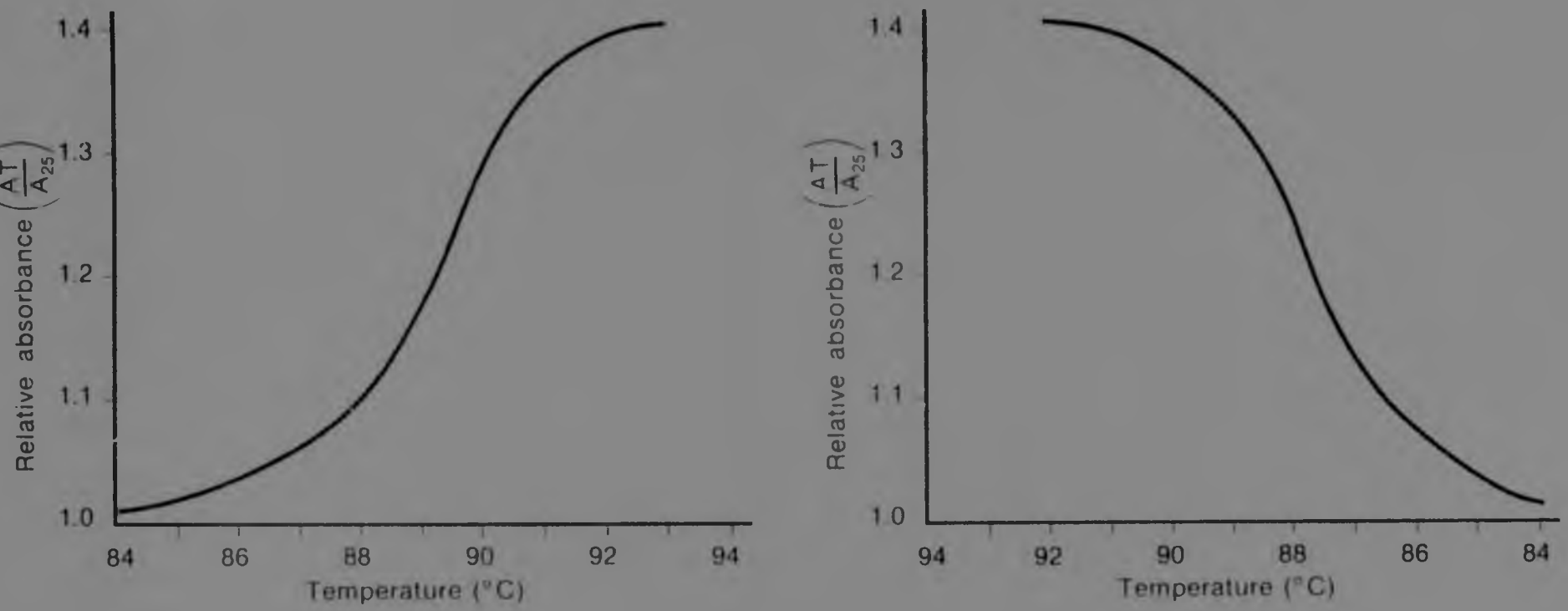


Fig. 29 Thermal denaturation and reassociation of E coli DNA in the presence of caffeine.

TABLE XVI INFLUENCE OF THE PHLEOMYCIN AMPLIFIERS ON THE Tm OF E coli DNA

ANALOGUE	T_m
2-α-Carbamoylmethylthio-6,9-dimethylpurine	89.5
2-Carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine	90.2
2-Carbamoylmethylthio-6,8,9-trimethylpurine	90.0
2-α-Carbamoylethylthiobenzothiazole	90.3
2-α-Carbamoylethylthio-s-triazolo-[1,5-1]-pyrimidine	89.8
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	90.7
2-α-Carbamoylethylthiobenzoxazole	90.2
2,5-Biscarbamoylmethylthio-7-methylthiazolo-[5,4-d]-pyrimidine	89.9
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	90.0
3-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[4,3-c]-pyrimidine	90.4
2-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[1,5-c]-pyrimidine	89.9
4-Carbamoylmethylthio-1-methyl-1H-pyrazolo-[3,4-d]-pyrimidine	89.8
1,3,7-Trimethylxanthine (caffeine)	88.1
DNA (control)	90.0

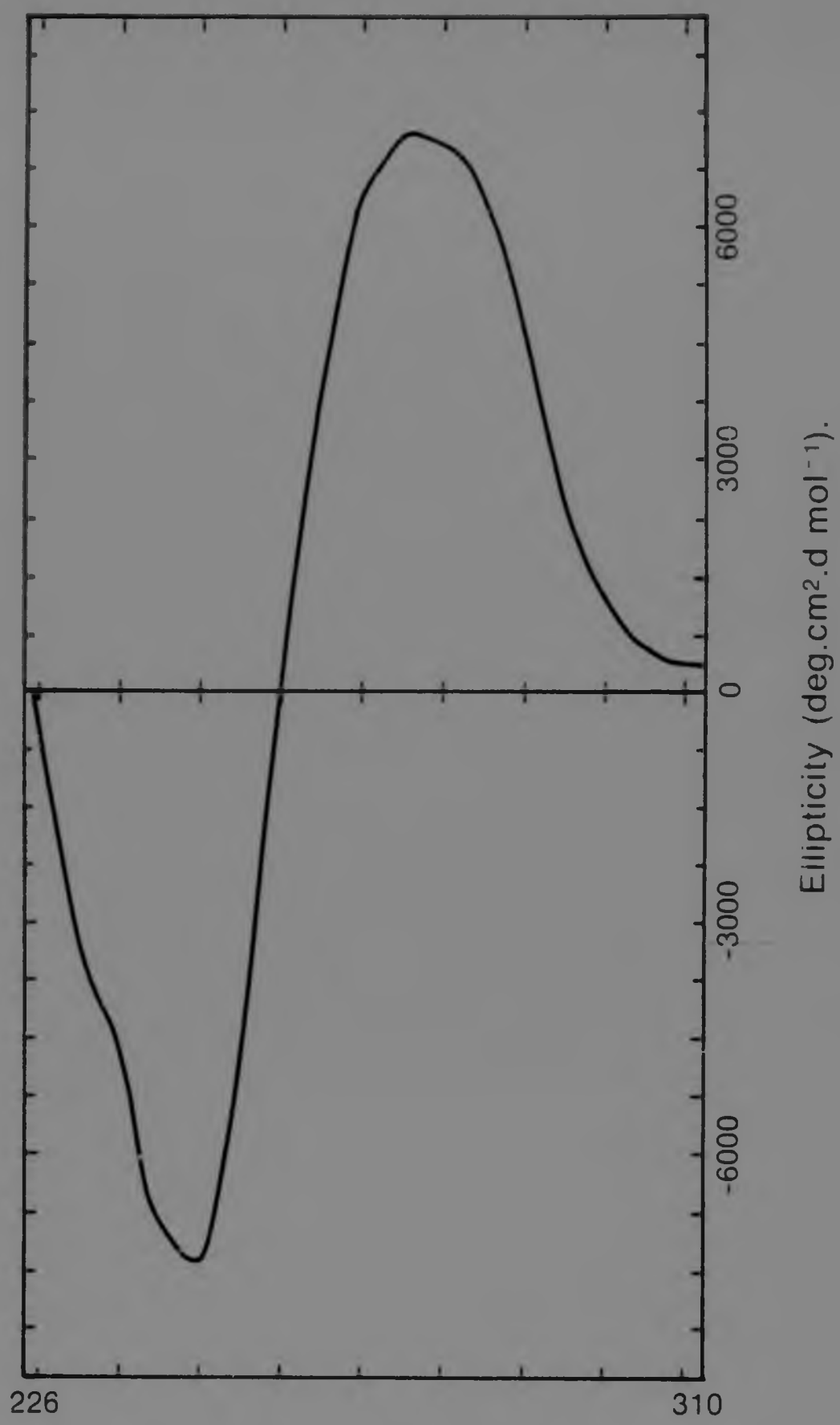


Fig. 30 Circular dichroism spectrum of E.coli DNA

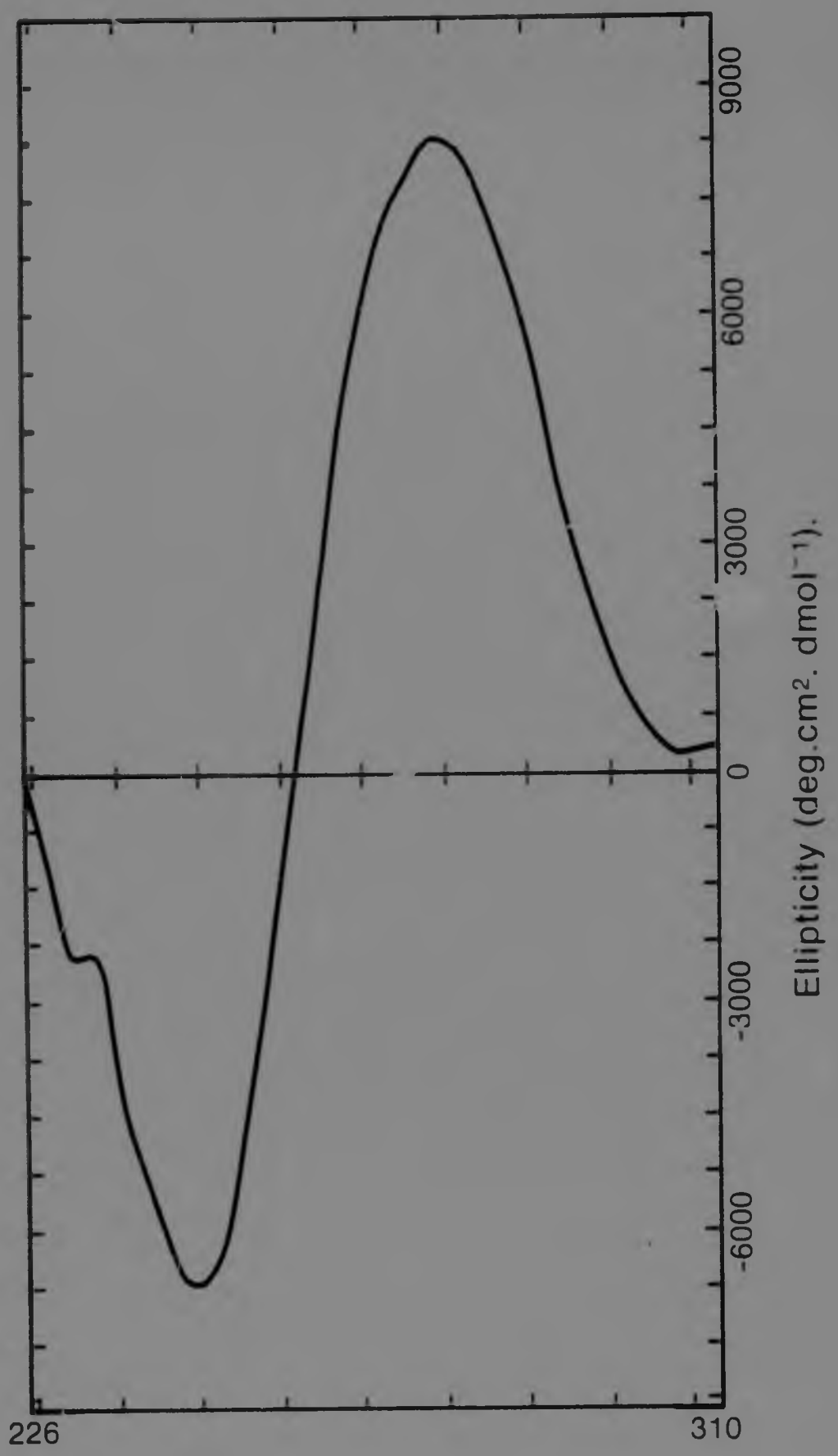


Fig. 31 Circular dichroism spectrum of E.coli DNA heated to 40° C.

