STUDIES ON DNA BIOSYNTHESIS IN HALOBACTERIUM CUTIRUBRUM

by

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SUMMARY

1) A synthetic medium is described in which Halobacterium cutirubrum grows as well as in complex media.

2) The cells, when grown in the synthetic medium, incorporate exogenous thymidine linearly for periods of at least 3 hours. Similar results are obtained whether the cells are grown in the presence or absence of thymidine, so that the uptake does not depend upon an inducible permease. The intracellular pools of intermediates between thymidine and DNA are small and fixed.

3) Thymidine incorporation ceases after degradation of the nucleoside to thymine by a constitutive thymidine phosphorylase. Thymine is only incorporated in the presence of a deoxyribose donor.

4) Nucleotides added to the growth medium are rapidly dephosphorylated prior to entry into the cell.

5) H.cutirubrum NRC 34001 does not perform dark repair of DNA either after u.v. irradiation or during normal growth. Cultures irradiated with u.v. are readily photoreactivated but do not recover viability in the dark. No increase in the rate of DNA synthesis is observed in the surviving cells after u.v. irradiation. At early times during normal semi-conservative replication newly incorporated thymidine is found only in the hybrid DNA.

6) The kinetics of the labelling of the thymidine nucleotide pools and DNA in H.cutirubrum by [^{3}H]-thymidine and of the passage of radioactivity from prelabelled pools into DNA show that dTTP is the precursor of DNA biosynthesis. Since dark repair biosynthesis of DNA does not occur in these cells, it is concluded that dNTP's are the immediate precursors of replication in vivo.

7) It is suggested that these organisms would be useful for the study of photoreactivation and DNA replication.

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ABBREVIATIONS

The following abbreviations are used in the text.

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
AMP, CMP, GMP, UMP, and dTMP	adenylic, cytidylic, guanylic, uridylic, and thymidylic acids
dTDP, and ADP	thymidine and adenosine diphosphates
dTTP, and ATP	thymidine and adenosine triphosphates
RNase	ribonuclease
DNase	deoxyribonuclease
Pi	inorganic orthophosphate
u.v.	ultraviolet
t.l.c.	thin layer chromatography
PEI	polyethyleneimine

Temperature is expressed as degrees Celsius throughout and is indicated only by the symbol °

INTRODUCTION

1.1. General Remarks.

The discovery of the nucleic acids was a result of the investigations of Miescher (Davidson, 1972). Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are constituents of plant, animal, and bacterial cells, and of viruses; DNA is confined to the nucleus, while RNA is found also in cytoplasm. Moreover, experiments indicated that within the interphase nucleus and during mitosis, DNA was apparently located in the chromosomes (Davidson, 1972). The significance of the latter observation was greatly enhanced by the transformation experiments on pneumococci.

Griffith (1928) observed that the pathogenicity of pneumococci is caused by the nature of the capsular polysaccharide of these bacteria. 'Smooth' glistening colonies are formed on solid media by the pathogenic bacteria, and 'rough' colonies are formed by the non-pathogenic type as these had lost their ability to form encapsulated cells. When heat-killed pathogenic pneumococci were injected into mice along with the living non-pathogenic variant, the mice died, suggesting that the pathogenic variant caused transformation of the non-pathogenic type.

The work of Avery, MacLeod, and McCarty (1944) identified the transforming principle as DNA. DNA extracted from encapsulated 'smooth' strains of pneumococcus could,

on addition to the culture medium, cause transformation of unencapsulated 'rough' cells into the smooth encapsulated type. These smooth cells could propagate indefinitely in the same form producing more DNA with the same capabilities. The pneumococcal DNA had induced the property of capsule synthesis, and the pathogenic variant so formed could initiate its own reduplication. These two functions were previously associated with the gene. It has since been established that DNA is indeed the hereditary molecule, containing the genetic information of the cell in its particular base sequence (Hotchkiss, 1955).

The importance of the preservation of the intact DNA molecule from generation to generation cannot be over-emphasised. It is thus not surprising to find that most cells are capable of both repair and replication of DNA, so that the genetic information it contains can be correctly maintained. The replication of DNA has been the subject of a vast amount of research, but a clear picture of the replicating machinery has not yet emerged. This process is very complex and some of the factors that are involved will be discussed in section 1.2.

The DNA molecule is the only macromolecule that can be correctly repaired by the cell. Repair mechanisms have been identified that correct damage to DNA caused by u.v. irradiation, chemical (mutagenic) agents, X-rays. The

effects of u.v. irradiation have been known since 1928 (Stent), but it was not until the 1960's that the nature of the lesion was identified (section 1.3).

Bacteria contain only one chromosome and so offer a simple system for studying DNA biosynthesis: both repair synthesis and replication. However, since both dark repair and replication occur in most bacteria commonly utilised, a system lacking repair could provide an understanding of replication without the complications of repair. Such a system would greatly simplify the studies on DNA replication.

1.2. DNA Replication.

1.2.a. Structure of DNA and semi-conservative replication.

The monomeric units of the DNA molecule are nucleotides, which are made up of three components : 1) a purine or a pyrimidine base, 2) a 5-carbon sugar, deoxyribose, 3) a phosphate esterified to carbon 5 of the sugar. The purine bases are adenine and guanine; the pyrimidine bases are cytosine and thymine. In some DNA molecules the bases are modified; the modification involves either methylation or glucosylation. The nucleotides are arranged as a long unbranched polymer, formed by a phosphodiester linkage between the 5'-phosphate of one nucleotide and the 3'-hydroxyl of the sugar of the next. Two right handed helical chains are coiled around the same axis, with the bases on the inside and

the phosphate on the outside. The coiling of the two chains is such that they cannot be separated except by unwinding. The two chains are of opposite polarity in the sense that the internucleotide linkage in one strand is 5' -> 3', and the other is 3' -> 5'. Hydrogen bonds as well as base stacking forces hold the two helical chains together (Davidson, 1972).

Only specific base pairs are bonded together, namely adenine with thymine, and guanine with cytosine (Watson & Crick, 1953). This equivalence of adenine with thymine, and guanine with cytosine, was first noted by Chargaff (1951), and is of utmost importance to the formation of the DNA double helix. Modified bases can also enter into base pair formation, provided that they can form hydrogen bonds with their partner. The sequence on any one chain is not restricted, but it determines the sequence of bases on the other strand. The complementary feature of the chain can be recognised as important in preserving the hereditary information stored in the molecule (Watson & Crick, 1953).

The structure elucidated by Watson and Crick is the B structure, and is a relatively open structure that exists in solutions of low ionic strength. The A structure, found at high ionic strengths (Tunis & Hearst, 1968) is also a right handed helix, but in this case the bases are tilted. Interest lies in the A structure because it is the structure of the double stranded RNA, or DNA-RNA hybrids in solution;

the latter may play a role in initiation of DNA replication.

Despite the stability of the double helix, the DNA molecule is not rigid, but it undergoes internal deformation. Tritium exchange experiments (Hanson, 1971), show that small segments of the double helix can swing apart and protrude into the external medium, depending on the environment of the DNA molecule. The double helical secondary structure can be further constrained to give rise to the tertiary structure such as the super-coiled or open cyclic molecules. Not all naturally occuring DNA is double-stranded: single stranded DNA occurs is some viruses. In these cases the first step in replication involves the conversion of single stranded DNA to double stranded DNA.

The complementary feature of the double helix (for every adenine there is a thymine, and for every guanine a cytosine), immediately suggests a mechanism for self-duplication, where the two chains come apart and each chain acts as a template for the formation of a new complementary chain. Two new pairs of chains are formed and the sequence of the parent molecule are duplicated exactly (Watson & Crick, 1953b). This mechanism, referred to as semi-conservative replication,was demonstrated for Escherichia coli, both by caesium chloride density gradient centrifugation (Meselson & Stahl, 1958), and autoradiography (Cairns, 1963), and is now generally accepted as the method by which

the whole DNA molecule is reproduced. Replication should be distinguished from repair synthesis. In repair (section 1.3 only a portion of the DNA strand is reproduced, but the complementary feature of DNA is also important.

Since it was established that DNA replication is semi-conservative, several basic concepts have been put forward to explain the mechanism of the process, but the details of the replication are not yet clearly understood. On the basis of what is known, it can be said that DNA replication must involve the separation of the two strands (section 1.2.b). In viral and bacterial chromosomes, replication starts at a unique point and proceeds by the addition of nucleotide monomers in a 5' -> 3' direction. It is also believed that replication is discontinuous(section 1.2.e) and that these short pieces are formed as an extension of an RNA fragment (section 1.2.f). Finally all gaps are sealed and two new DNA molecules are formed. If synthesis does occur in short pieces during replication, it is not unreasonable to assume that some of the factors that influence the process will influence the repair process (section 1.3).

1.2.b. The unwinding of the double helix.

Ever since it was proposed that the DNA molecule is made up of two right handed helices coiled around the same axis and that they separate during replication, the mech-

anism of the unwinding of the two strands has been sought. The two strands have to be separated without rotation of the whole molecule through a large number of turns.

Levinthal and Crane (1956) showed that unwinding a DNA molecule of 4×10^7 daltons over a period of 100 secs., would increase the energy required for replication by 0.1%. This energy could be made available from the polymerisation process and the driving force for replication would then be at the replicating fork.

A second model arose from the finding that the bacterial chromosome is circular while replicating (Cairns, 1963). The point of attachment of the parent and daughter molecules, where replication begins and ends, could be the site of an active unwinding mechanism. This swivel, the nature of which was never explicit, could result from alternate action of endonuclease and ligase which could respectively break and rejoin the molecules (Cairns & Davern, 1970).

More recently the isolation of certain proteins, the unwinding proteins, that appear to work in conjunction with the polymerase, supports the postulate that the double helix must unwind prior to replication. These proteins, T4 gene 32 (Alberts & Frey, 1970), M13 gene 5 (Oey & Knippers, 1972), and E.coli unwinding proteins (Sigal et al., 1972), bind single stranded DNA and cause denaturation in the region to be replicated. The E.coli unwinding protein stimulates the

activity of DNA polymerase II *in vitro*, but the physiological role of this polymerase is not clear. *E.coli* unwinding protein has been recognised as a factor in the *in vitro* synthesis of replicative phages (section 1.2.g), in which they stimulate the initiation of replication.

1.2.c. The direction of replication.

It has been known for some time that the replication of the chromosome does not occur in a random manner, but rather starts from a given point and proceeds in either one or two directions. The autoradiographic experiments of Cairns (1963) demonstrated that the replicating *E.coli* chromosome was circular, and interpretation of the differential grain densities led to the notion that replication starts at one point - the origin - and proceeds in one direction. This point of view was further substantiated by the genetic experiments on *E.coli* Hfr (Nagata, 1963) and *Bacillus subtilis* (Yoshikawa & Sueoka, 1963). Subsequent studies have shown that replication is not unidirectional in these bacteria, but is bidirectional.

The possibility of bidirectional replication in *E.coli* was first proposed by Caro and Berg (1968) from interpretation of gene dosage data. Several other investigators (Masters & Broda, 1971; Mckenna & Masters, 1972; Hohlfield & Vielmetter, 1973), using autoradiography, genetic analysis,

or labelling with 5-bromouracil followed by analysis of the newly synthesized strand, showed that replication is indeed bidirectional for E.coli. Wake (1973) and Hara and Yoshikawa (1973) also demonstrated bidirectional replication for B.subtilis using autoradiographic and genetic analysis. Replication was also shown to be bidirectional in eukaryotes : complex mammalian chromosomes (Huberman & Tsai, 1973), Drosophila (Kriegstein & Hogness, 1974), and animal virus SV40 (Danna & Nathans, 1972).

While it appears that replication is bidirectional for prokaryotes and eukaryotes, this is not true for all chromosomes. The work of Chattoraj and Inman (1973) on phage 186 DNA and Lovett et al. (1974) on plasmid ColE1 DNA showed that these chromosomes replicate unidirectionally. On the other hand λ phage replicates bidirectionally (Inman & Schnos, 1970). The difference in the mode of replication of these phages is not believed to be host controlled since the E.coli host was the same in both studies.

It appears then that for prokaryotes and eukaryotes replication is indeed usually bidirectional. In prokaryotes there is one origin but several replication forks, while in eukaryotes many origins are located at different positions along the chromosome.

1.2.d. The 5' -> 3' direction and models of in vivo replication

The enzymes isolated so far that catalyse the synthesis of DNA, add the nucleotide monomers in the 5' -> 3' direction (section 1.4). These enzymes, DNA polymerase I, II and III, share two basic features (Kornberg, 1974) : they catalyse the phosphodiester bridge formed between the 5'-phosphate group of the incoming deoxynucleotide and the 3'-hydroxyl of the growing end of the DNA primer chain; and the nucleotide selection is by a base pair matching to a template chain. The latter characteristic is in agreement with semi-conservative replication of the DNA duplex. If these enzymes are indeed the enzymes of replication, two questions concerning DNA replication arise :

1) since growth is in a 5' -> 3' direction, how can replication of two antiparallel strands take place?