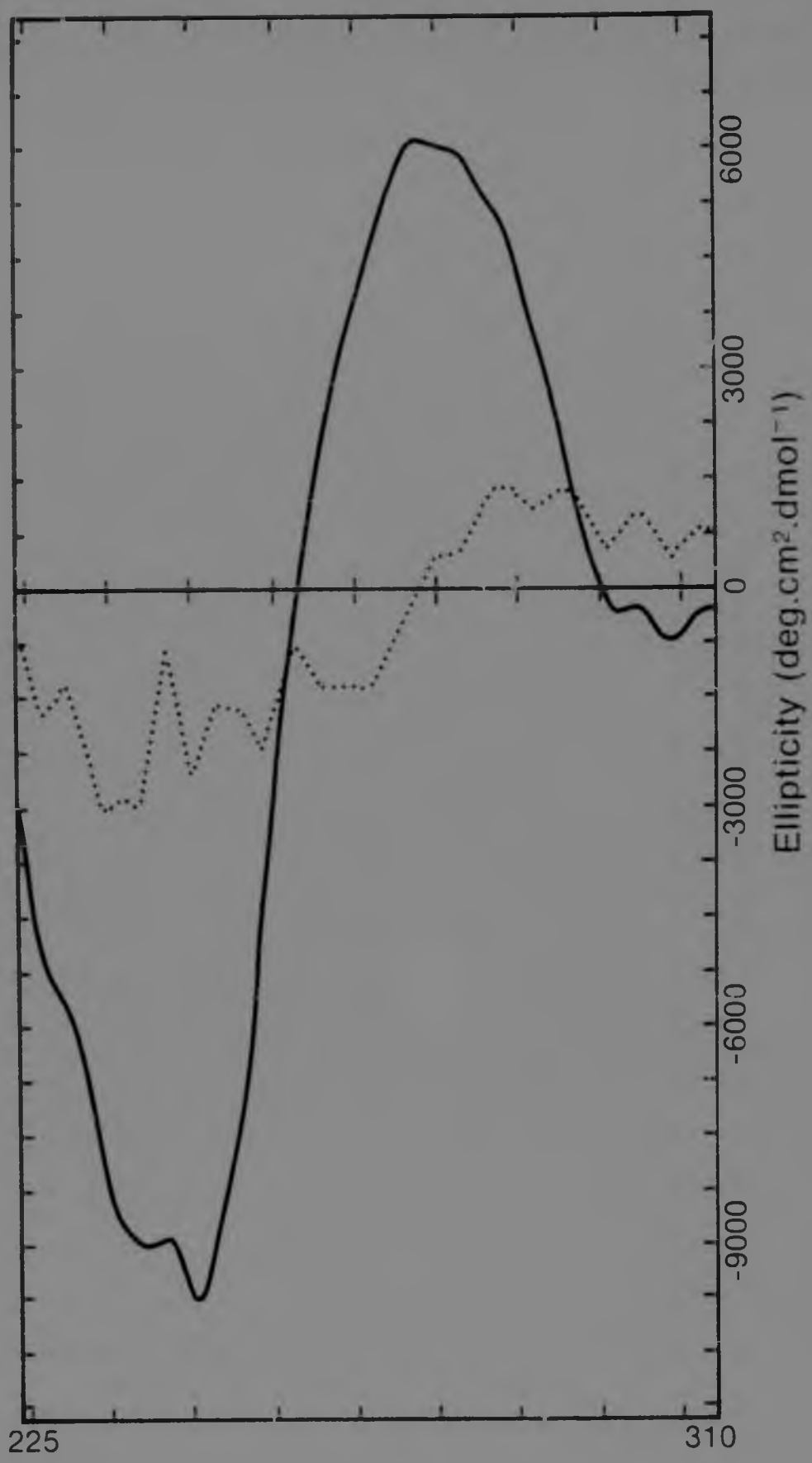


Fig. 32 Circular dichroism spectra of E.coli DNA (—) and phleomycin (·····).

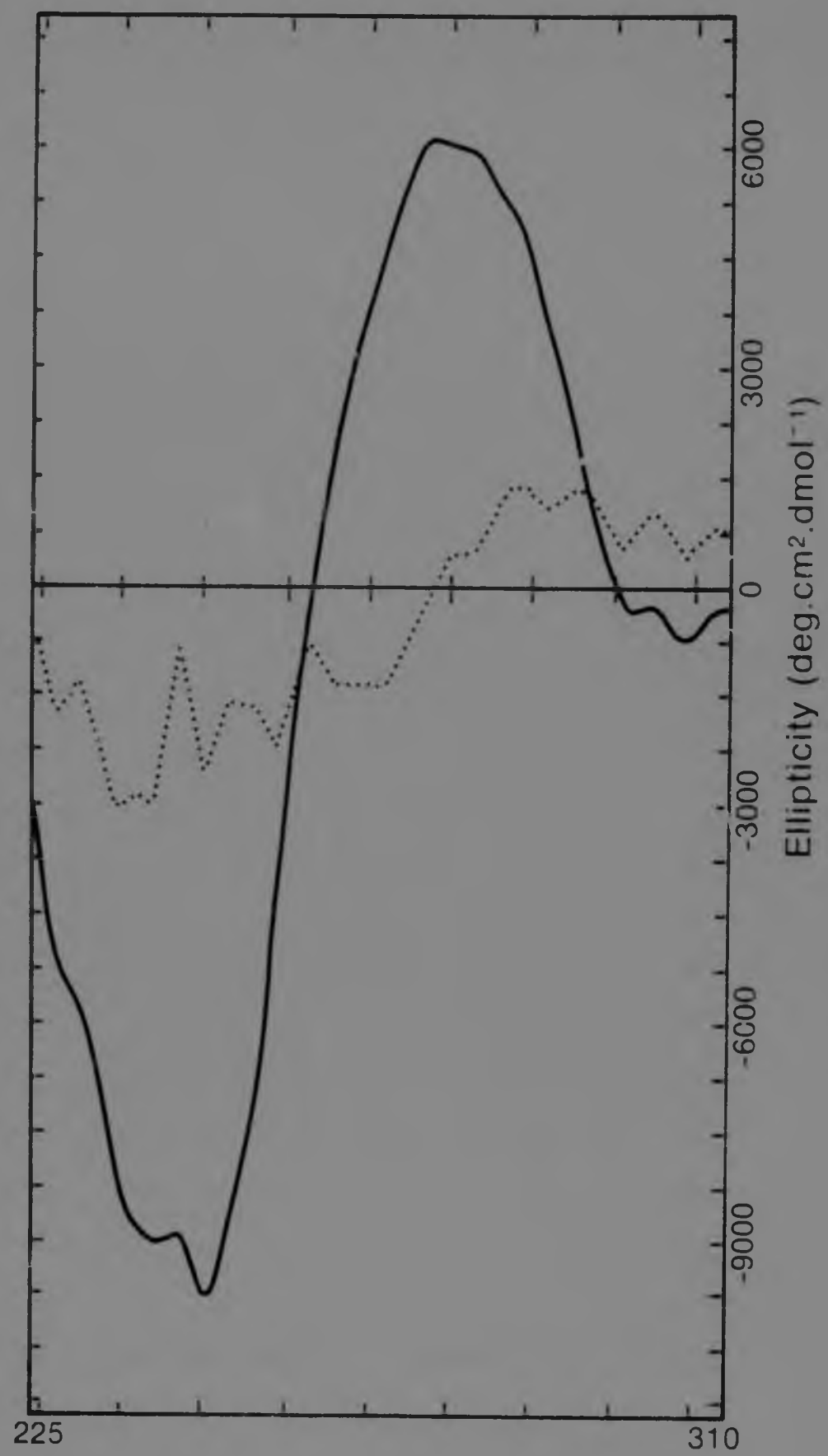


Fig. 32 Circular dichroism spectra of E.coli DNA (—) and phleomycin (·····).

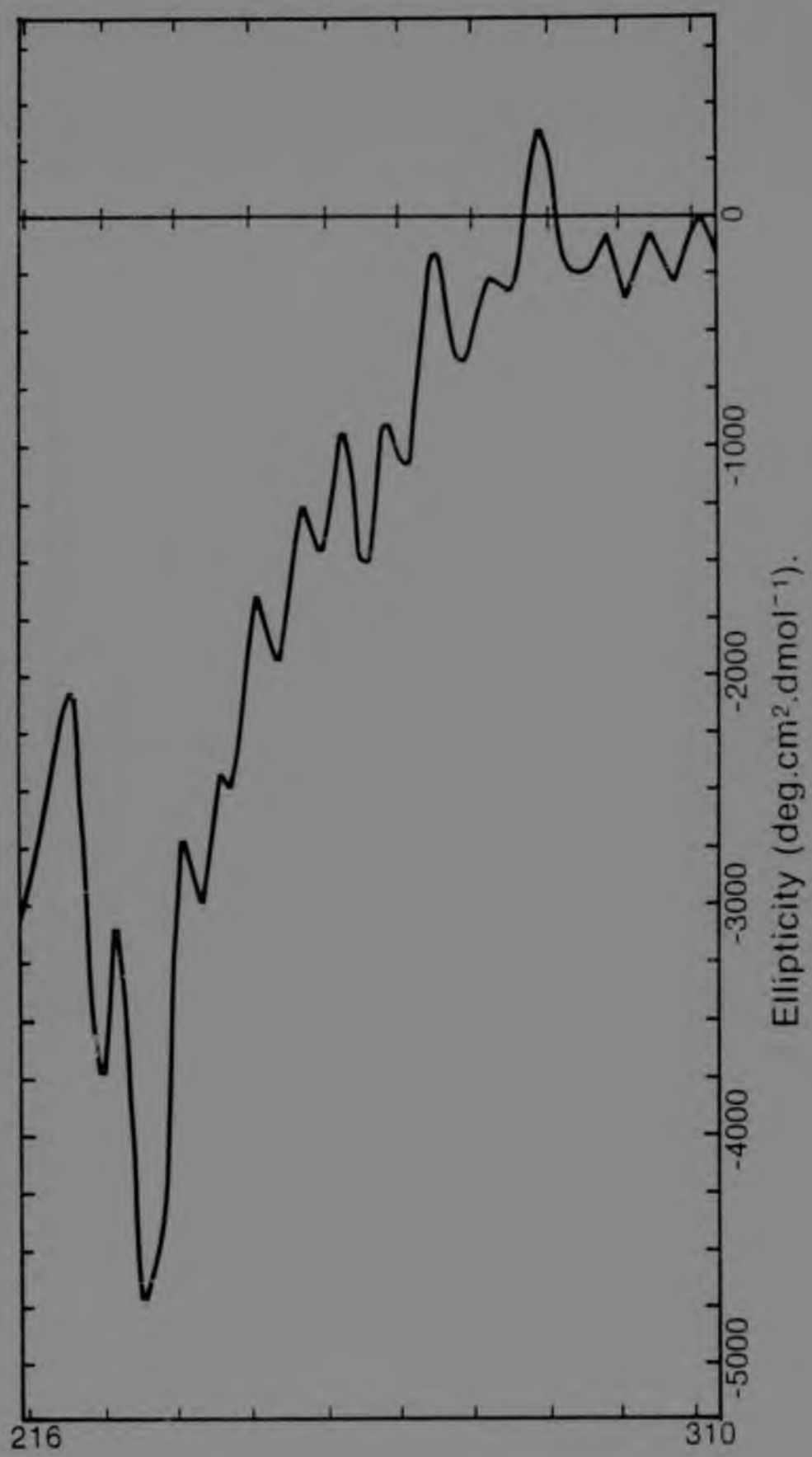


Fig.33 Circular dichroism spectrum of E.coli DNA treated with 1 mM 2-α - carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine.