2) since none of the three polymerases isolated so far can initiate DNA synthesis, how does this occur in vivo?

A second type of DNA polymerase that is capable of directly adding nucleotide precursors to the 5'-terminus, would, of course, explain the growth of both chains in the 5' -> 3' direction. However, extensive searches for this enzyme have proved unsuccessful (Mitra & Kornberg, 1966).

The 'knife and fork' model, put forward (Guild, 1968; Kornberg, 1969; Richardson, 1969) to explain the action of DNA polymerase I in vivo, suggests that the inert duplex

chromosome is first nicked, possibly at a specific site, and replication then proceeds by covalent extension at the 3'-hydroxyl end. The 5'-end of the molecule is either further degraded or maybe peeled off. Replication then proceeds for some distance and switches to the complementary strand as template to form a fork. The fork is cleaved by an endonuclease. A repetition of this process leads to small pieces of DNA in the region of the replicating fork, which are then sealed by ligase action. Although such pieces have been isolated (section 1.2.e), this model falters because no 'fork' that has not been nicked by endonuclease action has been detected so far (Dressler, 1975). In this model the primer is the nicked DNA strand, and the need to initiate synthesis of a new strand in vivo does not arise.

The presence of single stranded regions, if only transiently, at the replicating fork has been put forward to explain the replication of both strands in the 5' → 3' direction. These single stranded regions have been observed for λ phage (Inman & Schnos, 1971), and T7 chromosomes (Wolfson & Dressler, 1972). It is suggested that DNA synthesis involves the direct elongation of one daughter strand in the growing point. This elongation is accompanied by the unwinding of the parental double helix to expose a region of single strand DNA which is then converted to the duplex state by a discontinuous mechanism involving DNA fragments.

A variation of the replicating fork model is the rolling circle model put forward for ϕX174; a nick in one strand serves as the starting point for the covalent extension of the 3'-hydroxyl terminus (Gilbert & Dressler, 1968). As replication proceeds the 5'-end of the strand is displaced as a growing length of single strand and anchored in some manner. In this model, the parental molecule serves as primer for initiation.

The D-loop (Berk & Clayton, 1974; Grossman et al., 1973), as a model of replication of mitochondrial DNA is yet another solution to the problem. In this model, the super coiled duplex circle is transiently relieved so that a new strand can be initiated at a fixed point, using a complementary strand as template. The template strand balloons out as the D-loop, and at a later stage synthesis is initiated on the displaced single strand. Progress of replication thus involves a staggered copying of the strands, each in a unidirectional manner.

1.2.e. Discontinuous synthesis of DNA.

When E.coli cells are pulse labelled with [^{3}H]-deoxy-thymidine, the label is found in nascent short chains of DNA as determined by the sedimentation constant. If the pulse labelling is followed by a chase of unlabelled thymidine, then the radioactivity is recovered in the large molecular

fraction (Sakabe & Okazaki, 1966).

A similar result is obtained with T4 DNA (Okazaki et al., 1968). These results led Okazaki (1968) to propose the model of discontinuous synthesis for DNA in which short stretches of DNA are synthesized at the replicating point and subsequently connected to the older portion of growing strands by the formation of phosphodiester linkages. The enzyme responsible for joining these short chains is polynucleotide ligase and when thermosensitive ligase mutants are tested at the non-permissive temperature these short chains accumulate (Sugimoto et al., 1968).

Whether both strands replicate via short segments, or synthesis is discontinuous on only one strand and continuous on the other, is still not resolved. Sugimoto et al. (1968b), have shown that synthesis is discontinuous on both strands, since all the nascent DNA found in short chains anneal to an equal extent on both the template strands. The direction of growth of these short chains was determined by measuring the kinetics of release of $[^3H]$ and $[^{14}C]$ label after exonucleolytic degradation, and the results are consistent with growth in the 5' -> 3' direction along both template strands (Okazaki et al., 1969). These fragments (Okazaki fragments or pieces) have been found in viral, bacterial, and animal systems. The size of the fragments in bacterial systems is 1000-2000 nucleotides and the sedimentation rate is 10S.

The possibility that the Okazaki fragments are the products of nuclease action on newly synthesized DNA has been raised by Werner (1971a). He showed that using [^{3}H]-thymine results in the appearance of label in the large DNA and that [^{3}H]-thymidine is incorporated into the Okazaki pieces and concluded that these Okazaki pieces are possible repair fragments rather than replication fragments.

The possibility that these fragments are not produced to repair breaks by endonucleolytic action was tested for T4 DNA by Iwatsuki and Okazaki (1970). When chloramphenicol is added at an early time after infection, synthesis of T4 phage induced endonuclease is inhibited. Under these conditions, nascent short chains accumulate in ligase deficient mutants implying that the pieces are not products of long DNA strands.

The *in vitro* experiments of Olivera and Bonhoeffer (1972) showed that DNA synthesis involved two classes of DNA pieces both of which accumulate in the presence of nicotinamide mononucleotide (NMN), an inhibitor of DNA ligase. Since these two classes of pieces, long and short, are complementary (Hermann *et al.*, 1972) it is suggested that they are copied from both strands of DNA, and could represent either a difference in the rate of joining of nascent fragments or continuous synthesis on one strand and discontinuous synthesis on the other.

The above suggestion is supported by the pulse labelling experiments of Iyer and Lark (1970) in E.coli strain 15 T^-: only half of the nascent pulse label was found in DNA fragments; the electron microscopic evidence of Wolfson and Dressler (1972); and the experiments of Louarn and Bird (1974) for the newly synthesized DNA in E.coli lysogenic for phage λ: Their results indicated that synthesis is discontinuous on the 3' -> 5' strand , for both pol A^+ and the pol A^- strain (the pol A locus codes for DNA polymerase I) and continuous in the 5' -> 3' strand for the pol A^+ mutant. In the pol A^- strain, however, discontinuous synthesis occurs in both strands, though less frequently in the 5' -> 3' strand. Jacobson and Lark (1973) also observed two classes of short DNA chains in E.coli: one is the classical Okazaki piece; the other does not behave like a replication intermediate but has a similar size range as the Okazaki piece. Unlike the Okazaki fragment, these pieces do not accumulate when ligase action is inhibited. It appears then that DNA synthesis may be discontinuous, but not always on both strands.

1.2.f. Initiation of DNA synthesis.

As mentioned in section 1.2.d. none of the polymerases isolated so far can initiate DNA synthesis. An important distinction must be made between the origin and initiation of the overall process of replication, located at the fork, and the

initiation of the DNA chain. Very little is known about the factors that create the origin and set the replication in motion. However, the initiation of DNA synthesis has received much attention and it is believed to involve RNA synthesis.

While the DNA polymerase can only extend primer chains, RNA polymerase is capable of recognizing a specific DNA sequence and of initiating RNA synthesis. As early as 1961, it was reported (Maaloe and Hanawalt, 1961; Hanawalt et al., 1961) that protein and/or RNA synthesis was necessary to initiate, but not to maintain DNA synthesis. In 1972, Lark identified the dna G locus in E.coli, the gene product of which initiates synthesis of short DNA chains. This product has recently been described as an RNA polymerase (Zechel et al., 1975). RNA synthesis has been implicated in the replication of DNA of E.coli (Lark, 1972; Sugino et al., 1972); B.subtilis (Sarkar, 1975), the bacteriophages λ (Dove et al., 1971), M13 (Brutlag et al., 1971), ϕX174 (Schekman et al., 1972), the mitochondria of HeLa cells (Grossman et al., 1973), and mouse cells (Hunter & Francke, 1973), and polyoma virus (Magnusson et al., 1973).

The RNA is linked to the nascent DNA or short Okazaki pieces (Sugino et al., 1972), and the size of the RNA is estimated at 50 - 100 nucleotides (Hirose et al., 1973). The RNA-DNA link is a 3'→5' phosphodiester bond (Keller, 1972; Sugino & Okazaki, 1973), that can be removed by ribonuclease H,

an enzyme that specifically degrades RNA hybridised to DNA. The RNA is located at the 5'-end (Keller, 1972; Hirose et al., 1973; Reichard et al., 1974) and it is proposed that the specific deoxynucleotide involved in the linkage is dCMP (Hirose et al., 1973).

It has been suggested that RNA priming of DNA synthesis occurs to prevent the possibility of error when initiation begins on an open stretch of DNA. These errors are caused because the absence of adjacent bases that provide for $\pi - \pi$ interactions produce more wobble. Since the RNA fragment is excised and the gap filled by a repair-like process, any mismatched base pair can be corrected (Dressler, 1975).

1.2.g. In vitro replication systems.

The molecular mechanisms involved in DNA replication are very complex. The construction of an in vitro replicating system has proven to be a very difficult task, since more than the polymerisation step is involved. Not only are several proteins operating together, but they must also interact with a fragile template. In vitro replication studies have utilised cells rendered permeable to nucleotide precursors by toluene, (Moses & Richardson, 1970), sucrose (Wickner & Hurwitz, 1972), partial lysates such as the cellophane disc lysate (Smith et al., 1970), and membrane complexes (Knippers & Sterling, 1970), but a

complete understanding of the biochemistry of replication requires a soluble enzyme system.

Some of the enzymes involved in DNA synthesis have been purified. They are the DNA polymerases (section 1.4.), DNA ligase (section 1.2.e.) and DNA unwinding proteins (section 1.2.b.). Other proteins involved in DNA replication have been identified, only in so far as they are products of a gene locus, from studies utilizing temperature sensitive mutants. These mutants sustain DNA replication at the"permissive"temperature, but are deficient under a "restrictive" temperature. A soluble extract from E.coli developed to perform the first stage of replication of the single-stranded phages have been described. These phages M13 (Schekman et al., 1974) and ϕX174 (Schekman et al., 1975) are tiny viruses whose chromosomes contain only 8 - 10 genes. The first step in their replication involves the conversion of single stranded DNA to duplex DNA or RF II. The enzyme requirements for the reconstituted reactions are given in table 1.1.

The in vitro reconstruction of an E.coli replication system has also been described (Kornberg et al., 1974)using a DNA free soluble system (Wickner et al., 1972). and the folded chromosome or nucleotide as template (Stonington & Pettijohn, 1971). These folded chromosomes have been isolated as a compact structure containing small amounts of RNA and protein, and their solutions have low viscosities. Kornberg et al.(1974)

Table 1.1.

Proteins required for conversion of M13 and φX174 viral strands to RF

Replication stage	Proteins required
M13[1] : Single strand to RF II	RNA polymerase E.coli unwinding protein Polymerase III ┐ Copolymerase III ┘ Polymerase III holoenzyme Polymerase I DNA ligase
φX174[2]:Single strand to RF II	dna B protein dna C protein DNA polymerase III holoenzyme dna G protein Protein i[3] Protein n[3] Unwinding protein Spermidine DNA polymerase I DNA ligase

1. Kornberg (1974) p.218.
2. Schekman et al. (1975).
3. Protein i and n have no defined genetic locus.

were able to obtain semi-conservative synthesis of DNA in this soluble system. The newly synthesised DNA remains associated with the folded chromosome but on treatment with alkali is released as small DNA fragments.

Thus it can be said that DNA replication involves the interaction of several proteins with a DNA template. It occurs by extension of a primer. The need for an RNA polymerase in the soluble enzyme system would suggest that RNA may be the primer, but a clearer picture of the process is still needed.

1.3. Repair of DNA

1.3.a. Introduction

As mentioned in section 1.1., the maintenance of the correct base sequence in the DNA molecules is important since this sequence carries the information required for the survival of the species. Thus in addition to DNA replication, most organisms are capable of repairing DNA. The complementary nature of the strands permits retrieval of the genetic information when one strand is damaged. Damage to DNA can occur from u.v. light, X-rays, and chemical (mutagenic) agents such as the alkylating agents which modify bases. Some mutagenic agents such as the nitrogen mustards produce guanine-guanine crosslinks.

The damage to DNA by u.v. light causes dimerization of pyrimidine bases adjacent to each other. These dimers are not the only photoproducts of u.v. irradiation on DNA, but they

are the best understood of DNA lesions. The rate of dimer formation seems to be dependent on the surrounding bases. Of the three possible types of pyrimidine dimers, the thymine-thymine dimer is most readily formed; consequently thymine rich DNA is sensitive to u.v. irradiation. The adjacent thymine residues are joined through their respective 5 and 6 carbons in a cyclobutane ring and can no longer form hydrogen bonds with adenines in the opposing strand. The secondary structure in that region is consequently distorted and unless the dimer is removed replication and transcription are prevented or disturbed.