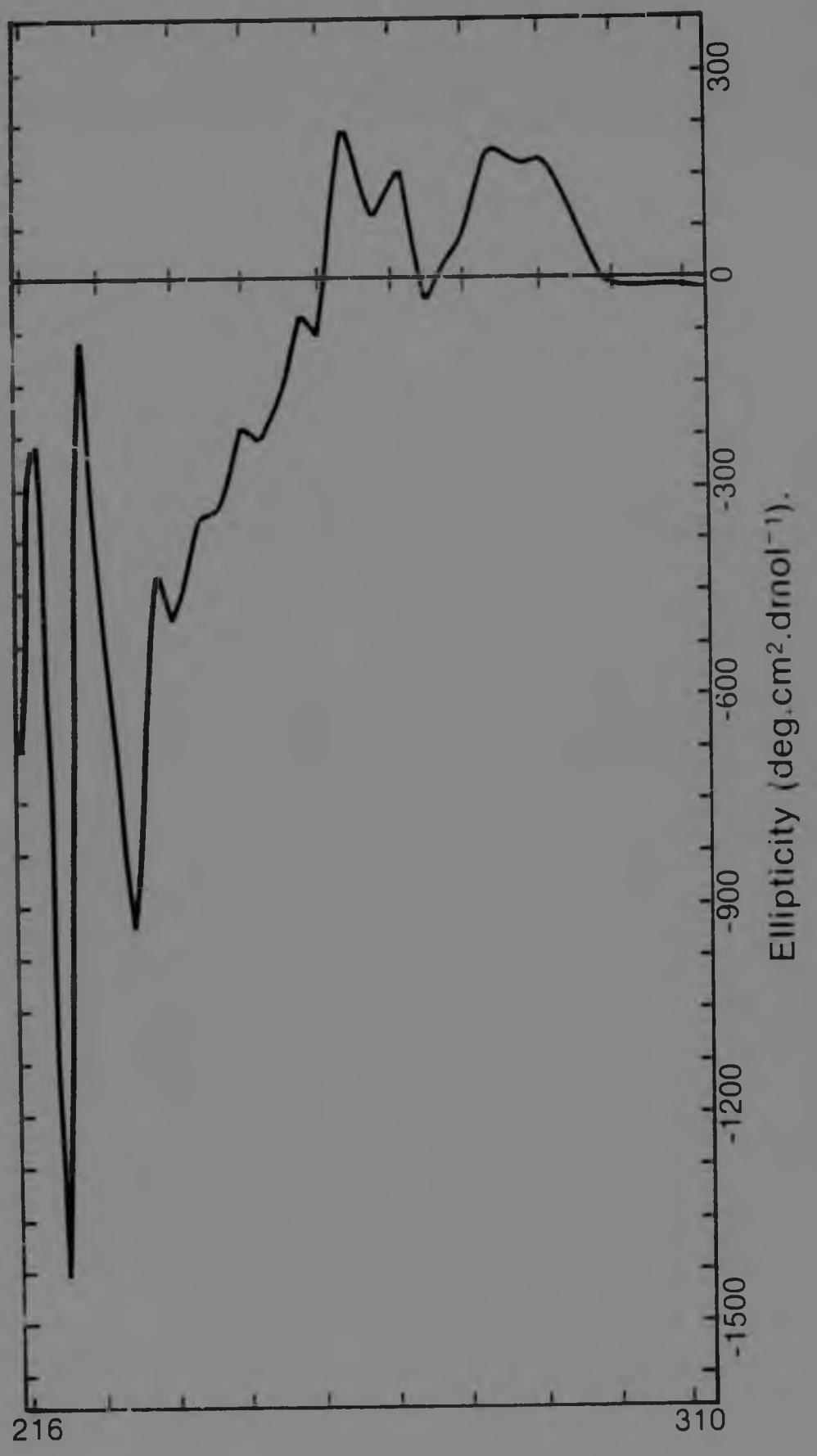


Fig. 34 Circular dichroism spectrum of caffeine

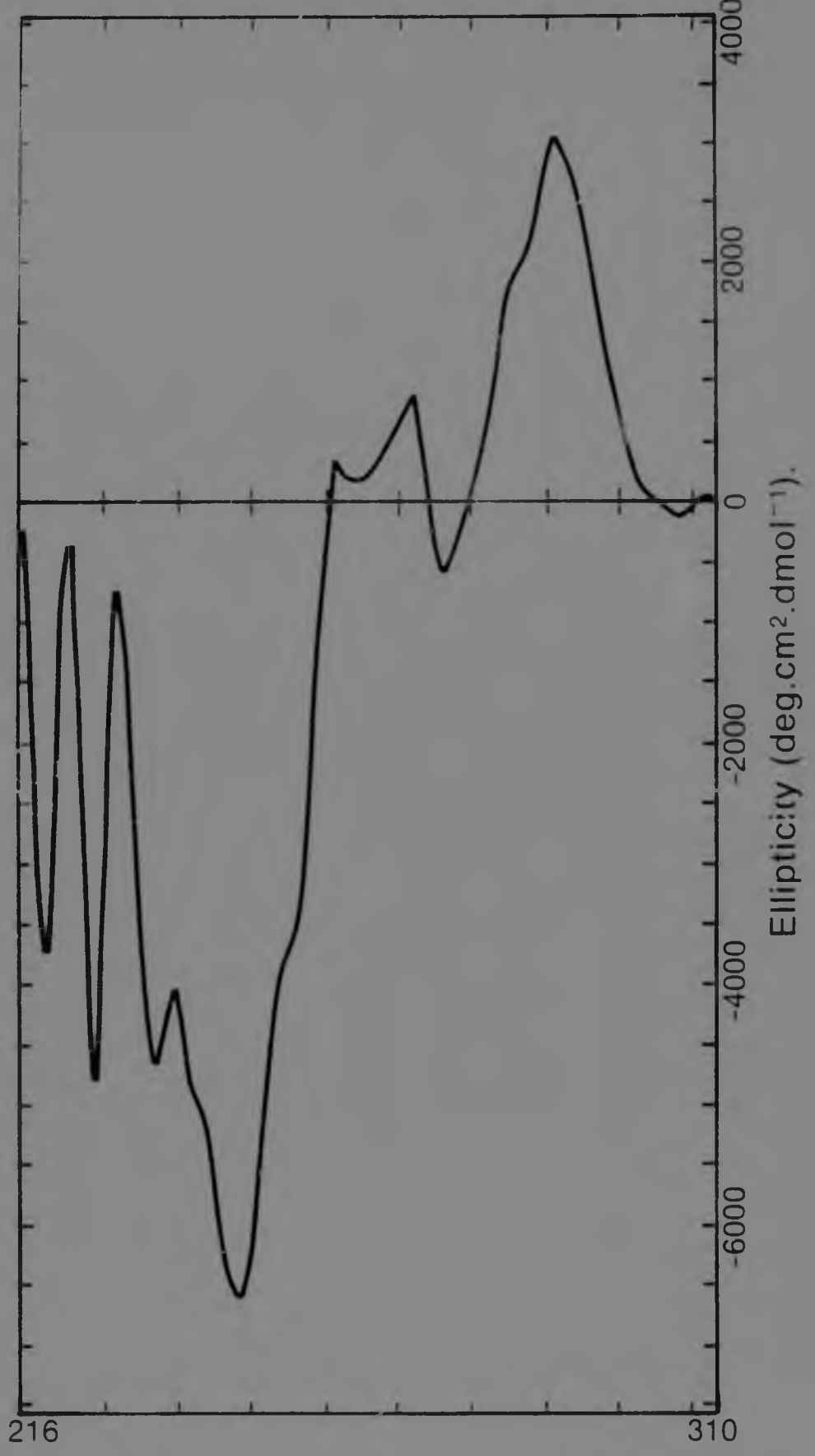


Fig. 35 Circular dichroism spectrum of DNA treated with 0.125mM caffeine.

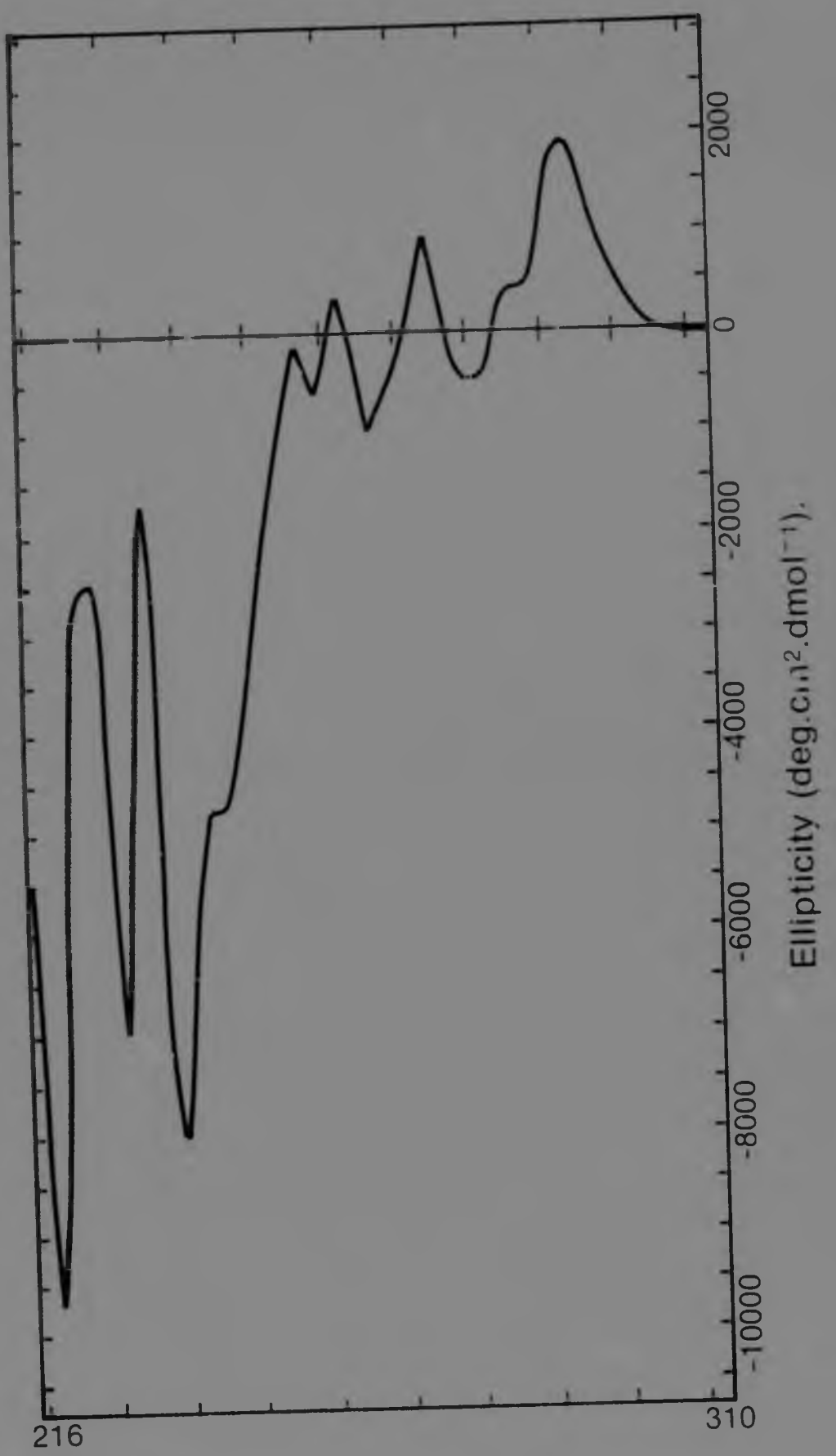


Fig. 36 Circular dichroism spectrum of DNA treated with 0.25mM caffeine

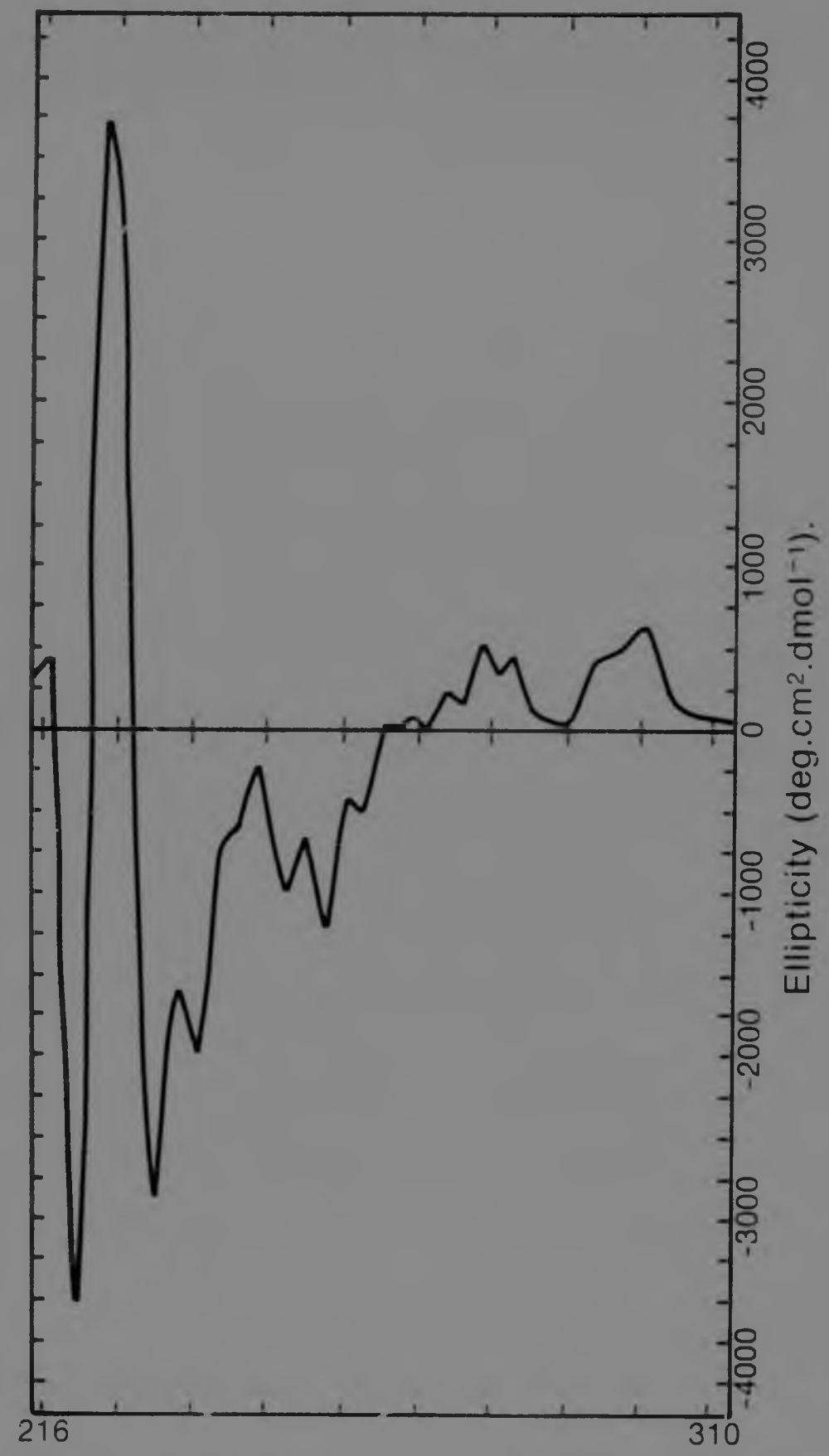


Fig. 37 Circular dichroism spectrum of DNA treated with 1mM caffeine.

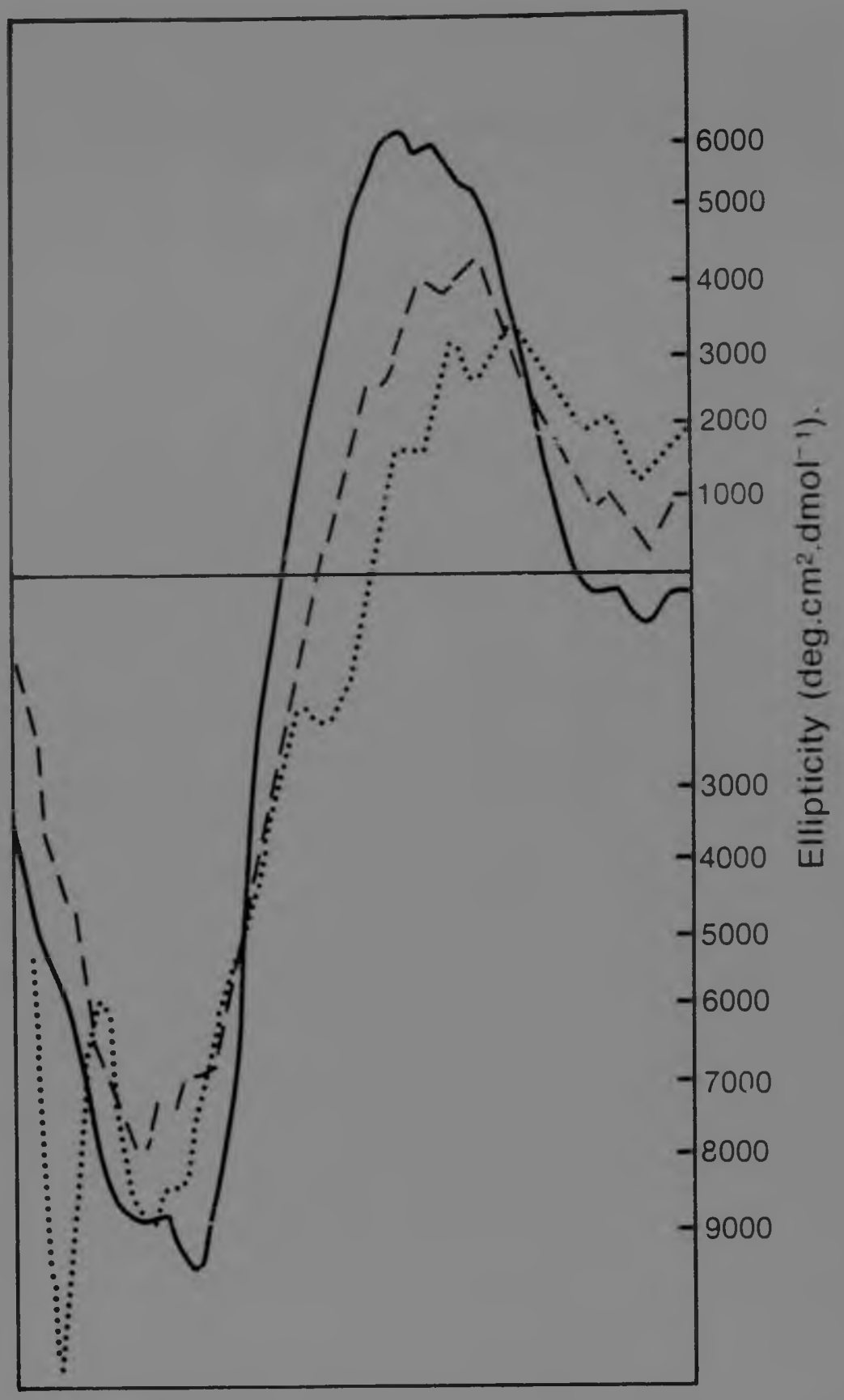


Fig. 38 Circular dichroism spectra of DNA (——), DNA treated with 20µg/ml phleomycin (— —), and DNA treated with both 20µg/ml phleomycin and 0.1mM 2-α - carbamoylmethylthio-6,9-dimethylpurine (····).

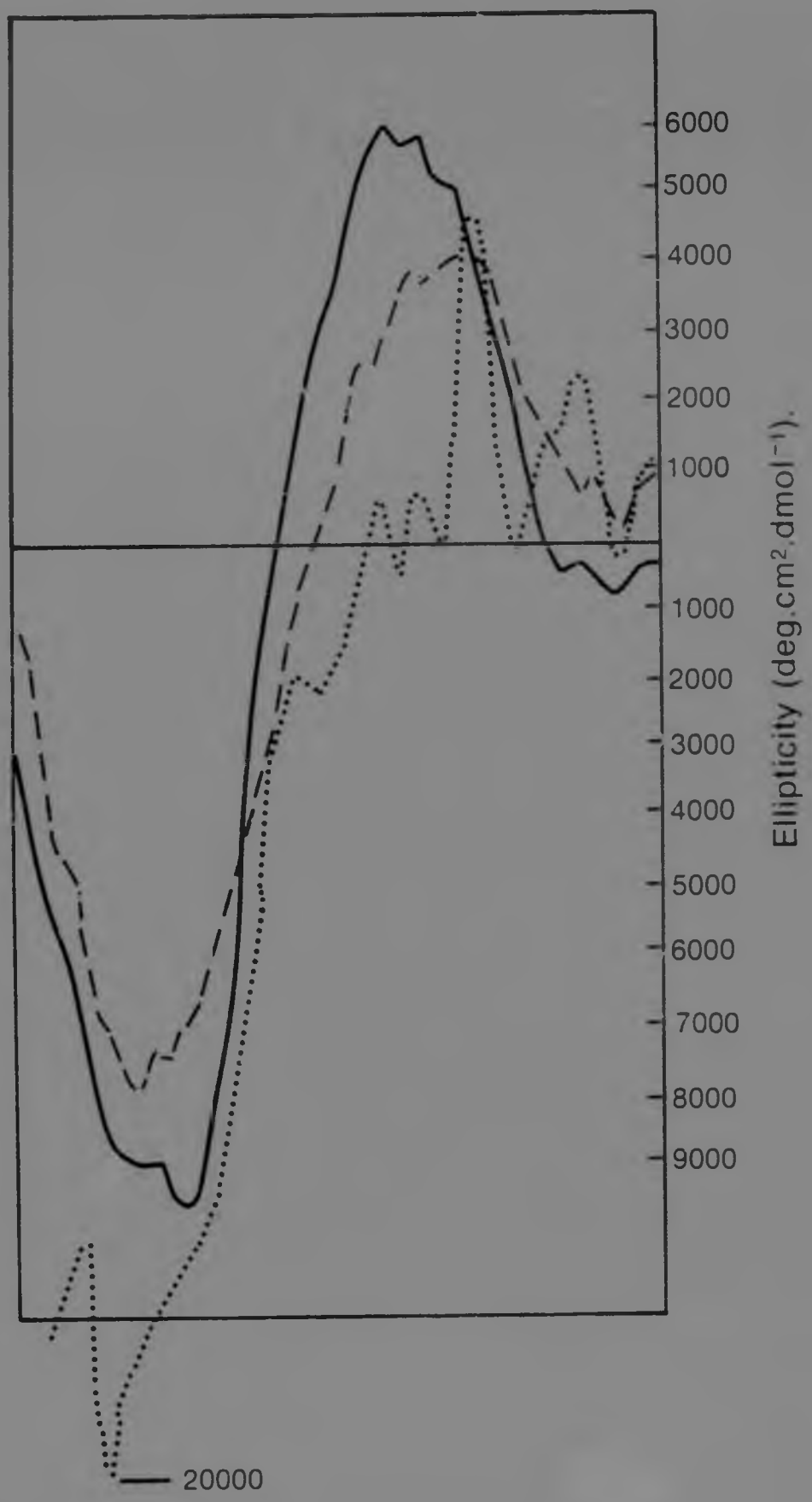


Fig. 39 Circular dichroism spectra of DNA (——), DNA treated with 20ug/ml phleomycin (– –), and DNA treated with both 20ug/ml phleomycin and 0.1mM 2-carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine (·····).

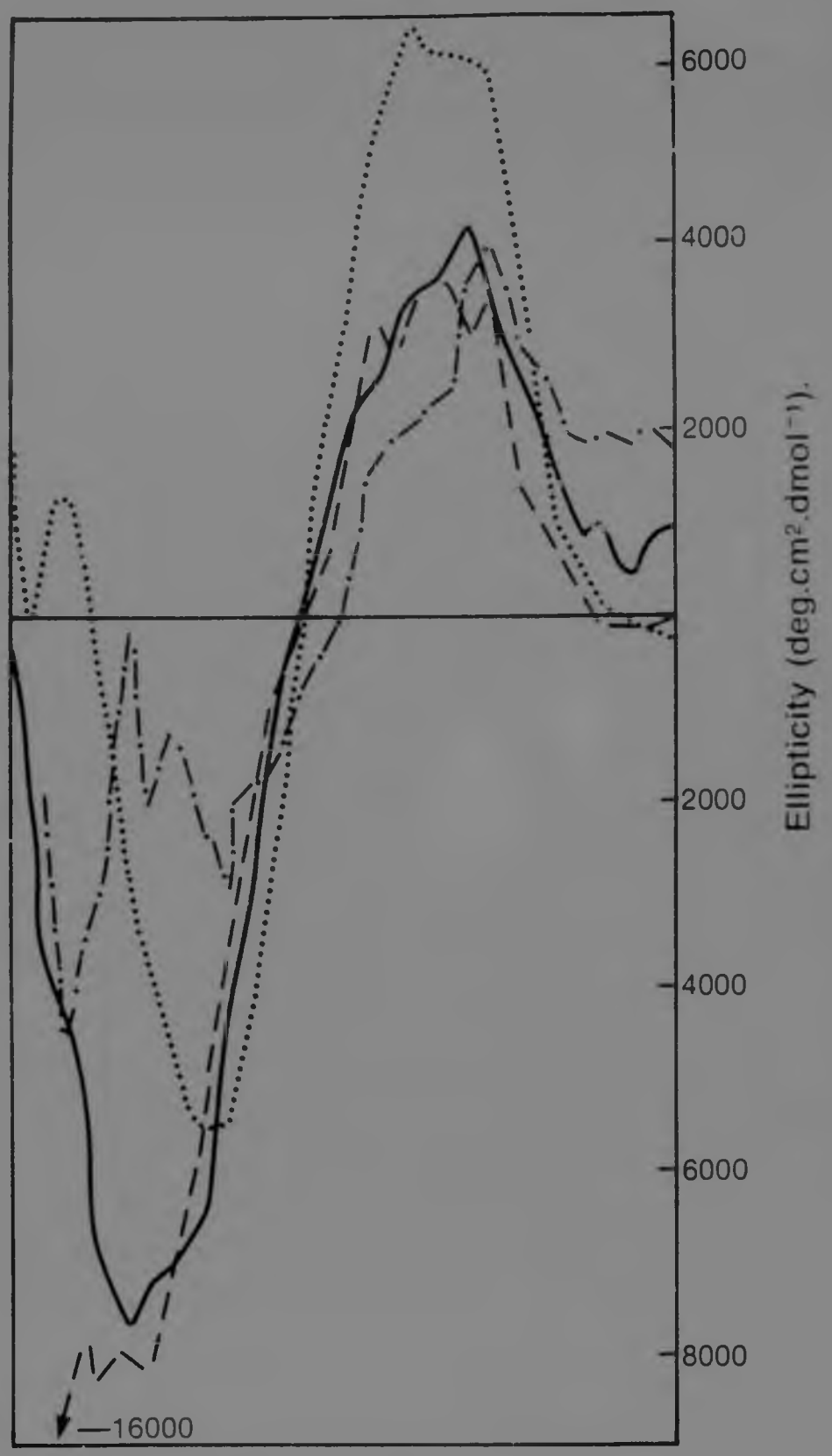


Fig. 40 Circular dichroism spectra of DNA (·····), DNA treated with 20μg/ml phleomycin (——), DNA treated with 0.1mM 2-α-carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine (—·—·), and DNA treated with both 20μg/ml phleomycin and 0.1mM 2-α-carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine (— —).