Bacteria have at least three repair systems for handling base damage like the thymine-thymine dimer. Present insight into these systems has been derived mainly from studies on u.v. induced lesions in E.coli. The repair systems are:

(1) Photoreactivation: a light-dependent enzyme catalyses the in situ reversal of pyrimidine dimer to pyrimidine monomers. The phr gene controls the production of the photoreactivating enzyme.

(2) Excision repair: the damaged portion of the DNA molecule is excised and a new piece is synthesized, inserted, and the strand resealed. This form of repair is controlled by the uvr gene.

(3) Post replication repair: when new DNA is synthesized using a dimer containing duplex as the template, the newly

synthesized strand contains gaps. In bacteria these gaps are filled by exchanges between sister duplexes and the recombinational genes play a role in this repair process.

Both excision repair and post replication repair are generally referred to as dark repair.

1.3.b. Photoreactivation

The photoreactivation process is specific for repair of pyrimidine dimers : thymine, cytosine and uracil dimers are all monomerized (Setlow et al., 1965). In this process, a specific photoreactivating enzyme or photolyase interacts with the dimer containing region of DNA in situ and, when irradiated with 300 nm - 500 nm light, cleaves the dimer to regenerate the original pyrimidines thus restoring biological activity of the DNA (Rupert, 1963). This form of repair is virtually error free (Witkin, 1968).

The photoreactivation process is complicated by indirect photoreactivation. The latter is not enzyme dependent and is believed to be an indirect repair process. The reaction seems to depend on (1) wavelength, (2) dose, (3) dose rate of reactivating radiation, and (4) temperature (Jagger & Stafford, 1965).

Although the photoreactivating enzyme has been identified in a variety of bacterial, fungal, and eukaryotic sources (Cook & McGrath, 1967), attempts to study the detailed

mechanism of action of the enzyme have been hampered by the small number of enzyme molecules present in the cells. Only recently has the enzyme been purified to electrophoretic homogeneity from yeast, (Boatwright et al., 1975), Streptomyces griseus, (Eker & Fichtinger-Schepman, 1975), E.coli, (Sutherland et al., 1973). The action spectra of the enzymes appear to be dependent upon the source of the enzyme, for example, it is 360 nm - 385 nm in yeast and E.coli, and 435 nm for S.griseus. It has been postulated (Eker & Fichtinger-Schepman, 1975) that the enzyme utilizes light at these wavelengths through the mediation of a chromophore or cofactor, but the nature of this is not clear. This could be compared to the role of chlorophyll in photosynthesis.

Until recently, the enzyme had not been found in placental mammals such as man. However, Sutherland (1974) has recently isolated the enzyme from human leukocytes. The enzyme has also been isolated from murine and human fibroblasts (Sutherland et al., 1974). The specificity of the enzyme for pyrimidine dimers enables its use as a diagnostic tool: if u.v. induced damage is photoreactable, pyrimidine dimers are probably the major cause of damage. Since u.v. light causes skin cancer in humans, which in the extreme case of xeroderma pigmentosum (a rare disease in which affected individuals develop malignant growth in the regions of skin exposed to sunlight) leads to death, the role of dimers

in the induction of such cancers in man can be evaluated. Some xeroderma pigmentosum cells contain low levels of the photo-reactivating enzyme (Sutherland et al., 1975).

1.3.c. Excision repair.

The excision of pyrimidine dimers from the DNA is an important mechanism for cellular repair of ultraviolet damage. The overall picture of excision that emerges from studies in E.coli and Micrococcus luteus indicates a multi-process. First the u.v. endonuclease detects the site of the lesion and makes an incision near the pyrimidine dimer. Further degradation by an exonuclease, removes the lesion within a short oligonucleotide, and the gap resulting is repaired by a DNA polymerase using the intact complementary strand as a template for replication. The 3'-end of the nicked strand serves as the primer for the polymerase action. This step is similar to replication but it is not yet known whether or not the enzyme utilised for both replication and repair are the same. Finally the newly synthesized piece is joined to the main strand of the DNA by a ligase, thereby restoring a normal DNA strand.

Excision repair seems to be an accurate, error free system of repair (Witkin, 1968). Unlike photoreactivation, it is not specific for u.v. damage. The u.v. endonuclease from E.coli and M.luteus act on DNA containing other types of

damage such as those produced by nitrogen and sulphur mustards, mitomycin C, psoralen (Howard-Flanders, 1973). These alkylating agents cause inter-, as well as intra-strand cross links which are released from the DNA in wild type bacteria but not in excision defective mutants (Lawley & Brookes, 1965).

This system is prevalent in prokaryotes and eukaryotes. In plants (Wolff & Cleaver, 1973) and some placental animals (Trosko et al., 1965), it is present to a limited extent. Until recently it was suggested that excision repair was generally absent from plants, but Howland (1975) recently reported that wild carrot protoplasts are capable of carrying out dark repair. Mice, hamsters and other rodents, can perform excision repair only to a limited extent, if at all (Hanawalt, 1975). Irradiated cells are capable of making high molecular weight DNA. Since these animals are also unable to photoreactivate the u.v. lesions, it is believed that they have developed an efficient means of bypassing dimers so that excision or photoreactivation is not necessary (Painter, 1974). There is evidence to suggest that the enzymes of excision repair found in bacteria are also present in humans (Cleaver, 1970). Some xeroderma pigmentosum cells are deficient in excision repair (Cleaver, 1968).

1.3.d. Post replication repair.

When DNA is synthesized after u.v. irradiation of excision defective mutants, it has lower molecular weight than that synthesized by unirradiated cells, suggesting that synthesis is interrupted by the dimers and resumed again beyond the dimers (Rupp & Howard-Flanders, 1968). This results in gaps along the newly synthesized strands.

One problem that arises with this model is how and where replication resumes after it has been blocked by a dimer. Since DNA synthesis is not initiated by DNA polymerase but requires a primer, possibly RNA (section 1.2.f.), it is possible that synthesis is resumed at the next primer site (Hanawalt, 1975). The gap size has been estimated to be about 3×10^5 daltons, similar to the replication units of discontinuous synthesis. This suggests that the polymerase is omitting a whole Okazaki fragment when the template contains a dimer, (Setlow & Setlow, 1972).

The missing base sequence can be reconstructed by recombinational or post-replication repair (Rupp et al., 1971) in bacteria. The possible ways in which these gaps are filled in bacteria are :

1) exchange of pre-existing DNA without de novo repair synthesis
2) exchange of pre-existing DNA with de novo repair synthesis.

It has been suggested that occasional errors in gap filling can give rise to u.v. mutagenesis and this repair system is

error prone, (Witkin, 1968). In bacteria it is believed that recombinational repair is important for tolerance to u.v. light, since recombinational enzymes appear to be needed for the full normal radiation resistance of cells.

In mammalian systems, it is suggested that the gaps are filled by de novo synthesis (Lehman, 1972a) implying that recombinational events do not always occur. The possibility that gaps do not occur in mammals has been raised by Painter (1974), who could not detect them in his studies with HeLa cells. However they have been reported in mouse L cells (Fujiwara, 1975), human cells (Buhl & Regan, 1974), and hamster cells (Meyn & Humphrey, 1971). Since the lesion responsible for the gaps in the mammalian systems is photoreactivable, it is believed that it is the pyrimidine dimer that is blocking the synthesis (Buhl et al., 1974). If these gaps are equivalent in size to the Okazaki fragment, then it is not unreasonable that the mechanism of normal chain elongation could be applied here.

1.4. DNA polymerases.

Repair, replication and recombination are the three processes involving DNA that occur in the living cell. Both excision repair and replication involve the de novo synthesis of DNA. Three enzymes, DNA polymerase I, II, and III, have been isolated from E.coli (Kornberg, 1974) and B.subtilis

(Otto, 1973) that are capable of synthesizing DNA. Only the E.coli DNA polymerases will be discussed in this section. A notable feature of these enzymes is their ability to build a DNA chain with remarkable accuracy according to the specifications of the template. It is possible that these three enzymes may have a special role in either replication or repair or in both of these processes.

The reaction catalysed by a DNA polymerase is :

$$(dNMP)_n + dNTP \Longrightarrow (dNMP)_{n+1} + PP_i$$

and it represents the polymerisation reaction of both repair and replication. In this reaction a phosphodiester bond is formed between the 3'-hydroxyl group of the growing DNA chain called the primer, and the 5'-phosphate of the incoming deoxynucleoside 5'-triphosphate. None of the polymerases isolated so far can initiate DNA synthesis, however, they all require a primer. In replication, it is suggested that RNA is utilised as the primer (section 1.2.f), while in repair the 3'-end of the damaged DNA strand serves as the primer. The overall direction of chain growth by the polymerases is in the 5' -> 3' direction, and they do not add nucleotides in the 3' -> 5' direction. Various models have been put forward to explain how two anti-parallel strands replicate simultaneously in the DNA molecule (section 1.2.d).

The incoming deoxynucleotide is selected by base pair matching to a DNA chain called the template. The E.coli and

T4DNA polymerases do not extend chains in which the 3'-terminal nucleotide is not paired with the template. One of the activities of the DNA polymerases, the 3' -> 5' exonuclease activity (see below), excises any mismatched bases and thereby serves a corrective function (Brutlag & Kornberg, 1972) that guarantees the accurate base pair matching.

When DNA polymerase I was first isolated (Lehman et al., 1958), it was believed to be the enzyme of replication. However the isolation of polAI mutants (mutants that contain low levels of polymerase I activity) by deLucia and Cairns (1969) suggested that this enzyme was not solely responsible for chromosome replication. These mutants grow normally but appear to be defective in their capacity to repair DNA damaged by u.v. irradiation and drugs like methylmalonyl sulphonate. This suggests that the enzyme may have a role in repair.

The reduced levels of polymerase I in the mutants made possible the isolation of polymerase II (Knippers, 1970; Kornberg & Gefter, 1970) and polymerase III (Gefter et al., 1971). All three polymerases require the presence of all four deoxynucleoside triphosphates, Mg^{++}, and a DNA template and primer. Responses to temperature, ionic strength, pH, buffer ions, detergents, sulfhydryl and other chemical agents, vary among the polymerases and serve to distinguish between E.coli polymerases I, II, and III (Kornberg, 1974).

Table 1.2. summarises some of the properties of the three DNA polymerases from E.coli.

The properties of DNA polymerase I have been studied quite extensively. The enzyme is a simple polypeptide chain of molecular weight 109,000, and has the following activities : 1) 5' -> 3' growth of the DNA chain; 2) hydrolysis of a DNA chain from 3'-OH end; 3) hydrolysis of the DNA chain from the 5'-end; 4) pyrophosphorolysis of a DNA chain from the 3'-end; 5) exchange of inorganic pyrophosphate. There appear to be at least five major binding sites in the active centre of the molecule : 1) DNA template site, 2) DNA primer site, 3) 3'-OH DNA primer terminus site, 4) deoxy-nucleotide triphosphate binding site and 5) site of the 5' -> 3' exonuclease activity.

Limited proteolysis of DNA polymerase I produces a small fragment (molecular weight 36,000 daltons), that retains only the 5' -> 3' exonucleolytic activity (Setlow & Kornberg, 1972). The large fragment (molecular weight 76,000 daltons) contains the polymerase and the 3' -> 5' exonuclease activities. These two fragments show no measurable affinity for each other, but the activity of the whole enzyme is restored when a mixture of the large and small fragments are used. This suggests that the single polypeptide chain contains two separate active sites. It has been shown (Lehman & Chien, 1973) that this 5' -> 3' exonuclease activity persists in

Table 1.2.

DNA polymerases from E.coli [1,2]

property	DNA polymerase I	DNA polymerase II	DNA polymerase III
1. molecular weight	109,000	120,000	180,000
2. direction of synthesis	5'->3'	5'->3'	5'->3'
3. exonuclease activity	5'->3' 3'->5'	3'->5'	5'->3' 3'->5'
4. template primer:			
intact duplex	no	no	no
primed single strands	yes		
nicked duplex	yes		
duplex with gaps	yes	yes	yes
5. pyrophosphorolysis	yes	yes	yes
6. molecules/cell (estimated)	400	100	10
7. structural gene	pol A	pol B	pol C

(1). Kornberg, (1974), page 127

(2). Livingston et al., (1975) and Livingston & Richardson, (1975)

the polA mutants isolated by deLucia and Cairns (1969). While polymerase I (Kornberg, 1969) and III have all five activities (Livingston & Richardson, 1975), the 5' -> 3' exonucleolytic activity has not been found in polymerase II. Polymerase III appears to have at least two subunits, the molecular weights of which are not certain. Wickner et al., (1973) claim they are each 90,000, while Livingston and Richardson (1975) claim they are 140,000 and 40,000 respectively.