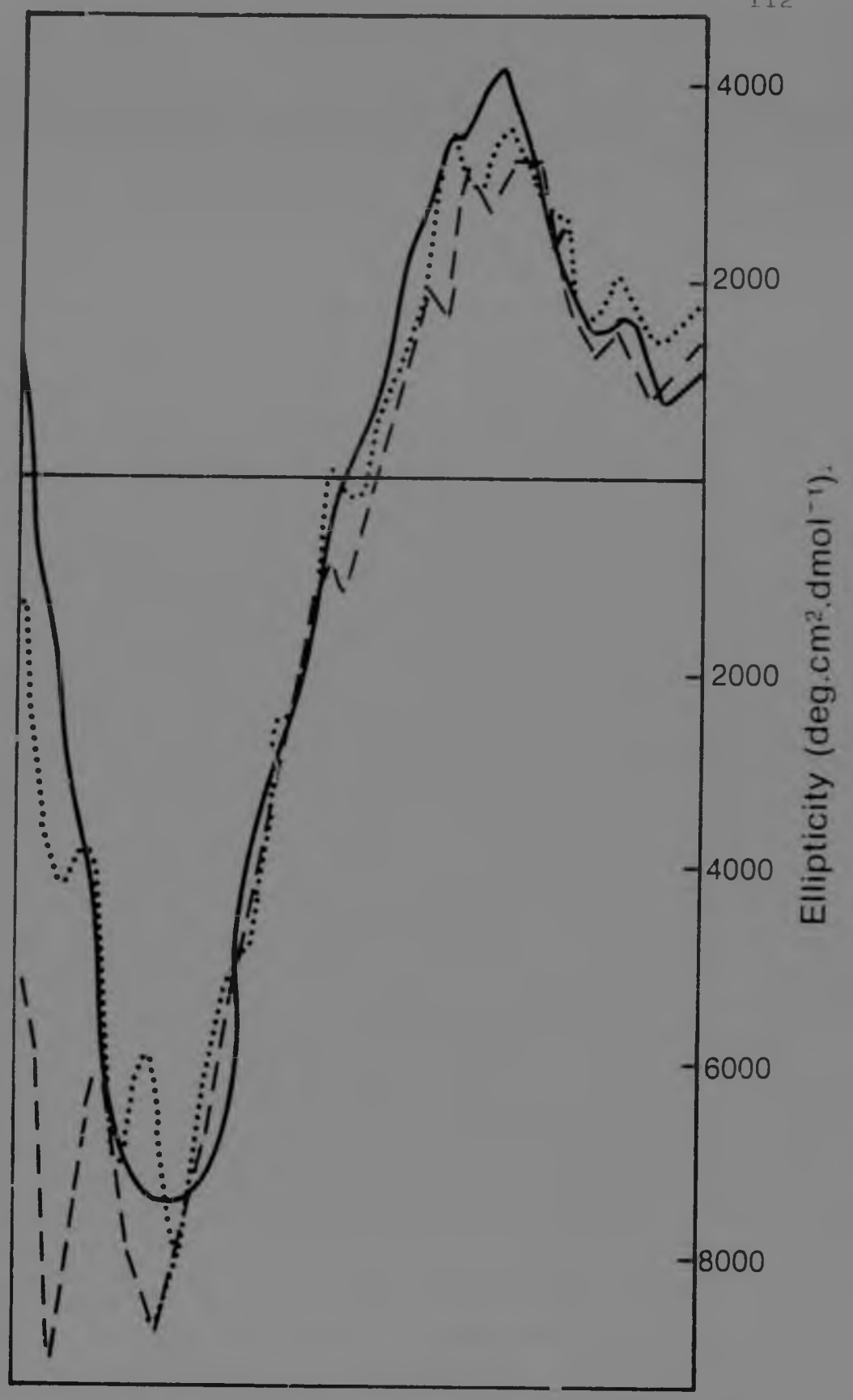


Fig. 41 Circular dichroism spectra of DNA treated with 20µg/ml phleomycin (———), DNA treated with 0.1mM 3-carbamoylmethylthio-s-triazolo-[4,3-α]-pyrimidine (— — —), and DNA treated with both 20µg/ml phleomycin and 0.1mM 3-Carbamoyl-methlthio-s-triazolo-[4,3-α]-pyrimidine (·····).

5.3.4 VISCOSITY OF DNA

The results are summarised in Table XVII.

The investigation on the viscosity of E.coli DNA in the presence of the phleomycin amplifiers reported here shows that the viscosity of the DNA is unchanged in the presence of the purine analogues. Ts'O et al. (108) also showed no change in the viscosity of DNA in the presence of 300 mM purine. This concentration was high enough for a change in T_m of 9° as reported by them. Many workers (101,109) have indicated that the viscosity of DNA increases significantly in the presence of various cyclic cationic dyes such as acridine orange. Based on this and other observations, they suggest that these compounds are intercalated between adjacent nucleotide pair layers by extension and unwinding of the deoxyribose-phosphate backbone. The results reported here suggest that the phleomycin amplifiers do not alter the macromolecular shape of the DNA at the concentrations tested.

5.3.5 ELECTRON MICROSCOPY OF DNA

5.3.5 a OBSERVATION OF PURIFIED NUCLEIC ACID

DNA molecules were observed in relaxed conformations under the experimental conditions described. This is illustrated in figures 42-44. The DNA was well spread with an absence of tangles and allowed ready tracing of strand contour and continuity. Rotary shadowing the grids with gold-palladium readily amplified the diameter of the strands so that they could be seen in an electron microscope.

When the atomic beam is directed at a low angle to the plane of the grid, the nucleic acid strand projects up from the plane and casts a wide shadow

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When the atomic beam is directed at a low angle to the plane of the grid, the nucleic acid strand projects up from the plane and casts a wide shadow

TABLE XVII INFLUENCE OF VARIOUS ANALOGUES ON THE VISCOSITY OF *E coli* DNA

ANALOGUE	VISCOSITY (cSt)
Untreated DNA	0.938
2-α-Carbamoylmethylthio-6,9-dimethylpurine	0.930
2-Carbamoylmethylthio-8-hydroxymethyl-6,9-dimethylpurine	0.938
2-Carbamoylmethylthio-6,8,9-trimethylpurine	0.941
2-α-Carbamoylethylthiobenzothiazole	0.938
2-α-Carbamoylethylthio-s-triazolo-[1,5-a]-pyrimidine	0.934
3-α-Carbamoylethylthio-s-triazolo-[4,3-a]-pyrimidine	0.938
2-α-Carbamoylethylthiobenzoxazole	0.930
2,5-Biscarbamoylmethylthio-7-methylthiazolo-[5,4-d]-pyrimidine	0.934
3-Carbamoylmethylthio-s-triazolo-[4,3-a]-pyrimidine	0.941
3-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[4,3-c]-pyrimidine	0.934
2-Carbamoylmethylthio-5,7-dimethyl-s-triazolo-[1,5-c]-pyrimidine	0.930
4-Carbamoylmethylthio-1-methyl-1H-pyrazolo-[3,4-d]-pyrimidine	0.938
1,3,7-Trimethylxanthine (caffeine)	0.938



Fig.42. Electron micrograph of DNA purified from E.coli (magnification: 8000 X)



Fig.43. Electron micrograph of DNA purified from E.coli (magnification: 15 000 x)



fig.44. Electron micrograph of DNA purified from E.coli (magnification: 20 000 x)



Fig.45. Electron micrograph of E.coli DNA treated with 20µg/ml phleomycin (magnification: 15 000 x)



Fig.46. Electron micrograph of E.coli DNA treated with 1 mM 2-carbamoylmethylthio-8-hydroxymethyl -6,9-dimethylpurine (magnification: 15 000 x)

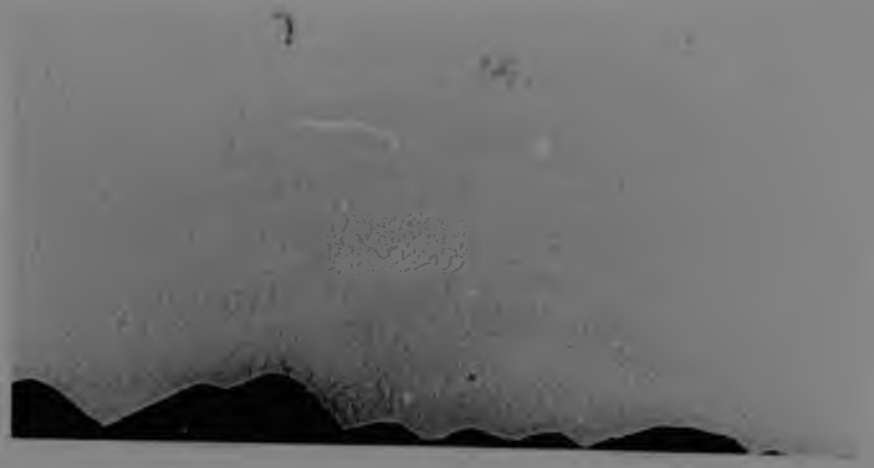


Fig.47. Electrom micrograph of E.coli DNA treated with 20µg/m. phleomycin and 500µM 2-carbamoylmethythio-8-hydroxymethyl-6,9-dimethylpurine (magnification: 15 000 x)

which may then be observed by contrast. Once the sample had been shadowed to give suitable contrast, DNA was easily viewed at a magnification of 5 000 to 10 000 fold.

5.3.5 b EFFECT OF PHLEOMYCIN AND/OR PHLEOMYCIN AMPLIFIER ON DNA

Denaturation of DNA resulting in distinct strand separation or localised single-stranded regions along the DNA was not observed in DNA samples treated with phleomycin and/or phleomycin amplifiers. Concentrations as high as 20 µg phleomycin and 500 µM phleomycin amplifier were used (figures 45-47).

Similar results were obtained for DNA samples treated with intercalating agents such as ethidium-bromide and quinicrine.

5.3.5 c DISCUSSION

Purified nucleic acids can be spread satisfactorily if they are prevented from lateral aggregation (106). This has been accomplished by

(i) spreading them in a monofilm of small molecules such as benzyldimethylalkylammonium chloride (BAC).

(ii) adsorbing them to immobile, positively charged residues on the specimen film surface or

(iii) stiffening them through intercalation by some small molecule.

The visualization of pure nucleic acids indicate little about their structure. However, it is possible to observe the single-stranded regions along its length as studied in experiments concerning

DNA replication (104) and heat denaturation (107). Recent advances have made it possible to visualize molecules of RNA polymerase that have been bound to DNA. DNA complexes with the smaller repressor molecules are not easy to photograph. To visualize the consequences of *in vitro* complexing of protein molecules and nucleic acids may be important in illustrating many reaction mechanisms.

The interaction of phleomycin and/or phleomycin amplifiers with DNA was not observed. Visualisation of DNA treated with ethidium-bromide did not indicate any changes in DNA structure as reported by Koller *et al.* (110). The results indicate that the compounds tested may interact like intercalating agents. Strand separation resulting in denaturation of DNA was not observed. If any distortion of DNA did result, it could perhaps have been only a few nucleotides long at various loci along the DNA. This may not have been possible to visualize under the conditions employed.

Stern *et al.* (79) have shown that phleomycin can act directly upon DNA producing single strand breaks. Hence, electron microscopy examination of DNA was carried out on DNA treated under the same conditions described, namely, DNA incubated with phleomycin at 37°C in the presence of dithiothreitol. Visualisation of DNA treated in this manner also indicated no changes in the gross structure of DNA. In other experiments, the method of Grigg *et al.* (10) was adopted in treating DNA. In this case too, incubation of DNA with phleomycin before the addition of dithio threitol resulted in no charges in the contour of DNA. Hence the interactions of these compounds with DNA was best illustrated using circular dichroism measurements.

5.4 DISCUSSION

A number of techniques had been employed to study the interaction of DNA with the phleomycin and/or phleomycin amplifiers. Evidence for the interaction of the phleomycin amplifiers with DNA was only yielded by circular dichroism. Thermal denaturation and re-association of DNA in the presence of these compounds had no influence on the helix-coil transition of DNA. However, these compounds could only be tested at a maximum concentration of 0.3 mM. Concentrations above 0.3 mM could not be used because of their high extinction at 260 nm. If the compounds tested are presumed to change the Tm of DNA, then these changes may probably have been too small to be observed at a temperature as high as 90°. Also, the concentrations tested may have been too low to elicit any observable changes. That these compounds interact with DNA is quite clear as can be concluded from their ability to inhibit the excision repair mechanism of UV-irradiated E.coli and also their ability to induce significant changes in the circular dichroism pattern of native DNA. The circular dichroism changes observed are quite large, but these changes could not be interpreted because of their complexity. However,superimposing the CD spectra of DNA, analogue and phleomycin did not contribute to a final spectra in an additive fashion. New spectral peaks resulted which is a strong indication of interaction. The only possible conclusion that could be made was that amplifier concentrations as low as 0.1 mM induced changes in the circular dichroism of native DNA. Furthermore, concentrations as low as 0.1 mM influenced the circular dichroism of DNA treated with low concentrations

of phleomycin. High concentrations of either phleomycin or amplifier resulted in complete loss of both positive and negative bands.

Viscosity measurements revealed no changes in the viscosity of DNA when treated with the compounds at a concentration of 1 mM. Hydrodynamic properties of polymers are sensitive to chain conformation. Hence, it can be concluded that the overall conformation and macromolecular shape of the DNA was not changed by these compounds. The amplifiers were not tested at concentrations greater than 1 mM because of their poor aqueous solubility.

Direct visualisation of nucleic acids has proved to be an important technique to study a variety of problems. Some of the results yielded include the direct measurement of single-stranded DNA (102), the analysis of partially denatured DNA (103), the replication and recombination complexes (104), and the physical mapping of genes in hybrid molecules (105). In this study, the interaction of phleomycin and/or phleomycin amplifiers with DNA was investigated. Native DNA molecules were observed in relaxed conformations under the expermental conditions described. Denaturation of DNA resulting in distinct strand separation or localized single-stranded regions along the DNA was not observed in DNA samples treated with phleomycin and/or phleomycin amplifiers. Concentrations as high as 20 µg phleomycin and 1 mM phleomycin amplifier were used. Similar results were obtained for DNA samples treated with intercalating agents such as ethidium bromide and quinicrine. Hence, no change in the overall conformation and contour of DNA was observed. Denaturation of DNA by intercalating agents was also not observed by many workers (110).

It can thus be concluded from the results described above that evidence for the interaction of the phleomycin amplifiers with DNA was yielded only by circular dichroism measurements. No changes at the macromolecular level were observed and perhaps these compounds induce new transitions in the DNA molecule as noted by changes in the circular dichroism measurements.

CHAPTER SIX

CHEMICAL SYNTHESIS OF NEW ANALOGUES

6.1 INTRODUCTION

A number of new purine analogues have been characterised as inhibitors of hypoxanthine guanine phosphoribosyl transferase (EC.2.4.2.8) (chapters 2 and 3, this thesis). A series of other analogues, based on the heterocyclic nucleus oxazole, were synthesised since 2-α-carbamoylethylthiobenzoxazole was found to be one of the inhibitors. Imidazole derivatives based on the oxazoles were also synthesized. Although no specific interaction of the phleomycin amplifiers with HPRT could be demonstrated, further analogues of one of the inhibitors were sythesised to establish a structure-function relationship. Inhibitors of HPRT are of biological significance and inhibitors are needed (109) to

(i) evaluate the role of this enzyme in maintaining steady state pools of purines in various tissues and tumours.,
(ii) potentially treat schistosomiasis and malaria, diseases in which the parasites lack the de novo biosynthetic pathways.,
(iii) potentiate the action of other agents which inhibit de novo biosynthesis, effects of which may be "bypassed" due to HPRT; and
(iv) possibly mimic in animals the genetic disease which occurs in man as a result of HPRT deletion , i.e. the Lesch-Nyhan syndrome.

6.2 MATERIALS AND METHODS

All regents used were of standard grade.

6.2.1 Preparation of 2-∝-Cartamoyethylthiobenzoxazole

6.2.1 a Reaction

[A] (i) Propionic acid ⟶ ∝-Bromopropionic acid.

(ii) ∝-Bromopropionic acid ⟶ ∝-Bromopropionylchloride

(iii) ∝-Bromopropionyl chloride ⟶ ∝-Bromopropionamide

[B] ortho-aminophenol ⟶ 2-Mercaptobenzoxazole

[C] ∝-Bromopropionamide + 2-Mercaptobenzoxazole ⟶ 2-∝-Carbamoylethylthiobenzoxazole

6.2.1 b Method

(i) ∝-Bromopropionic acid

64 g of freshly distilled, dry propionic acid and 150 g of dry bromine were placed in a 500 ml flask equipped with a reflux condenser the top of which was connected with a trap and an absorption vessel containing water.

The condenser was momentarily removed and 1.5 ml phosphorus trichloride cautiously added.

Once the reaction had commenced and hydrogen bromide was being smoothly evolved, the mixtur was heated to 50-70°C.

Towards the end of the reaction, the temperature was raised to 100°C. The reaction was complete when all the bromine had reacted (approximately 4 hours).

The reaction mixture was then distilled under vacuum to yield 105 g ∝-bromopropionic acid at 95-97°C/10 mm Hg.

(ii) ∝-Bromopropionyl chloride(116)

37.5 g of redistilled thionylchloride was placed in a 100 ml Claisen flask fitted with a dropping funnel containing 22 g of ∝-bromopropionic acid.

The flask was heated gently, and the ∝-bromopropionic acid added during the course of 30-40 minutes. The hydrogen chloride evolved was absorbed in a water trap.After all the acid had been introduced. the reaction mixture was heated for a further 30 min. Distillation of the product yielded 25 g ∝-bromopropionyl chloride at 98°C and 10 mm Hg.

(iii) ∝-Bromopropionamide

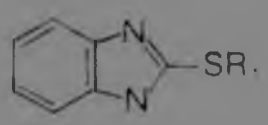
3 g ∝-bromopropionyl chloride in 15 ml benzene was cooled while stirring to 4°C.

Dry ammonia was then bubbled into the mixture whereupon a white precipitate appeared. The product was filtered and the residue evaporated to dryness.

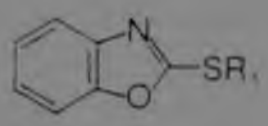
∝-Bromopropionamide was then extracted with ether in a soxhlet apparatus.

(iv) 2-Mercaptobenzoxazole (117)

A mixture of 33 g (0.3. mole) ortho-aminophenol, 19 g of potassium hydroxide, 26 g (i.e.0.34 mole) carbon disulphide and 45 ml of water in a one litre flask was heated under reflux for 3 hr. 12 g activated charcoal was then added cautiously and after the mixture had been heated at a reflux temperature for 10 minutes the charcoal was removed by filtration. The filtrate was then heated to 70°C while stirring and 300 ml of warm tap water (60-70°C) added, followed by 25 ml of acetic



(i) Benzimidazole derivative



(ii) Benzoxazole derivative

Where R is:

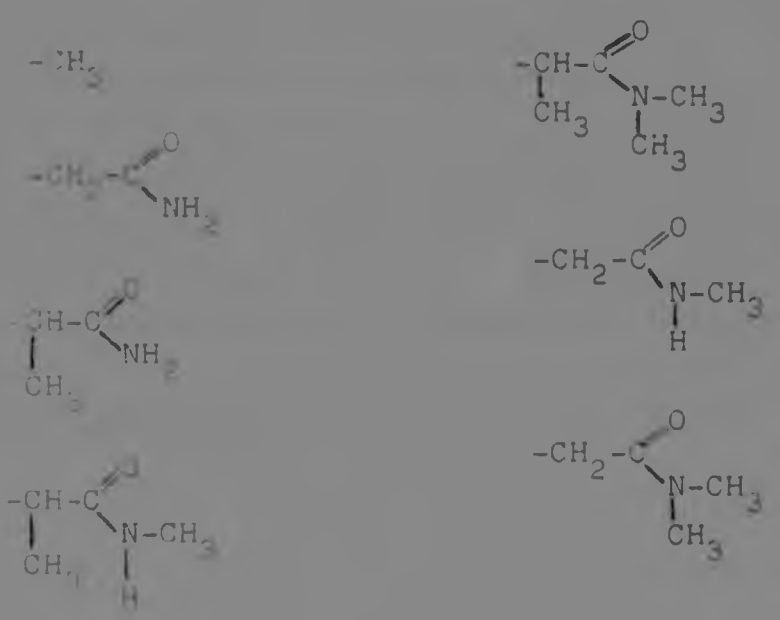


Fig. 4.1: Structures of the benzoxazole and benzimidazole derivatives synthesised

acid in 50 ml water. The product separated on cooling as glistening white crystals, and the mixture was refrigerated to complete crystallisation. The product was dried overnight at 40°C to yield 32 g 2-mercaptobenzoxazole.

(v) 2-∝ Carbamoylethylthiobenzoxazole [113.121]

A mixture of 0.01 mole 2-mercaptobenzoxazole. 0.01 mole ∝-bromopropionamide and 0.01 mole sodium hydrogen carbonate in 50 ml water was heated for 10 minutes with agitation. The mixture was then refrigerated whereupon 2-∝-carbamoylethylthiobenzoxazole crystallised as white needle shaped crystals.

6.2.2 Other alkylating agents prepared

Using a similar procedure, the following alkylating agents were prepared using methylamine or dimethylamine as the aminating agent instead of ammonia:

1. N,N-dimethyl-2-bromopropionamide
2. N-methyl-2-bromopropionamide
3. ∝-Bromoacetamide
4. N,N-dimethyl-2-bromoacetamide
5. N-methyl-2-bromoacetamide
6. The alkylating agents chloroacetamide and methyliodide were purchased from Merck

6.2.3 2-Mercaptobenzimidazole

This was prepared by a similar procedure as 2-mercaptobenzoxazole, using o-phenylenediamine instead of o-aminophenol.

6.2.4 S-Alkylation Procedure

By the procedure of 2-∝-Carbamoylethylthiobenzoxazole and appropriate combinations of benzoxazole

or benzimidazole with the alkylating agents described above the various compounds were obtained.

6.3 RESULTS

6.3.1 Chemical syntheses

(a) 2-Mercaptobenzoxazole

Melting point : 194°C (aqueous ethanol)

m/e : 151 ; 150 ; 118 ; 90 ; 76.

(b) 2-Carbamoylmethylthiobenzoxazole

Melting point : 163°C (aqueous ethanol)

m/e : 208 ; 164 ; 150 ; 76.

CHN : Found C,52.3 ; H,4.2 ; N,13.4.
$C_9H_8N_2O_2S$ requires C,51.9 : H,3.9 N,13.5% .

(c) 2-Carbamoylethylthiobenzoxazole

m.p. : 124° (water)

m/e : 222, 178, 150, 118, 76

CHN : Found C,54.4 ; H,4.3 ; N,12.8.
$C_{10}H_{10}N_2O_2S$ requires C,54.1 ; H,4.5 ; N,12.6%.