Polymerase I is very important in excision repair (Glickman, 1974), and in its absence polymerase III can be used (Youngs & Smith, 1973). The role of polymerase II in repair is not very clear, though it can carry out repair in the absence of both polymerases I and III (Tait et al., 1974; Masker et al., 1973). The evidence thus indicates that all three enzymes have a role in repair. The isolation of thermosensitive dna E (the gene locus of polymerase III) mutants that fail to replicate their DNA at 42° (Gefter et al., 1971) led to the suggestion that polymerase III is indispensible for replication. The rate of nucleotide addition by DNA polymerase III is twenty times faster than polymerase I, and the total activity of this enzyme per cell can account for the rate of DNA replication. The soluble enzyme systems described recently (section 1.2.g) also require a polymerase III holoenzyme in replication.

The isolation of a thermosensitive pol I mutant, conditionally lethal at the elevated temperature (Olivera & Bonhoeffer, 1974; Konrad & Lehman, 1974), suggests that polymerase I has a role in replication. This mutant appears to be defective in the 5' -> 3' exonuclease activity, the latter being important in the joining of Okazaki pieces, (Okazaki, 1971). Thus, although the role of the DNA polymerases in replication is not clearly defined, the evidence indicates that they may have a function in this process.

1.5. The acid soluble precursor.

The studies on polydeoxynucleotide synthesis in cell free extracts that led to the discovery of polymerase II and III utilised the 5'-deoxynucleoside triphosphates as substrates. These studies were influenced by the work on DNA polymerase I, the substrate for which is indeed the 5'-deoxynucleotide triphosphate. The diphosphate was shown to be inert in replacing the triphosphates for this enzyme (Kornberg, 1957). No direct evidence that the triphosphates are the immediate intracellular precursors of DNA in vivo has been put forward. It is quite possible that even though the polymerisation reaction of repair and replication are similar, they may not be identical. The studies of Price and his collaborators (1967), utilizing a thymine-requiring strain of E.coli, measured the incorporation of ^{32}P into free

nucleotides and nucleotidyl units of DNA. Their findings indicated that the in vivo precursors are activated 5'-deoxyribonucleotides, but the nature of these was not clear. They were presumed to be triphosphates.

Werner (1971b) measured the passage of thymine and thymidine into E.coli thymine requiring mutants and suggested that DNA replication and repair synthesis draw their precursors from different pools which can be labelled differently by thymine and thymidine, respectively. He questioned the assumption that the triphosphates were indeed the precursors of replication based on his measurements on the kinetics of incorporation of thymine and thymidine into dTMP, dTTP, and DNA. He suggested that while triphosphates were the precursors of repair, they were not the precursors of replication. From a mathematical analysis of Werner's data, Rubinow and Yen (1972) concluded that dTDP seems more likely to be the direct precursor of DNA synthesis. More recently, Pollock and Werner (1975), using the in vitro cellophane disk DNA synthesizing system of Schaller et al., (1972), suggested that neither dTDP nor dTTP are direct precursors, but that both are converted to some activated form of nucleotide prior to incorporation into DNA.

Studies in a thymine requiring mutant of B.subtilis (Harris, 1973) showed that DNA repair and replication had different precursor pools, but that thymine was the precursor

of repair, and thymidine the precursor of replication. Although these conclusions were diametrically opposed to those of Werner (1971), their essential similarity is that it cannot be taken for granted that the substrates of repair and replication are the same.

In contrast, the studies of Fridland (1973) on the rate of loss of radioactivity from prelabelled thymidine nucleotide pools in human lymphoblasts provide evidence to support the role of triphosphates as the DNA precursor in replication. In these studies it was not established whether the DNA was involved in semi-conservative or non-conservative synthesis and no attempt was made to establish that the incorporated radioactivity was present in the DNA and not in artefacts other than RNA that have been found in previous studies of thymine and thymidine incorporation by animal cells (Counts & Flamm, 1966; Dobson & Cooper, 1971; Morley & Kingdon, 1972).

Thus, the evidence that the acid soluble precursor of DNA synthesis in vivo is the deoxynucleoside triphosphate is lacking.

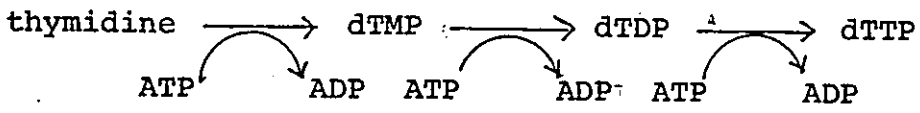
1.6. Metabolism of thymine and thymidine.

Attempts to provide evidence on the nature of DNA precursors in vivo cannot utilise exogenous nucleotides since these are rapidly dephosphorylated prior to entry into the cell (Lesley & Graham, 1956; Lichtenstein , 1960; Liebman &

Heidelberger, 1955). Since incorporation into DNA is the major anabolic fate of labelled thymine, it is useful as a measure of DNA synthesis.

Exogenous thymine is not incorporated into DNA in wild type cells, but the existence of thymine-requiring mutants implies that the enzymes must exist for conversion of thymine to dTMP. In contrast, thymidine is rapidly incorporated into DNA of wild type cells, but the incorporation stops due to the rapid breakdown of thymidine to thymine by an inducible thymidine phosphorylase (Kammen, 1967). Addition of ribo- and deoxyribonucleosides permits the incorporation of thymidine into DNA by wild type cells. The deoxyribonucleosides inhibit the breakdown of thymidine by thymidine phosphorylase. The addition of deoxyribonucleosides to the growth medium also permits extensive incorporation of exogenous thymine into DNA since they serve as a source of deoxyribose-1-phosphate necessary for the formation of thymidine.

Once within the cell, thymidine is phosphorylated stepwise to dTTP by a series of ATP-dependent kinases (Henderson & Paterson, 1973):



This endogenous pathway for the utilisation of thymidine as precursor for DNA synthesis forms the basis of the kinetic studies for the measurement of incorporation of thymidine into the

[redacted]eotide pools and DNA.

1.7. Halophiles (Larsen, 1962, 1967; Kushner, 1968).

1.7.a. General description

The studies of DNA synthesis presented here utilised the extreme halophile Halobacterium cutirubrum. Halophilic organisms are those requiring salt concentrations above 0.5M for growth; while the extreme halophiles are those requiring above 3.0M. The limits of the various classifications appear in table 1.3. The extremely halophilic bacteria include the rod shaped halophiles in the genus Halobacterium and the halophilic cocci in the family Micrococcaceae. In the eighth edition of Bergey's manual (1974) Gibbons has classified both the rod shaped halophiles and the halophilic cocci in the family Halobacteriaceae. The rod shaped halophiles in the genus Halobacterium now contain only two species H.salinarium, and H. halobium. H.cutirubrum is now considered to be the same species as H. salinarium. In this study the old nomenclature H. cutirubrum is being used. The halophilic cocci have been reclassified in the genus Halococcus.

One of the first questions asked about halophiles is whether they have an active pumping mechanism for excluding salts so that their interior environment is of low ionic strength. These organisms do indeed have an active sodium pump that maintains a low sodium concentration relative to

Table 1.3.

Classification of bacteria according to their salt requirement for growth

class	example	M NaCl
non-halophiles	*Escherichia coli*	0 - 0.4
halotolerant bacteria	*Bacillus cereus*	0 - 1.7
	Staphylococcus aureus	0.5 - 3.5
moderate halophiles	*Micrococcus halodenitrificans*	0.5 - 3.5
	Vibrio costicola	0.5 - 3.5
extreme halophiles	*Halobacterium cutirubrum*	3.0 - saturation
	Halococcus morrhuae	3.0 - saturation

the exterior, but their overall internal environment has as high an ionic strength as the exterior. Nevertheless, the intracellular ionic composition is different from that of the external medium as shown in table 1.4. The anion moiety of the salt within the cell is dominantly Cl^-, but the surprising point is that the cation moiety is mainly K^+; the concentration of KCl in the cell comes close to its solubility limit in water. The possibility that K^+ is bound by an intracellular matrix was suggested, since an activation of the Na^+ pump in whole cells does not result in K^+ (Ginzburg, 1969). However the results of Lanyi and Silverman (1972) suggested that K^+ in H. cutirubrum is not bound. In this kind of environment many protein derivatives and enzymes are usually inactivated, but extreme halophiles are equipped with a metabolic apparatus that can function in saturated salt solutions. Most of the approximately 30 enzymes tested, from both halobacteria and halococci, require high salt for maximum activity.

Another aspect of extreme halophilism is the influence of salt on the structural components of the cell. When the strongly saline environment of the halococci is diluted with water, the cell structure is not affected. In the halobacteria the situation is quite different: these organisms are lysed at concentrations less than 10%.

Table 1.4.

Intracellular ionic concentrations in various bacteria [1]

	Staphylococcus aureus	Vibrio costicola	Halobacterium salinarium	Halococcus morrhuae
NaCl in medium (M)	0.15	1.0	4.0	4.0
KCl in medium (M)	0.025	0.0004	0.032	0.032
Na^+ in cells	0.098	0.684	1.37	3.17
K^+ in cells	0.680	0.221	4.57	2.03
Cl^- in cells	0.008	0.139	3.61	3.66

(1) Christian,J.H.B., Waltho, J. (1962) Biochim Biophys Acta 65 506-508

1.7.b. Nucleic acids of halophiles

The high molecular weight nucleic acid of halophiles do not differ significantly from those commonly found in other bacteria (Larsen, 1967). The extreme halophiles possess, however, a separate satellite DNA, comprising 11 - 36% of the total DNA. The satellite DNA of the extreme halophiles if episomal, is unusually large. An exception is the spirillum, SL-1, an anaerobic, photoautrophic extreme halophile (Moore & McCarthy, 1969).

The hybridization experiments of Moore and McCarthy (1969b) showed that the halobacterium strains tested have considerable sequence homology, but are quite different from the halococci or the moderate or nonhalophilic bacteria; the extent of divergence of base sequence for the major and minor components amongst the different species is indistinguishable. The base composition of the halobacteria and the halococci, determined by caesium chloride equilibrium density gradient, showed a high G+C content, 66 - 68% in the major component and 57 - 60% in the minor component. The high G+C content was also determined in the halococci from the melting point temperatures and the values ranged from 60.5-65.8% (Kocur & Bohacek, 1972). Since the natural habitat of these bacteria is salt flats, it is possible that they have evolved a low A+T content to avoid thymine-specific damage from ultraviolet radiation in sunlight (Singer & Ames, 1970).

1.7.c. Growth requirements of extreme halophiles

The extreme halophiles have a complex nutritional requirement. They do not utilize exogenous carbohydrates, but rather amino acids as the carbon source. Carbohydrates may stimulate growth but the medium does not become acid (Gochnauer & Kushner, 1969). Dundas et al. (1963), composed a chemically defined medium, consisting of inorganic salts, 10 amino acids and cytidylic acid, for growth of Halobacterium salinarium. Four amino acids: valine, methionine , isoleucine, and leucine, were essential for growth. Growth of strains of halococci and some strains of halobacteria was not supported by this medium.

The medium of Onishi et al.(1965), supported growth of all extreme halophiles tested by them, both halobacteria and halococci. This medium comprised fifteen amino acids, two nucleotides (adenylic acid and uridylic acid), and glycerol. The addition of 0.2% - 0.5% NH_4Cl reduces the lag phase. The requirements for ammonium ions is not specific and their stimulatory effect is related to the utilization of amino acids (Onishi & Gibbons, 1965). The medium was later modified to include increased quantities of K^+ (Gochnauer & Kushner, 1969). For H.cutirubrum, arginine, valine, lysine and leucine were essential for growth. It has been reported that arginine completely disappears from the medium before the beginning of exponential growth (Ducharme et al., 1972).

1.7.d. The pigment of halobacteria

Cells of the halobacteria owe their pigmentation to the presence of C_{50} bacterioruberins (Kelly et al., 1970) and C_{40} carotenes, isoprenoid compounds (Kushwaha et al., 1972) and retinal (Oesterhelt & Stoeckenius, 1971).

The presence of glycerol in the synthetic medium causes the cells of the extremely halophilic bacteria to lose the bacterioruberins, but at the same time increases the formation of β-carotenes (Gochnauer et al., 1972). Such cells are purple, and acetone extracts of these cells contain retinal. The nature of the pigment is also influenced by the extent of aeration. Cells grown aerobically produce the red pigment, but when grown anaerobically, the membrane is purple: the chromophore consisting of a retinal-protein complex. This purple membrane was first found in H.halobium (Oesterhelt & Stoeckenius, 1971) and has since been shown to occur in H.cutirubrum (Kushwaha & Kates, 1972), but the composition of the purple membrane of the two species differs. Under anaerobic conditions the purple membrane appears to function as a light driven proton pump and the cells utilize the resulting chemiosmotic gradient for ATP synthesis (Oesterhelt & Stoeckenius, 1973).

The pigment of these bacteria has been implicated in their tolerance to u.v. irradiation. As early as 1922, Browne suggested that "the brilliant pigmentation is a protection

against the sunlight in the salina of the tropics where the sea salt is reduced" but did not report experiments to support his view.

Dundas and Larsen (1962) reported that on exposure to light from a tungsten lamp the growth of H. salinarium was not affected. However the growth of colorless mutant was strongly inhibited. The extremely halophilic bacteria occur in waters of high salinity and are characteristic inhabitants of solar evaporation ponds. The results of Dundas and Larsen (1962) suggest that the carotenoid pigment may play a role in their protection against sunlight in their natural environment. Another carotenoid containing bacterium Rhodopseudomonas spheroides, (Sistrom et al., 1956) a non-halophilic bacterium, is also unaffected by sunlight, but a mutant lacking the pigment is killed. It would appear then that the carotenoid pigment may have a role in the protection of the bacteria against the deleterious effects of sunlight.

In 1972, Hescox and Carlberg, reported that H. cutirubrum is indeed more tolerant to u.v. light than the commonly used bacterium E. coli. The inhibition of pigment formation by diphenylamine did not affect their radiation sensitivity as measured by their survival curves, but did affect their recovery from u.v. irradiation by photoreactivation. The cells treated with diphenylamine, although capable of photoreactivation, were less efficient in their recovery than the untreated cells which

were photoreactivated fully. These authors suggested that while the pigment did not appear to protect the organisms from sunlight they may serve an energy transfer role to augment the photo-reactivation process. They stated, without presenting any evidenc that no dark repair was apparent.

1.8. Purpose of the work

The work of Dundas and Larsen (1962) implied that the carotenoid pigment of H.salinarium may be solely responsible for its protection against irradiation. The fact that the growth of colorless mutants was inhibited would suggest that the bacteria may not have any other method of protection: the carotenoid pigment and the low A+T content (section 1.7.b.) being the only factors that ensure their survival in their natural environment. The later work of Hescox and Carlberg (1972), on H.cutirubrum, suggested that the pigment may indeed play a role in the photoreactivation process. This implied that these organisms may not have any dark repair and thus could be a good system for studying replication. However, to use this organism for any studies on replication it was important to establish definitively that H.cutirubrum was lacking in dark repair.

The search for any polymerase in replication requires knowledge of the in vivo precursor. As stated earlier in section 1.5., repair and replication draw their precursors from different pools within the cell and the general assumption that the deoxynucleotide triphosphates were indeed the in vivo precursors of replication has been questioned. If H.cutirubrum was lacking dark repair, then any precursor of DNA synthesis would be the precursor of replication.

Since no information of the nucleotide metabolism in the

extreme halophiles or the ability of the bacteria to incorporate exogenous precursors into their nucleic acids is available, it was first important to establish the conditions for studying the incorporation of the DNA precursor. The work of Onishi et al. (1965), led to the belief that these organisms are capable of incorporating exogenous nucleotides, contrary to the case in other well studied cells. If this were true, then it is possible that the metabolism of nucleotides in these cells may be atypical.

Therefore, the specific objectives of this work were:

(1) the establishment of conditions for studying the incorporation of a DNA precursor.

(2) the proof of the lack of dark repair.

(3) the determination of the immediate DNA precursor in the DNA replication machinery.

2. EXPERIMENTAL

2.1. Materials

Halobacterium cutirubrum NRC 34001 was obtained from Dr. D.J. Kushner. This strain was originally isolated from salt buffalo hides (Lochhead, 1934). The amino acids in the growth medium (L-alanine, L-arginine, L-cysteine hydrochloride monohydrate, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine), were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. The other components $CaCl_2.2H_2O$; $CuSO_4.5H_2O$; $FeCl_2.4H_2O$; KCl; KH_2PO_4; KNO_3, $MgSO_4.7H_2O$; $MnSO_4.H_2O$; NaCl, sodium citrate dihydrate; K_2HPO_4; $ZnSO_4.7H_2O$; glycerol, KOH; were obtained from Fisher Scientific Co.,Ottawa, Ontario, Canada. Difco agar, casamino acids and yeast extract were obtained from Canlab,Montreal, P.Q., Canada.

Streptomyces griseus protease (Pronase), was obtained from Calbiochem, La Jolla, California, U.S.A.; protease-free pancreatic ribonuclease A (Type I-A), and electrophoretically purified pancreatic deoxyribonuclease I were obtained from Sigma Chemical Co. Non-radioactive thymine, nucleosides, deoxynucleosides, nucleotides, and deoxynucleotides were obtained from P.L. Biochemicals Inc., Milwaukee, Wis., U.S.A. or Sigma Chemical Co.

MN polygram 300 plastic-backed cellulose and polyethyleneimine cellulose t.l.c. sheets were purchased from Brinkman Instruments (Canada) Ltd., Rexdale, Ontario, Canada. Lithium chloride, ethyl acetate, formic acid were obtained from Fisher Scientific Co.

Schwarz-Mann caesium chloride for density gradient centrifugation was bought from Picker X-ray Engineering Ltd., Ottawa, Ontario, Canada.

The radioactive materials [^{3}H]-thymine, [^{3}H]-thymidine, [^{14}C]-thymidine, [^{3}H]-deoxyadenylic acid, [^{3}H]-thymidylic acid, [^{3}H]-5-bromo-2'-deoxyuridine, PCS liquid scintillation counting fluid were obtained from Amersham/Searle Corp., Oakville, Ont., Canada. Xylene was obtained from Fisher Scientific Co.

2.2. Methods

2.2.1. Maintenance of cell culture

Stock cultures of H.cutirubrum NRC 34001 were maintained on slants of Gochnauer and Kushner's (1969) complex medium, containing 2% (w/v) agar. They are grown at 37^{o} for 7 days and stored in the refrigerator. Transfers were made at 4 - 6 month intervals.

2.2.2. Growth of liquid cultures

Liquid cultures were grown in sterile conditions in

culture flasks fitted with stainless steel closures. They were incubated in either a New Brunswick Scientific Model G25 gyratory incubator shaken at 250 rpm at 37^{o} , or a New Brunswick Aquatherm gyratory water bath shaken at 250 rpm and 37^{o} or 22^{o} . In all cases, where 50 ml media was used, the inocula were 10% (v/v) relative to the volume of fresh culture media. For preparation of the inocula, the bacteria were passaged at least twice in the appropriate liquid medium and a pre-culture was then grown for 48 hours. The inocula contained approximately 2.5×10^{8} bacteria per 50 ml of fresh medium. Growth of the liquid cultures was followed by determining the number of viable bacteria and/or by measuring the turbidity of the bacterial suspension at 660 nm, relative to 25% (w/v) aqueous NaCl, with a Zeiss PMQ II spectrophotometer.

Viable cell counts were determined in sterile disposable plastic Petri dishes (10 cm. diameter) containing 2% (w/v) agar in approximately 15 ml Gochnauer and Kushner's (1969) complex medium. 0.1 ml aliquots of serial dilutions of the culture in sterile 25% (w/v) aqueous NaCl were spread over the surface of the nutrient agar. The dishes were placed in plastic bags to prevent dessication and incubated at 37^{o} in a moist atmosphere. Colonies were counted after 5 - 7 days until no more appeared, and each dilution was assayed in duplicate. Similar results were obtained if viable counts were done on 2% (w/v) agar in synthetic medium, but the latter is expensive

and colonies hard to see, hence Gochnauer and Kushner's medium was used routinely unless otherwise stated.

The composition of the different media used in these studies are:

(1) Gochnauer and Kushner's medium (all ingredients are in gm/1000 ml):- casamino acids, 7.5; yeast extract, 10.0; sodium citrate dihydrate, 3.0; KCl, 2.0; $MgSO_4.7H_2O$, 20.0; NaCl, 250.0; $FeCl_2.4H_2O$, 23.0 mg. The pH of the medium is adjusted to 6.5 - 6.8 with NaOH.

(2) The medium of Onishi et al.(1965) (all ingredients are in mg/100 ml):- Aminoacids: DL-alanine, 43; L-arginine, 40; L-cystine, 5; L-glutamic acid, 130; glycine, 6; DL-isoleucine, 44; L-leucine, 80; L-lysine, 85; DL-methionine, 37; DL-phenylalanine, 26; L-proline, 5; DL-serine, 61; DL-threonine, 50; L-tyrosine, 20; DL-valine, 100. Nucleotides: adenylic acid, 10; uridylic acid, 10. Salts: NaCl, 25 gm; $MgSO_4.7H_2O$, 2 gm; KNO_3, 10; K_2HPO_4, 5; KH_2PO_4, 5; sodium citrate, 50; $MnSO_4.H_2O$, 0.03; $CaCl_2.7H_2O$, 0.7; $ZnSO_4.7H_2O$, 0.004; $FeCl_2$, 0.23; $CuSO_4.5H_2O$, 0.005; NH_4Cl, 500; glycerol, 100. The pH of the medium was adjusted to 6.5 with NaOH.

(3) A modification of the medium of Onishi et al.(1965) (all ingredients are in mg/100 ml):- Amino acids: L-alanine, 43; L-arginine, 40; L-cysteine hydrochloride monohydrate, 5; L-glutamic acid, 130; glycine, 6; L-isoleucine, 44; L-leucine, 80; L-lysine, 85; L-methionine, 37; L-phenylalanine, 26;

L-proline, 5; L-serine, 61; L-threonine, 50; L-tyrosine, 20; L-valine, 100. Nucleotides: adenylic acid, 10; uridylic acid, 10. Salts: NaCl, 25gm; $MgSO_4.7H_2O$, 2gm; KNO_3, 10; K_2HPO_4, 5; KH_2PO_4, 5; KCl, 100; sodium citrate dihydrate, 50; $MnSO_4.7H_2O$, 0.004; $FeCl_2$, 0.23; $CuSO_4.5H_2O$, 0.005. The pH of the medium was adjusted to 6.6 with (w/v) KOH before dilution to the final volume.

(4) During the course of the studies (sec.3.1.) a synthetic medium was developed that gave as good a growth as complex medium. This new growth medium (Grey & Fitt, 1976) was used for all the incorporation studies. The medium contained per ml:- Amino acids: L-alanine, 43mg; L-arginine, 40mg; L-cysteine hydrochloride monohydrate, 5mg; L-glutamic acid, 130mg; glycine, 6mg; L-isoleucine, 44mg; L-leucine, 80mg; L-lysine, 85mg; L-methionine, 37mg; L-phenylalanine, 26mg; L-proline, 5mg; L-serine, 61mg; L-threonine, 50mg; L-tyrosine, 20mg; L-valine, 100mg. Salts: $CaCl_2.2H_2O$, 0.07mg; $CuSO_4.5H_2O$, 5µg; $FeCl_2.4H_2O$, 0.23mg; KCl, 100mg; KH_2PO_4, 15mg; K_2HPO_4, 15mg; KNO_3, 10mg; $MgSO_4.7H_2O$, 2g; $MnSO_4.H_2O$, 30µg; NaCl, 25g; sodium citrate dihydrate, 50mg; $ZnSO_4.7H_2O$, 44µg; glycerol 0.1% (w/v). The pH of the medium was adjusted to 6.6 with 50% (w/v) KOH before dilution to the final volume.

When 50 ml media was used, the flasks were autoclaved at 121° for 15 minutes. When 600ml media was used the time of autoclaving was increased to 30 minutes.

2.2.3. Thin layer chromatography

2.2.3.a. Separation of thymidine and thymine

The separation of thymidine from thymine was carried out by thin layer chromatography on plastic backed cellulose sheets with the ethyl acetate:water:formic acid 65:35:5 (v/v/v). The method is Kammen's (1967) modification of the paper chromatography method of Fink et al. (1956). 20 µl samples of the mixture containing approximately 2800 cpm in aqueous 12 mM thymine - 10 mM thymidine was applied. Development was for 3 hours in a Shandon - Universal tank lined with Whatman No. 3 paper. The tank was previously equilibrated with the solvent system for at least 18 hours at room temperature. This equilibrium was essential for good resolution. The thymine and thymidine spots were identified by comparison with standards chromatographed at the same time, and were cut out and counted for radioactivity. The R_f's of thymine and thymidine relative to the solvent front were 0.85 and 0.74 respectively.