(d) 2-(Benzoxazol-2'-ylthio)-N-methylpropionamide

m.p. : 180° (aqueous ethanol)

m/e : 236, 206, 178, 150, 118, 76.

CHN : Found C,55.4 ; H,5.3 ; N,11.1.
$C_{11}H_{12}N_2O_2S$ requires C,55.9 ; H,5.1 ; N,11.8%.

(e) 2-(Benzoxazol-2'-ylthio)-N,N-dimethylpropionamide

m.p. : 136-138° (aqueous ethanol)

m/e : 250, 206, 178, 150, 118, 76

CHN : Found C,57.2 ; H,5.1 ; N,11.9%
$C_{12}H_{14}N_2O_2S$ requires C,57.6 ; H,5.6 ; N,11.2%

(f) 2-(Benzoxazole-2'-ylthio)-N-methylacetamide

m.p. : 99° (aqueous ethanol)

m/e : 222 ; 192 ; 164 ; 150 ; 118; 76

CHN : Found C,54.7 . H,3.9 ; N,12.2%

$C_{10}H_8N_2O_2S$ requires C,54.5 ; H,3.6 ; N,12.7%

(g) 2-(Benzoxazole-2'-ylthio)-N,N-dimethylacetamide

m.p. : 128° (aqueous ethanol)

m/e : 236 ; 192 ; 164 ; 150 ; 118 ; 76

CHN : Found C,55.1 ; H,5.3 ; N,11.1%

$C_{11}H_{12}N_2O_2S$ requires C,55.9 ; H,5.1 ; N,11.9%

(h) 2-Mercaptobenzimidazole

m.p. : 288°

m/e : 150 ; 117 ; 105 ; 76

(i) 2-Methylthiobenzimidazole

m.p. : 200° (aqueous ethanol)

m/e : 164 ; 149 ; 117 ; 105 ; 76

(j) 2-Carbamoylmethylthiobenzimidazole

m.p. : 208° (aqueous ethanol)

m/e : 207 ; 163 ; 149 ; 117 ; 105 ; 76

CHN : Found C,54.7 ; H,4.8 ; N,19.3%

$C_9H_9N_3OS$ requires C,54.3 ; H,5.0; N,19.0

(k) 2-Carbamoylethylthiobenzimidazole

m.p. : 175° (aqueous ethanol)

m/e : 221 ; 177 ; 149 ; 117 ; 105 ; 76

CHN : Found C,59.7 ; H,5.2 ; N,18.3%

$C_{10}H_{11}N_3OS$ requires C,54.3 ; H,5.0 ; N,19.0%

6.3.2 Biological testing

6.3.2 a Effects on HPRT activity

The influence of these analogues on the HPRT activity from human erythrocytes was tested. The enzyme assay used is as described previously (Chapter 2, this thesis). None of the analogues tested were found to be inhibitors of HPRT. The results are summarised in Table XVIII.

6.3.2 b Effects on the excision repair mechanism of UV-irradiated E.coli

The excision repair mechanism was studied by measuring the liquid holding recovery of UV-irradiated E.coli B. Table XIX shows the effects of these analogues on the liquid holding recovery of the irradiated cells. The analogues had no sinificant effect on the survival of unirradiated cells held in analogue containing buffer (Table XX). Similar results were obtained for the phleomycin amplifiers tested (Chaper 4, this thesis).

6.3.2 c Influence on the thermal melting of E.coli DNA

The influence of these analogues on DNA melting and reannealling was carried out as described previously (Chapter 5, this thesis). No change in Tm was observed at the concentrations used. The results are summarised in Table XXI.

6.3.2 d Influence on the optical activity of E.coli DNA

Any analogue incubated with native DNA induced significant changes in the circular dichroism spectrum of native DNA. DNA incubated with 1 mM analogue resulted in complete loss of both the

positive and negative bands. DNA treated with the analogues at a concentration less than 1 mM resulted in marked changes in the CD spectra of native DNA. The CD changes induced by the different analogues could not be inter-related.

6.3.2 e Influence on the hydrodynamic properties of DNA: Viscosity measurement

The viscosity of E.coli DNA in the presence of the benzoxazole and benzimidazole derivatives synthesised is unchanged. Concentrations as high as 1 mM were used for the investigations. Concentrations above 1 mM were not used because of poor aqueous solubility. The results are summarised in Table XXII.

6.3.2 f Electron microscopy

As in the case of the phleomycin amplifiers, none of the derivatives synthesised influenced the gross structure of DNA when incubated with the analogues.

TABLE XVIII EFFECTS OF DIFFERENT ANALOGUES ON HPRT ACTIVITY ISOLATED FROM HUMAN ERYTHROCYTES

ANALOGUE (0.2 mM)	ACTIVITY (n moles/min/ml)
2-Mercaptobenzoxazole	0.825
2-Carbamoylethylthiobenzoxazole	0.820
2-Carbamoylmethylthiobenzoxazole	0.830
2-(Benzoxazol-2'-ylthio)-N-Methylpropionamide	0.819
2-(Benzoxazol-2'-ylthio)-N,N-dimethylpropionamide	0.827
2-(Benzoxazol-2'-ylthio)-N-Methylacetamide	0.830
2-(Benzoxazol-2'-ylthio)-N,N-dimethylacetamide	0.814
2-Mercaptobenzimidazole	0.828
2-Methylthiobenzimidazole	0.819
2-Carbamoylmethylthiobenzimidazole	0.832
2-Carbamoylethylthiobenzimidazole	0.821

TABLE XIX EFFECTS OF VARIOUS ANALOGUES ON THE LIQUID HOLDING RECOVERY OF E.coli B CELLS IRRADIATED WITH UV FOR 12.5 SECONDS

ANALOGUE	% INHIBITION OF LHR
2-Mercaptobenzoxazole	52
2-Carbamoylethylthiobenzoxazole	90
2-Carbamoylmethylthiobenzoxazole	86
2-(Benzoxazol-2'-ylthio)-N-Methylpropionamide	86
2-(Benzoxazol-2'-ylthio)-N,N-dimetnylpropionamide	88
2-(Benzoxazol-2'-ylthio)-N-methylacetamide	76
2-(Benzoxazol-2'-ylthio)-N,N-dimethylacetamide	80
2-Mercaptobenzimidazole	50
2-Methylthiobenzimidazole	64
2-Carbamoylmethylthiobenzimidazole	68
2-Carbamoylethylthiobenzimidazole	60

TABLE XX EFFECTS OF VARIOUS ANALOGUES ON THE SURVIVAL OF THE CONTROL SUSPENSIONS OF UNIRRADIATED CELLS HELD IN ANALOGUE CONTAINING BUFFER FOR 3 HOURS

ANALOGUE	% SURVIAL
2-Mercaptobenzoxazole	96
2-Carbamoylethylthiobenzoxazole	94
2-Carbamoylmethylthiobenzoxazole	94
2-(Benzoxazol-2'-ylthio)-N-Methylpropionamide	93
2-(Benzoxazol-2'-ylthio)-N,N-dimethylpropionamide	89
2-(Benzoxazol-2'-ylthio)-N-methylacetamide	88
2-(Benzoxazol-2'-ylthio)-N,N-dimethylacetamide	91
2-Mercaptobenzimidazole	87
2-Methylthiobenzimidazole	87
2-Carbamoylmethylthiobenzimidazole	90
2-Carbamoylethylthiobenzimidazole	93

TABLE XXI EFFECTS OF VARIOUS ANALOGUES ON THE THERMAL MELTING TEMPERATURE OF E.coli DNA

ANALOGUE	Tm (°C)
2-Mercaptobenzoxazole	89.8
2-Carbamoylethylthiobenzoxazole	90.0
2-Carbamoylmethylthiobenzoxazole	90.2
2-(Benzoxazol-2'-ylthio)-N-Methylpropionamide	90.7
2-(Benzoxazol-2'-ylthio)-N,N-dimethylpropionamide	89.9
2-(Benzoxazol-2'-ylthio)-N-methylacetamide	90.0
2-(Benzoxazol-2'-ylthio)-N,N-dimethylacetamide	90.4
2-Mercaptobenzimidazole	89.9
2-Methylthiobenzimidazole	90.0
2-Carbamoylmethylthiobenzimidazole	89.8
2-Carbamoylethiobenzimidazole	90.0

TABLE XXII INFLUENCE OF VARIOUS ANALOGUES ON THE VISCOSITY OF DNA

ANALOGUE	VISCOSITY(cSt)
2-Mercaptobenzoxazole	0.934
2-Carbamoylethylthiobenzoxazole	0.934
2-Carbamoylmethylthiobenzoxazole	0.930
2-(Benzoxazol-2'-ylthio)-N-Methylpropionamide	0.934
2-(Benzoxazol-2'-ylthio)-N,N-dimethylpropionamide	0.941
2-(Benzoxazol-2'-ylthio)-N-methylacetamide	0.934
2-(Benzoxazol-2'-ylthio)-N,N-dimethylacetamide	0.938
2-Mercaptobenzimidazole	0.934
2-Methylthiobenzimidazole	0.938
2-Carbamoylmethylthiobenzimidazole	0.938
2-Carbamoylethylthiobenzimidazole	0.938

CHAPTER SEVEN

7.1 GENERAL DISCUSSION

The stated object of this study was to examine the effects of various purine and pyrimidine analogues on several aspects of nuceotide metabolism. The analogues tested were all reported as amplifiers of the antibiotic phleomycin. The influence of these analogues on nucleotide metabolism was investigated to obtain experimental evidence as to the mechanism of amplification of phleomycin. The findings of this study will be discussed in the following sequences: namely, influence of the amplifiers on -

i) The purine salvage enzyme, hypoxanthine guanine phosphoribosyl transferase (EC.2.4.2.8)
ii) The excision-repair mechanism of UV-irradiated E.coli, and
iii) The physical properties of DNA

7.1 a EFFECTS OF THE PURINE AND PYRIMIDINE ANALOGUES ON HYPOXANTHINE GUANINE PHOSPHORIBOSYL TRANSFERASE (EC 2.4.2.8)

As regards the effect of these phleomycin amplifiers on HPRT, it was found that no specific interaction of these analogues with the enzyme could be demonstrated. Only three of all the analogues tested were found to inhibit HPRT activity. Furthermore, detailed kinetic studies revealed different modes of inhibition. It should be borne in mind that these analogues could not strictly be called "inhibitors" since they could be acting as weak substrates for the enzyme. The analogues could not be tested as possible alternative substrates since neither the analogues nor

the second substrate, PRPP, could be obtained with a radioactive label. Inhibitors of HPRT are of biological significance and inhibitors are needed (114) to

i) Evaluate the role of this enzyme in maintaining steady-state pools of purines in various tissues and tumours
ii) Potentially treat schistosomiasis and malaria, diseases in which the parasites lack the de novo biosynthetic pathways
iii) Potentiate the action of other agents which inhibit de novo biosynthesis, effects of which may be "bypassed" due to HPRT; and
iv) Possibly mimic in animals the genetic disease which occurs in man as a result of HPRT deletion, i.e. the Lesch-Nyhan syndrome.

Since most analogues tested do not affect HPRT activity, it can be concluded that the mechanism of phleomycin amplification is not a result of the analogues being phosphoribosylated to their nucleoside forms. Hence, any influence on the nucleotide pool and ultimately DNA synthesis could be attributed to the analogues themselves rather than their nucleosides. However, promising inhibitors of HPRT were examined further since many inhibitors of HPRT such as 6-mercaptopurine and 8-azaguanine have proved to be of pharmacological importance. The study was then extended to in vivo studies. The inhibitors of HPRT were then tested on hepatoma cell cultures and all three active analogues were found to inhibit HPRT activity. Kinetic studies showed that this enzyme is active in hepatoma cell cultures and this activity is directly proportional to the number of cells present.

A complete loss of HPRT activity resulted when cells in culture were incubated with these analogues for one hour. Removing the medium and re-incubating the cells for 2 hours in analogue free medium did not restore HPRT activity. Presumably, during the 2 hours, the analogue or its metabolite) is still present in sufficient concentrations for inhibition. Furthermore, the analogues had no effect on cell number and HPRT activity was r[illegible]tected within 6 hours. The results indicate that the analogues effectively penetrate the cells and bind, possibly irreversibly, to the enzyme.