2.2.3.b. Separation of thymidine from thymidine nucleotides

The separation of thymidine (plus thymine) dTMP, dTDP, dTTP, was performed by thin layer chromatography on plastic backed polyethyleneimine cellulose sheets using a modification of the lithium chloride system of Randerath (1964). The modifications include both LiCl concentrations and time of development, and give an improved separation of the three thymidine nucleotides. Development solutions and times were:

0.2M LiCl, 2 min.; 1.0M LiCl, 6 min.; 1.6M LiCl, 22 min.

For good resolution, it was essential that the sheet be thoroughly dried over silica gel. Old or discoloured sheets were washed well with distilled water and dried. Known aliquots (10 - 60 µl) of samples were applied 2 cm. from the bottom of the sheets, as a thin band and chromatographed. The nucleotide spots were identified by comparison with standards, cut out and counted for radioactivity. The R_f's relative to the solvent fronts were:

Thymidine and thymine	1.0
dTMP	0.56
dTDP	0.45
dTTP	0.36

the nature of the separation obtained is shown in figure 2.1.

2.2.4. Liquid scintillation counting

The measure of radioactivity forms the basis of most of the work described. The samples, either in aqueous 5% (w/v) TCA or on solid support (PEI plates, cellulose, or Whatman #3 paper), were counted in 10 ml of a 2:1 (v/v) mixture of PCS and xylene. A Beckman LS-230 liquid scintillation counter was used and the approximate counting efficiency for ^3H in aqueous solution was 40 - 45% and 25 - 30% on t.l.c. strips as shown in table 2.1. In the latter case it was important that the strips be no larger than 2.5 x 0.7 cm or there was a

Fig. 2.1. The nature of the separation of thymidine from thymidine nucleotides by t.l.c. on PEI-cellulose

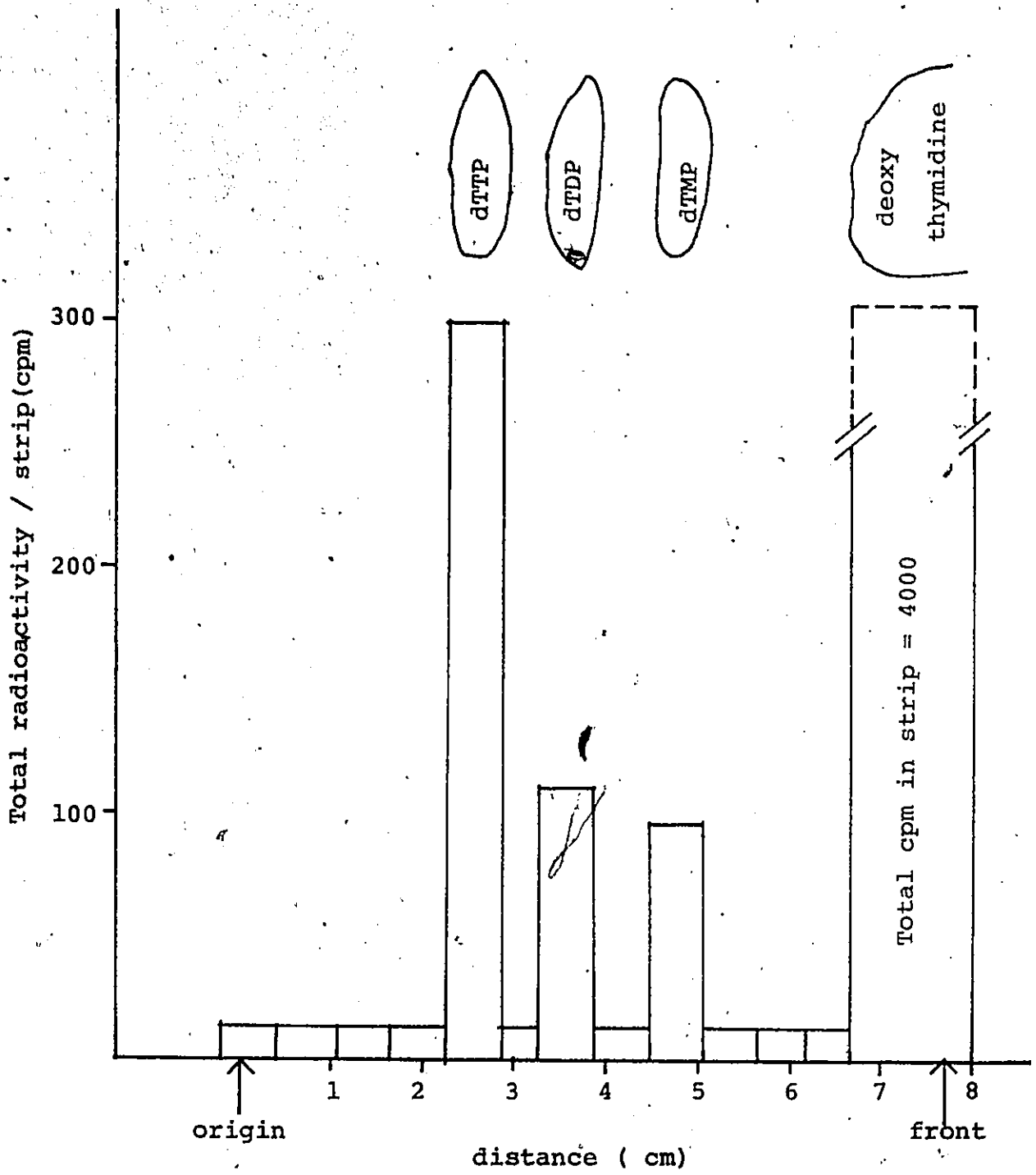

dTTP
dTDP
dTMP
deoxy thymidine
Total radioactivity / strip (cpm)
300
200
100
Total cpm in strip = 4000
1
2
3
4
5
6
7
8
origin
front
distance (cm)

Table 2.1.a.

Efficiency of counting of aqueous samples

The samples contained 2.2×10^4 dpm/µl in 5% v/v trichloroacetic acid. Known aliquots were counted as described in section 2.2.4.

Volume of sample (µl)	cpm x 10^4	% efficiency
10	.99	45
20	2.2	50
50	4.8	44
100	8.8	40

Average efficiency = 44.75 ± 5%

Table 2.1.b.

Efficiency of counting t.l.c. plates

The samples were applied as a thin band on PEI cellulose strips 2.5 x 0.7 cm in size. They were dried in a stream of cool air prior to counting. Sample A contained 2.2×10^2 dpm/µl in 5% trichloroacetic acid. Sample B contained 2.2×10^3 dpm/µl in 5% trichloroacetic acid.

Volume of sample (µl)	cpm	% efficiency
Sample A		
20	1158	26.3
20	1185	26.9
20	1291	29.3
20	1279	29.1
20	1255	28.5
Sample B		
20	1236 x 10	28.1
20	1312 x 10	29.8
20	1140 x 10	25.9

Average efficiency = 27.9 ± 2%

decrease in counting efficiency. In experiments where double labelling was done, a Searle Isocap 300 Liquid scintillation counter was used to obtain ^{3}H and ^{14}C cpm directly.

The thin layer chromatographic separation of thymidine and thymine from nucleotides required extraction of the TCA sample with ether. In these cases, chemiluminescence was observed. This was detected by the low values obtained in the channels $^{14}C/^{3}H$ ratio. After about 8 - 12 days this effect disappeared. It was therefore important to keep a close check on the channels ratio.

2.3.1. Detection of semi-conservative replication

A culture flask containing the synthetic medium supplemented with 68 µM-5-bromo-2'-deoxyuridine was inoculated with 5 ml of a culture of H.cutirubrum previously grown to a cell density of approximately 10^{10} cells/ml in the synthetic medium supplemented with 82.6 µM-thymidine. After 48 hours of growth at 37°, 2.5 ml of this culture was transferred to fresh synthetic medium (25 ml) containing 68 µM- 6-[^{3}H]-5-bromo-2'-deoxyuridine (spec. act. 3.7 Ci/mol) and incubation was continued for a further 31 hours. A sample (2 ml) was then withdrawn for treatment as descibed below and the remainder of the culture was centrifuged at 45 000 g_{max} for 5 min. The cells were resuspended in preincubated synthetic medium (25 ml) containing 82.6 µM- 2-[^{14}C]-thymidine (spec. act. 0.61 Ci/mol). Incubation

was continued at 37° and samples (2 ml) were withdrawn immediately after resuspension of the cells (zero time), 8 hours (1 generation) and 16 hours (2 generations). The samples were added to 25% (w/v) NaCl (10 ml) and the cells were immediately sedimented at 45 000 g_{max} for 2 min. The pellets were washed once by resuspension of the cells in 25% (w/v) NaCl (10 ml) followed by centrifugation and the washed cells were lysed carefully by gentle swirling in 1M-KCl (1 ml) for 5 min. at room temperature. The mixture was transferred quantitatively to a glass vial with the aid of 9 ml of distilled water and the suspension was mixed gently on a rotator until homogeneous (about 30 min.). CsCl (4.347g) was dissolved in 3.4 ml of the crude DNA solution and the refractive index of the latter was adjusted to 1.4020 at 25° using a Zeiss model A Abbé refractometer. The whole sample was passed three times through a 23 gauge hypodermic needle to shear the DNA (Sinclair, 1974) and was then placed in a Beckman polyallomer centrifuge tube (5/8 in x 3 in). The solution was overlayed with sufficient white paraffin oil (liquid petrolatum) to fill the centrifuge tube and centrifugation was performed at 20° and 110 000g_{max} for 66 hours in a Beckman Type 40 fixed angle rotor (Flamm et al., 1966) using a Beckman L2-65B preparative ultracentrifuge. The centrifuge was allowed to stop without the brake, the tubes were pierced and 7 drop fractions were collected using a fraction collector

and a peristaltic pump. The density of every fourth fraction was determined from its refractive index at 25° (Sober, 1970) and the acid-insoluble radioactivity of each fraction was measured using the method of Furano (1971). A 24 cm. diam. Whatman #3 filter paper, presoaked in 5% (w/v) trichloroacetic acid and dried, was marked in numbered rectangles (3cm x 4cm). Samples (150 µl) of the fraction were applied to the corresponding rectangles. The filter paper was air-dried and washed in a Buchner funnel three times with 300 ml of 5% (w/v) trichloroacetic acid and twice with 100 ml of ether, using filter pump suction to remove the last drops of wash fluid only. The paper was air-dried and cut into the rectangles whose radioactivity was counted as described (section 2.2.4), except that a Searle Isocap 300 liquid scintillation Counter was used to obtain the ^{3}H and ^{14}C cpm directly.

2.4.1. Irradiation experiments

Cultures of H. cutirubrum in the synthetic medium supplemented with 82.6 µM-thymidine were grown at 37° and 250 rpm until the cell density was approximately 10^{9} cells/ml. Samples (15 ml) of the cell suspension were transferred to an uncovered, sterile Petri dish placed on the platform of a lab-line Junior Orbit gyratory shaker (Canlab, Montreal, P.Q., Canada). The culture was swirled gently (100 rpm) at room temperature and irradiated for the indicated times with an ultraviolet lamp

(Mineralight, Ultra-violet Products Inc., San Gabriel, Calif., U.S.A.) suspended 24 - 27 cm above it. The dose rate in ergs/ mm^2/sec was measured with a Black-ray u.v. meter (Ultra-violet Products Inc.). Survival curves were determined by withdrawing samples (0.1 ml) at suitable intervals with sterile pipettes: they were then diluted serially with 25% (w/v) NaCl for measurement of the viable cell count.

2.4.2. Photoreactivation experiments

H.cutirubrum cells were irradiated as described above and samples (0.1 ml) were withdrawn immediately before and after irradiation for determination of the viable cell count. The Petri dish containing the remainder of the irradiated culture was placed on ice 10 cm below two white fluorescent desk lamps (General Electric F6T5-D) and irradiated while the culture was swirled gently (100 rpm) on a Lab-line Junior Orbit shaker. Samples (0.1 ml) were withdrawn at intervals for determination of the viable cell count.

2.5. Preparation of H.cutirubrum DNA

H.cutirubrum DNA was prepared by a modification of the method of Marmur (1961). All operations were performed on ice unless otherwise specified. The DNA was prepared from freshly harvested bacteria, grown in the complex media of Gochnauer and Kushner (1969). The bacteria were washed once with 25% (w/v) NaCl, 0.05M EDTA, pH 8.0, and then suspended in a mixture

of 0.15M NaCl, 0.1M EDTA, pH 8.0, and 2% (w/v) sodium dodecyl sulfate. The suspension was incubated at 75° for 10 min. and then cooled to room temperature. 5M $NaClO_4$ was added to a final concentration of 1M. The mixture was swirled for 30 min. on a rotary shaker with an equal volume of 24:1 (w/v) chloroform: isoamyl alcohol. The mixture was centrifuged for 5 min. at 10 000 rpm in stainless steel centrifuge tubes and the aqueous phases combined. Ethanol (2vol.) was added and the mixture cooled to -22°. The DNA was removed by gentle swirling around a glass rod then dissolved in 15 mM Na citrate-pH 7.0. Two additional deproteinizations were done and the DNA was then treated with 1) RNase (40 µg/ml) for ½ hour at 37°, and 2) self digested Pronase (40 µg/ml) for 1 hour at 37°, (Moore & McCarthy, 1969). Five further deproteinizations were then performed. When the final solution had occured, the DNA was selectively precipitated with 0.54 vol. isopropanol, 3.0M acetate, 1mM EDTA. Finally the DNA was washed free of acetate in ethanol and dissolved in 0.15M NaCl, 0.015M Na citrate, pH 7.0.

The protein contamination as determined by the micro-biuret reaction (Zamenhof, 1957) was not detectable. The RNA content, determined by the orcinol procedure (Dische, 1955) was less than 1%. The yield of purified DNA was determined by the diphenylamine reaction (Dische, 1955) and was approximately 0.5 mg/gm wet weight of cells.

3. RESULTS AND DISCUSSION

3.1. A synthetic medium for Halobacterium Cutirubrum

In order to study the precursors of DNA synthesis in H.cutirubrum it was necessary to develop a defined synthetic medium that would support growth of the bacterium satisfactorily. Previous studies with extreme halophiles (section 1.7.c.) led to the development of a synthetic medium that contained amino acids, a complex salt mixture including ammonium chloride and glycerol. The addition of the nucleotides, adenylic acid, cytidylic acid, guanylic acid, and uridylic acid, each at a concentration of 10 mg/100 ml, to the medium was stimulatory (section 1.7.c.). Since in other bacteria, e.g. E.coli, nucleotides are first dephosphorylated prior to entry into the cell (section 1.6.), we decided to investigate the medium further in order to determine the nature of the stimulatory effect of the nucleotides on the growth of H.cutirubrum.

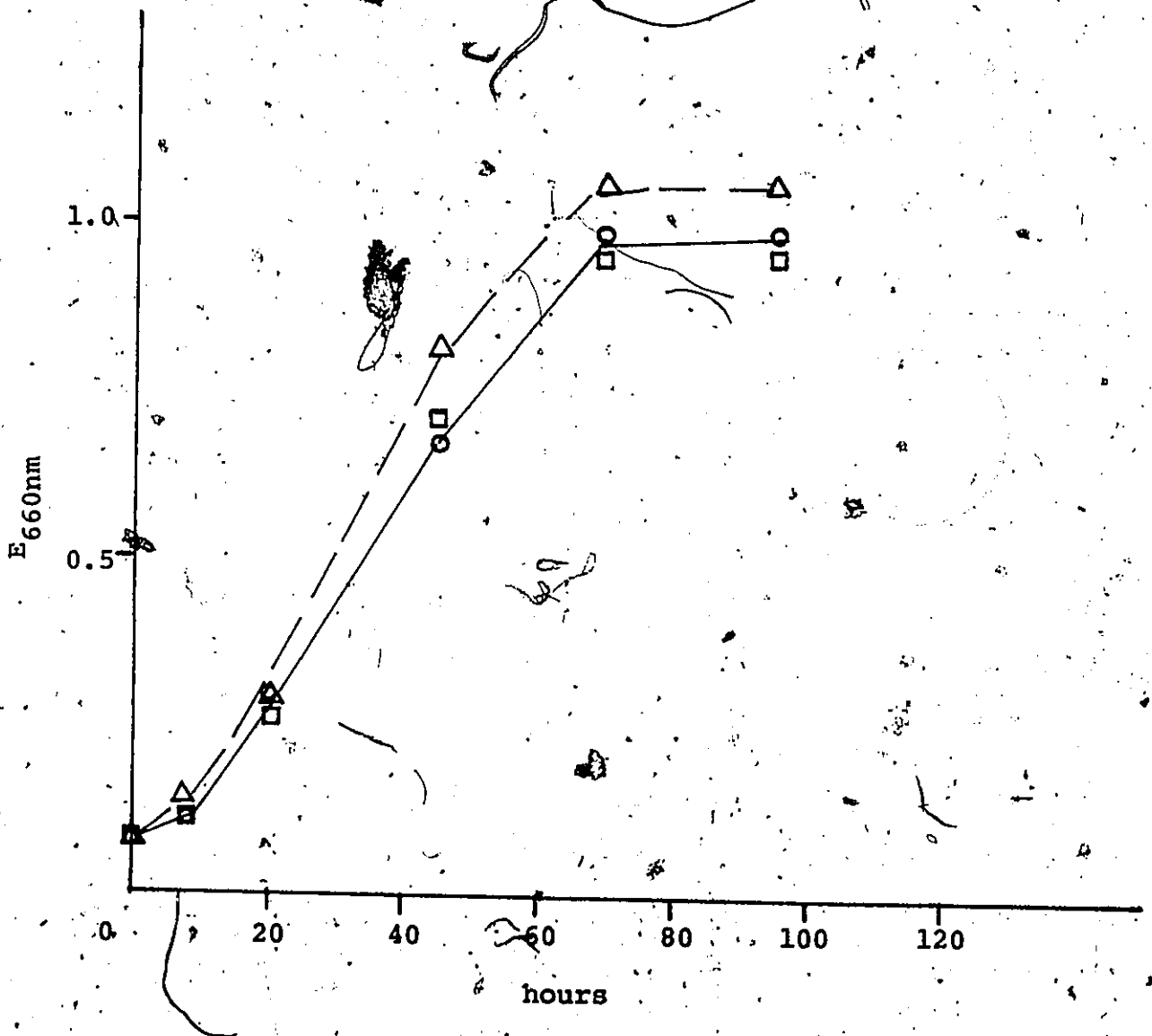
Fig. 3.1. shows the effect of NH_4Cl and glycerol on the growth of H.cutirubrum in medium 3, as decribed in section 2.2.2. It can be seen that while glycerol had a small stimulatory effect on the final cell concentration, NH_4Cl did not appear to have any. When 1% glutamine was substituted for NH_4Cl, nostimulatory effect was again observed, showing that ammonium ion had no effect on the growth of the bacteria. It is possible that the need for ammonium ion was eliminated because this

Fig. 3.1. Effect of NH_4Cl and glycerol on the growth of H.cutirubrum in medium 3 at 37°

o---o medium 3

Δ---Δ medium 3 + glycerol 0.1% (v/v)

□---□ medium 3 + 2% NH_4Cl or 1% glutamine


1.0
0.5
E_{660nm}
0
20
40
60
80
100
120
hours

medium contains only L-amino acids, and not a mixture of L- and DL- as in the medium of Onishi et al. (1965). , Onishi and Gibbons (1965) have suggested that the stimulatory effect of the ammonium ion is related to the utilization of amino acids, and was not a specific requirement of these bacteria.

The inorganic phosphate concentration in the medium of Onishi et al. (1965) is 10 mg/100 ml, and the contribution of potentially available phosphate to the bacterium from the nucleotides is approximately 11.3 mg / 100 ml. Fig. 3.2. shows the effect of inorganic phosphate on growth in the synthetic medium (section 2.2.2.) deficient in nucleotides. It can be seen that the increased phosphate concentration 30 mg/ 100 ml resulted in a higher cell density as detectable by O.D. at 660 nm. Phosphate concentrations of 15 mg/ 100 ml and 20 mg/ 100 ml gave essentially the same result as 30 mg/ 100 ml. At this optimum concentration (30 mg/ 100 ml) the addition of nucleosides and nucleotides have no significant effect on the growth.

In fig. 3.3. it can be seen that when nucleotides are added to the 10 mg / 100 ml Pi medium, so that they contribute a further 14.2 mg / 100 ml of potentially available phosphate, the bacterium grew as well as in the medium containing 15 - 30 mg / 100 ml Pi and no nucleotides. Nucleosides had no effect on growth.

These results, in fig. 3.2 and fig. 3.3., strongly suggest

Fig. 3.2. **Effect of Pi concentration on the growth of H.cutirubrum in synthetic meddium, deficient in nucleotides**

□---□ no added Pi

o---o 10 mg Pi/100ml

Δ---Δ 30 mg Pi/100ml

The Pi concentration refers to equal quantities of K_2HPO_4 and KH_2PO_4, as defined in section 2.2.2.