Further analogues of one of the inhibitors, the benzoxazole derivative, were synthesised in an attempt to establish a structure-function relationship. Structures of the analogues synthesised are summarised in Fig 48. None of the analogues synthesised proved to be inhibitors of HPRT, i.e. the modifications introduced must all have inhibited binding of the derivatives by the enzyme. One of the modifications introduced in the active derivative included changing the alkyl substituent on the sulphur atom. The heterocyclic parent structure benzoxazole was also modified so as to yield the benzimidazole derivatives.

The enzyme assay employed was a modification of that described by Wohlheuter (65). The assay is based on the separation of the radioactively labelled product [^{14}C]- nucleotide, i.e. IMP, from the radioactively labelled substrate, [^{14}C]-hypoxanthine by binding the former on an anion exchanger, in this case PEI-cellulose. The method of assay is simply, rapid and capable of measuring HPRT activity of samples of low protein content.

Hypoxanthine was quantitively removed by the washing procedure, and like the results of Wohlheuter, backgrounds of 200 counts per minute of a total of 22 000 counts per minute were left behind. The counting efficiency of the plates was found to be 84%.

The results of the purification procedure are in agreement with that described by Krenitsky (67). The enzyme yield was found to be 13.6% with a specific activity of 48.8 units per mg protein. Krenitsky et al. reported a yield of 16% with a specific activity of 55.6 units per milligram protein.

7.1 EFFECTS OF THE PURINE AND PYRIMIDINE ANALOGUES ON THE EXCISION REPAIR MECHANISM OF UV-IRRADIATED E.coli

The excision repair mechanism was studied by measuring the liquid holding recovery of UV-irradiated E.coli B as reported earlier. As described by Harm (27) most of the liquid holding recovery is found within 4 hours after irradiation, holding for still longer periods adds relatively little to the effects. Control suspensions of unirradiated cells, held in buffer for the same period of time, showed very little change in cell viability. Furthermore, the analogues had no significant effect on the survival of the control suspensions of unirradiated cells held in analogue containing buffer. All the phleomycin amplifiers tested were found to inhibit the liquid holding recovery, i.e. the excision repair of UV-irradiated E.coli B.

Grigg (10) has reported an association between phleomycin induced cell death and the inhibition

of dark repair processes. Furthermore, some phleomycin amplifiers, for example, 6.9-dimethyl-2-methylthiopurine (19) and caffeine (27) have been reported to be inhibitors of the excision repair pathway. The results reported in this thesis indicate no ordered pattern between phleomycin amplification activity and inhibition of excision repair. The results of the phleomycin amplifiers tested by Grigg (19) also indicated no ordered pattern. Many studies (10,19) have reported the inhibition of excision repair by caffeine which binds specifically to single stranded DNA (54). Domon et al. (56) suggested that this property is important in explaining inhibition of excision repair. Inhibition of excision repair by these phleomycin amplifiers may support the view that the analogues interact with DNA at the molecular level. Some of the inhibitors of excision repair reported by Grigg were also reported to have in common the ability to bind preferentially to single-stranded DNA. It was further suggested that the nature of binding is important for amplification activity. Compounds that bind weakly to denatured DNA without significantly altering the nature of the region of single-stranded DNA to which they are bound may be important for their amplification effect (19). Hence, in the third section of this thesis the interaction of the amplifiers with DNA was investigated using various physical techniques.

7.1 c EFFECTS OF THE PURINE AND PYRIMIDINE ANALOGUES AT THE MOLECULAR LEVEL

Firstly, the effect of these amplifiers on the Tm of E.coli DNA was investigated by measuring the optical density at 260 nm as a function of temperature. The Tm of E.coli DNA was found to be

90.5°C. This compares favourably with the reported values of 90°C (57). Of all the amplifiers tested, only caffeine influenced the helix-coil transition of E.coli DNA at a concentration of 0.3 mM. The amplifiers could not be tested at concentrations greater than 0.3 mM because of their high extinction at 260 nm. Hence, any interaction of the amplifiers with DNA could not be followed by changes of optical properties at 260 nm because of the highly UV absorbing substances added to the DNA.

The next technique used was circular dichroism. Circular dichroism (CD) measurements have the special feature that many of the added highly UV absorbing substances do not interfere with the measurements. The CD spectrum of DNA recorded in this thesis compares favourably with the spectra reported by other workers with one positive band at about 273 nm and one negative band at about 243nm. The principal contribution to the CD of the DNA arises from the conformational structure of the polymer. Loss of the double-stranded structures (due to thermal denaturation) decreases the amplitude of both bands. However, elevation of temperature in the premelting zone increases the positive CD band. All the amplifiers tested changed the CD spectra of DNA indicating the loss of optical activity. Concentrations as low as 100 µM indicated interaction with DNA. The intercalating agents, ethidium bromide and quinicrine, also altered the CD spectra of DNA in the same manner. Higher concentrations (up to 1 mM) of all the amplifiers results in loss of the amplitude of both bands. The results may be interpreted as a loss of the double-stranded structure of the DNA since similar CD spectra have been reported for thermally denatured DNA. At lower concentrations of amplifier (up to 0.3 mM) no changes in

Tm was measured at 260 nm although the CD spectra was altered. This could perhaps be considered as a change in conformation of the DNA without significantly altering the double-stranded nature of the DNA. At higher concentrations, strand separation may be favoured.

Hence, the interaction of these amplifiers with DNA was studied at the macromolecular level. Viscosity measurements revealed no changes in the viscosity of DNA when treated with the compounds at a concentration of 1 mM. Hydrodynamic properties of polymers are sensitive to chain conformation. Hence, it can be concluded that the overall conformation and macromolecular shape of the DNA was not changed by these compounds. The amplifiers were not tested at concentrations greater than 1 mM because of their poor aqueous solubility.

Fourthly, the gross structure of DNA treated with the phleomycin amplifiers was investigated using electron microscopy to examplify the results observed.

Electron microscopy has proved to be an important technique to study a variety of problems. Some of the information yielded by many workers include the direct measurement of single-stranded DNA (102), the analysis of partially denatured DNA (103), the replication and recombination complexes (104), and the physical mapping of genes in hybrid molecules (105). In this study, denaturation of DNA resulting in distinct strand separation or localised single-stranded regions along the DNA was not observed in samples treated with phleomycin and/or phleomycin amplifiers. Similar results were obtained for DNA samples treated with

intercalating agents such as ethidium-bromide and quinicrine. If any distortion of DNA did result, it could perhaps have been only a few nucleotides long at various loci along the DNA. This may not have been possible to visualize under the conditions employed. The DNA treated with phleomycin and/or phleomycin amplifiers or the intercalating agents results in marked changes in the CD spectra of DNA without changing the strand continuity as observed by electron microscopy may suggest that the compounds interact with DNA so as to change the conformation of DNA. This change in conformation may be sufficient to yield the DNA sensitive to the single-strand specific endonuclease suggested by Grigg (26).

7.1 d GENERAL CONCLUSIONS

The main aim of this project was to examine the effects of various purine and pyrimidine analogues on purine metabolism. The influence of these analogues on nucleotide metabolism was investigated to obtain experimental evidence as to the mechanism of amplification of phleomycin since all the analogues tested are good amplifiers of phleomycin.

As regards the effects of these phleomycin amplifiers on HPRT activity, no specific interaction of these analogues with the enzyme could be demonstrated. Only three of all the analogues were found to inhibit HPRT activity. Thus the mechanism of phleomycin amplification is not a result of the analogues being phosphoribosylated to their nucleotide forms and subsequently influencing the nucleotide pool. Hence any influence on the nucleotide pool and ultimately DNA synthesis could be attributed to the analogues themselves rather than their nucleotides.

Since inhibitors of HPRT are of biological significance, as outlined in chapter one, the inhibitors were tested *in vivo* in hepatoma cell cultures. All three analogues effectively penetrate the cells and bind, possibly irreversibly to the enzyme. Further analogues of one of the inhibitors, the benzoxazole derivative, were synthesised in an attempt to establish a structure-function relationship. None of the analogues synthesised proved to be inhibitors of HPRT, i.e. the modifications introduced must all have inhibited binding of the derivatives by the enzyme.

Secondly, all the analogues were found to inhibit the excision repair mechanism of UV-irradiated E.coli. Many workers (54) have suggested that the ability of compounds to bind specifically to single-stranded DNA may be important in explaining inhibition of excision repair. Inhibition of excision repair by these analogues may support the view that the analogues interact with DNA at the molecular level. Hence, the interaction of the analogues with DNA was investigated using various physical techniques.

The gross structure and macromolecular shape of the DNA was not changed by these analogues as can be concluded from thermal denaturation and re-association studies, viscosity measurements and electron mocroscopy. However, circula dichroism studies revealed direct interaction indicating conformational changes in the DNA molecules. The dramatic changes in the circular dichroism spectra could not be interpreted unequivocally. However, the conformational changes may be sufficient to induce localised denaturation rendering the DNA sensitive to the endonuclease suggested by Grigg (26).

8. APPENDIX

This thesis describes the effects of various phleomycin amplifiers on several aspects of nucleotide metabolism in an attempt to elucidate their mode of action.

The purine salvage enzyme hypoxanthine-guanine phosphoribosyl transferase was unaffected by most of the amplifiers, although three did show some inhibitory effect on its activity in vitro and in vivo. Detailed kinetic studies revealed both non-competitive and uncompetitive types of inhibition. This study was undertaken to ascertain whether the amplifiers alter the purine salvage pool by inhibition of HPRT or by acting as alternate substrates and thereby entering into the normal nucleotide pool. Incorporation of the inhibitors into DNA or into the phosphorylated purine pool could not be tested since neither the analogues nor the second substrate, PRPP could be obtained with a radioactive label. Since no correlation of activity as an amplifying agent and activity as an inhibitor of HPRT was obtained, it was concluded that phleomycin amplification is not a result of the analogues being phosphorylated to their nucleotide forms. Furthermore, the results indicate that amplification is not a result of the analogues interfering with the normal function of the enzyme so as to modify the nature of the purine salvage pool with significant consequences to cells whose DNA was under attack by phleomycin.

Secondly, all the amplifiers tested inhibited the excision repair of UV-damaged E.coli as measured by its liquid holding capacity. However no direct correlation between the degree of amplification emerged. The results may indicate that, as in the case of caffeine, the analogues bind selectively to single-stranded DNA, resulting in co-operative localised denaturation around UV lesions which is thought to be an explanation for caffeine inhibiting excision repair (26). One model (26) postulated to illustrate amplification of phleomycin by caffeine suggests a similar situation, namely that

phleomycin and caffeine induce localised cooperative denaturation in DNA resulting in the DNA being sensitive to an excision endonuclease which leads to DNA breakdown and cell death. Inhibition of excision repair by the amplifiers tested may support the view that the analogues interact with DNA at the molecular level.

Hence, in the third section, direct interaction of the amplifiers with DNA was investigated using various techniques:

(a) optical density
(b) circular dichoism
(c) electron microscopy
(d) viscosity

Measurements of helix-coil transition temperatures, viscosity and electron microscopy demonstrated no detectable effects of the amplifying agents, presumably because they were too insensitive. Circular dichroism studies revealed considerable changes in the optical activities of DNA on treatment with the amplifiers. Unfortunately there is no way as yet of interpreting these changes. However, the changes revealed direct interaction indicating conformational changes in the DNA molecule. The conformational changes may be sufficient to induce localised denaturation rendering the DNA sensitive to the endonuclease suggested by Grigg (26).

Theoretical work is at present being undertaken in collaboration with Dr Sawaryn of the University of Erlangen-Nuremberg in West Germany to study the conformational changes in DNA suggested (predicted) from the CD measurements. The crystal structures and the electronic configurations of the amplifiers are being calculated on an empirical level using a computer program developed by Dr Sawaryn. A search in the Cambridge crystallographic data file revealed that the structural and electronic data of the analogues has not yet been de-

termined. Once the structural data has been calculated, two modes of interaction of the amplifiers with DNA can be considered:

(a) Intercalation : Optimal structures of the intercalated complex would be determined by calculation of the interaction energy for those analogues found to interact by intercalation, and

(b) Covalent binding : An attempt would be made to describe a preferred reaction path for binding for those analogues found to interact by covalent binding.

Finally, the results from the theoretical calculations may suggest a molecular mechanism for phleomycin amplification in correlation with biological activity.

This thesis studies the effects of only one class of amplifiers, namely the purine and pyrimidine analogues. Many other classes of compounds, for example, the aromatic tricyclic compounds reported by Grigg et al (19) are good amplifiers of phleomycin. These compounds were not tested in this study because none of the results obtained for the purine analogues provided an unequivocal lead as to the mechanism of phleomycin amplification.

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