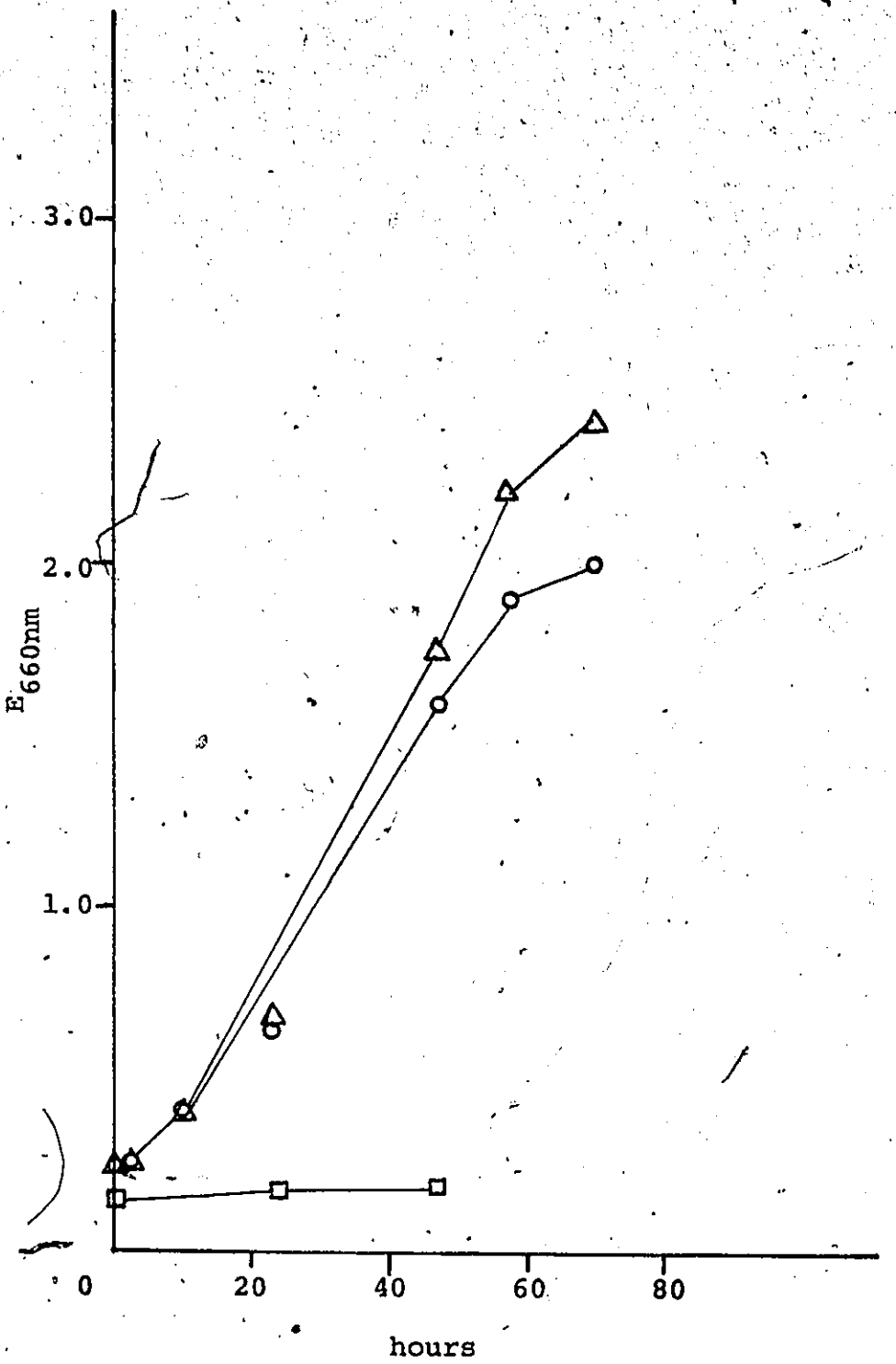
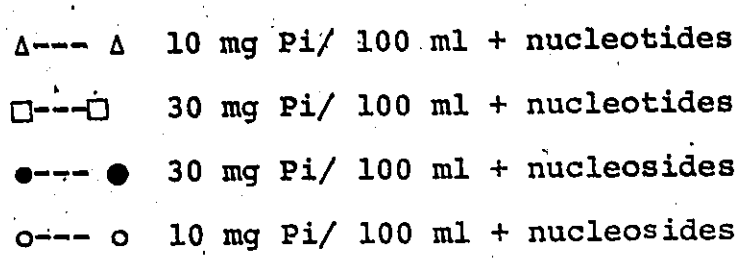
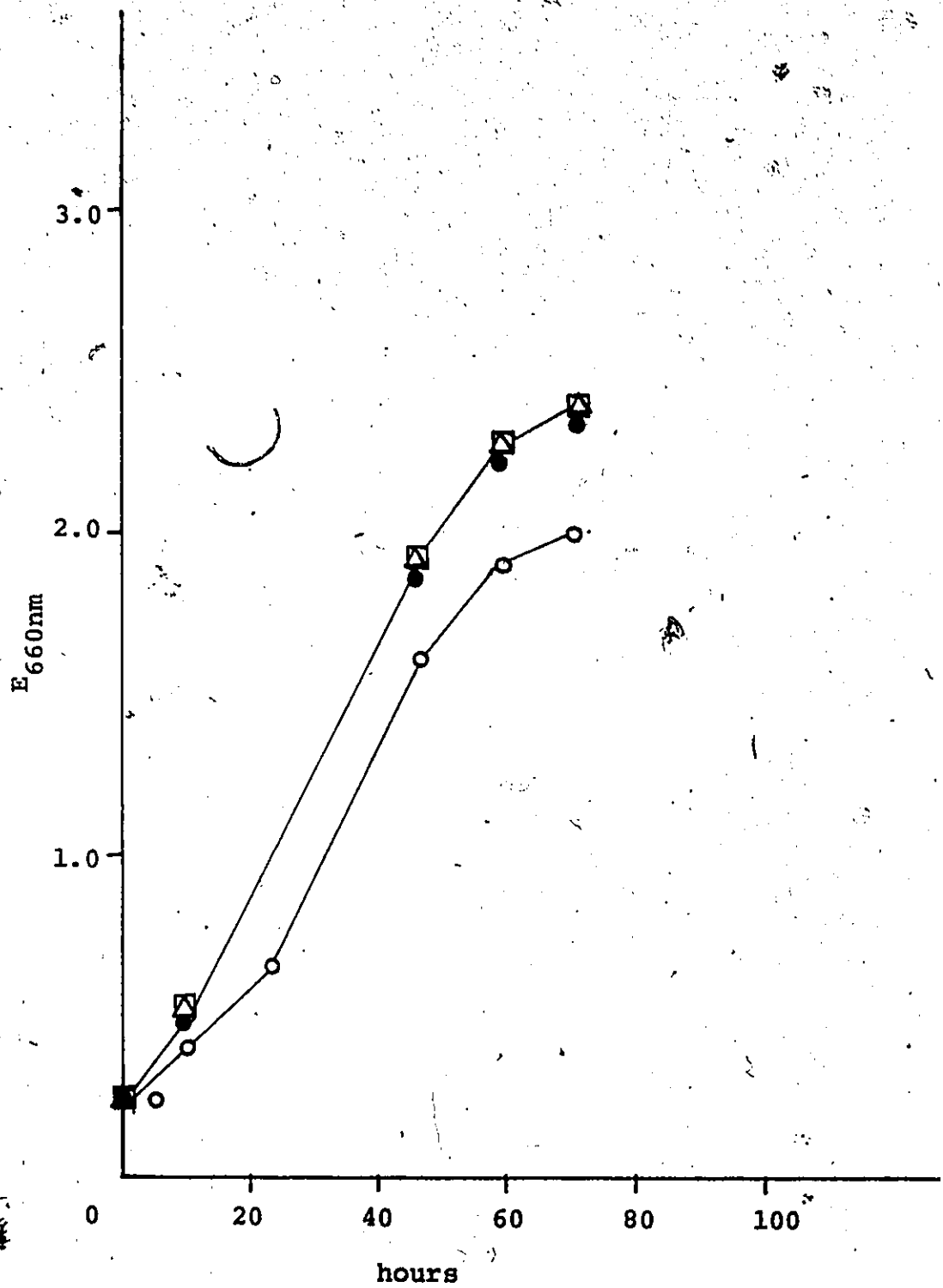

3.0
2.0
1.0
E_{660nm}
0
20
40
60
80
hours

Fig. 3.3. Effect of nucleotides and nucleosides on the growth of H.cutirubrum in synthetic medium



The nucleoside and nucleotide mixtures contained 0.1 mg/ml each of 5'-AMP, -CMP, -GMP, -dTMP, and -UMP or of the corresponding nucleosides, respectively. The Pi concentration refers to equal quantities of K_2HP_4 and KH_2PO_4, as defined in section 2.2.2.


3.0
2.0
1.0
E_{660nm}
0
20
40
60
80
100
hours

that the nucleotides merely serve as an additional source of phosphate in a medium deficient in this component. The results in table 3.1. show that the nucleotides AMP and dTMP present in the culture medium are rapidly dephosphorylated to their corresponding nucleosides. Under these conditions, where an excess of orthophosphate was present, both nucleotides were quickly degraded (after 10 min only about 50% of the nucleotide was present in the supernatant), so that even more rapid hydrolysis would be expected if the orthophosphate concentration were limited to growth. The more extensive degradation of dTMP after 30 min was probably due to the removal of thymidine from the medium by further degradation of thymine (see section 3.2.b.) or its incorporation into DNA (section 3.2.a.)with consequent displacement of the equilibrium.

It is clear then that the stimulatory effect of nucleotides observed by Onishi et al (1965) only occurs in orthophosphate deficient media and that H.cutirubrum resembles other well studied cells in being impermeable to nucleotides.

Fig. 3.4. compares the growth of H.cutirubrum in the complex medium of Gochnauer and Kushner (1969) and in the new synthetic medium (Grey & Fitt, 1976). There is no significant difference in either growth rate or final concentration of cells, and the generation times in the two media are very similar (table 3.2.).

The improved synthetic medium differs from those previously

Table 3.1. Breakdown of nucleotides in the supernatant

H.cutirubrum was grown as described in section 2.2.2., in the presence of either AMP (82.6 µM) or dTMP (82.6 µM). At 24 hours the cells were harvested (see fig.3.5.) and resuspended in fresh medium, prewarmed to 37°, pH adjusted to 7.1, and containing 82.6 nmoles $[^3H]$-AMP (sp. act. 22 Ci/mmol) or $[^3H]$-dTMP (sp. act. 25.6 Ci/mmol) to a final concentration of 20 µCi/ml. At indicated times 1 ml. samples were withdrawn into cold 25% (w/v) NaCl-0.02M-KCN (10 ml). The cells were collected by centrifugation at 45 000g_{max} for 5 min., then washed once in cold 25% (w/v) NaCl. The supernatant was diluted 1:100 in water and chromatography carried out as described in section 2.2.3.b., except that the development times were 0.2M LiCl for 2 min. and 1.0M LiCl for 10 min. The third step was omitted. Radioactivity was counted as described in the experimental section (section 2.2.4.).

	% found in the supernatant	
time (minutes)	adenosine	adenylic acid
1	16	84
2	22	78
5	29	71
7	56	44
10	52	48
15	56	44
20	52	48

Table 3.1.continued

time (minutes)	% found in the supernatant	
	thymidine	thymidylic acid
1	15	85
3	20	80
5	25	75
7	32	68
10	48	52
15	67	33
20	78	22
33	82	18

Fig. 3.4. Comparison of the growth of H.cutirubrum in the synthetic (●) and complex (▲) media

———————— O.D. 660nm

---------- viable cell count

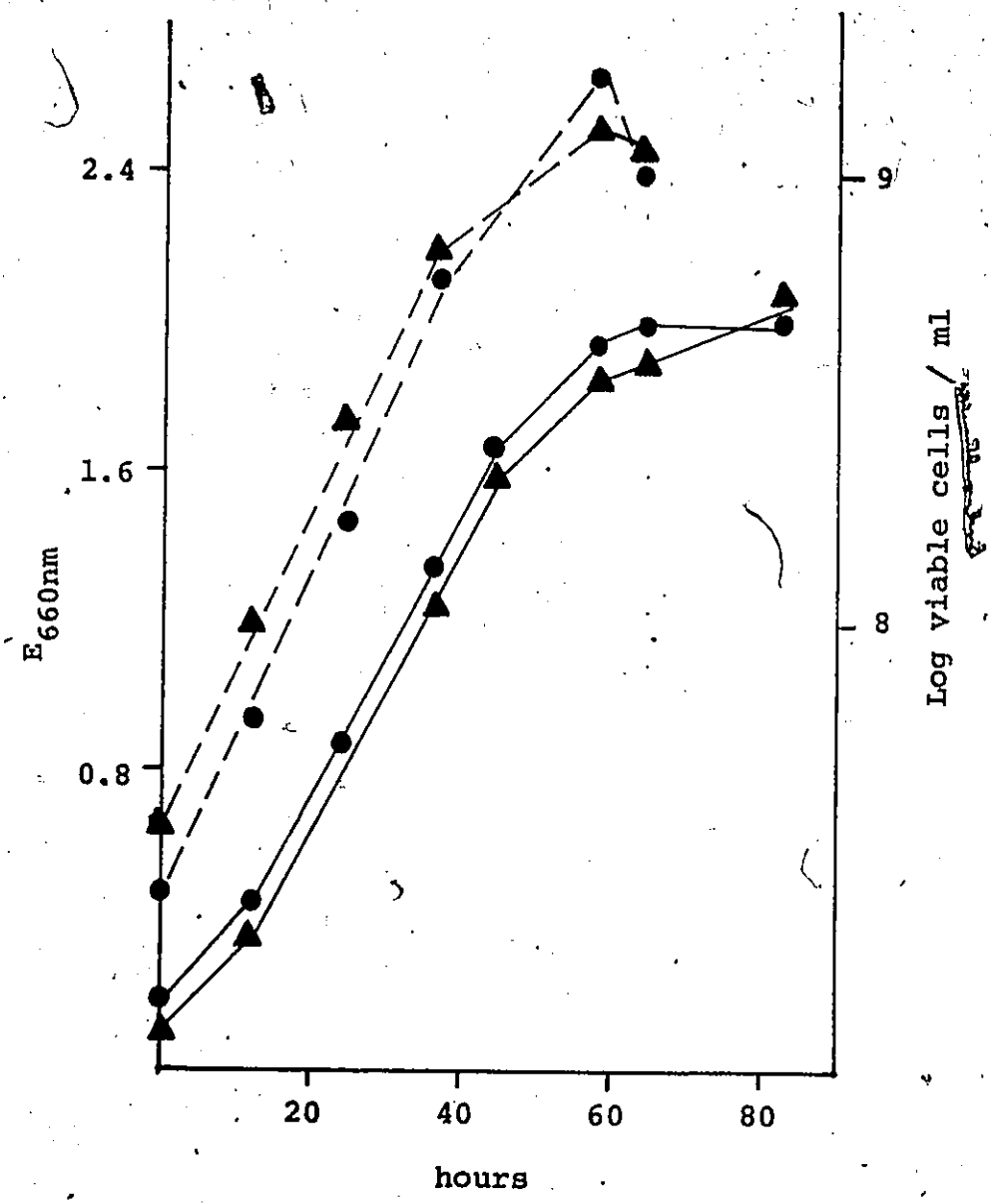

2.4
1.6
0.8
E_{660nm}
9
8
Log viable cells / ml
20
40
60
80
hours

Table 3.2. Generation times of H.cutirubrum in synthetic and complex media

Synthetic medium	7.6 ± 0.5 hours
Complex medium	7.7 ± 0.3 hours

The generation times were calculated from the following equation:

$$\text{generation time} = \frac{0.301\ t}{\log_{10} N_t - \log_{10} N_0}$$

where N_t = population at time t

N_0 = population at time 0

These results were calculated on the basis of data obtained from three growth curves.

described in the following respects:

(1) it contains only L-amino acids,

(2) it does not contain NH_4Cl,

(3) nucleotides are not present,

(4) the total inorganic phosphate concentration has been increased from 0.1mg/ml to 0.3mg/ml.

3.2. Exogenous thymine and thymidine as DNA precursors in H.cutirubrum

3.2.a. Incorporation of thymidine into DNA

The uptake of labelled thymidine provides a sensitive and convenient method for the measurement of DNA synthesis. The procedure used to determine the extent of incorporation of labelled thymidine into the cold acid insoluble product and thus the extent of DNA synthesis, is described in table 3.3. The results in table 3.3 show that the radioactive product was resistant to digestion by Pronase and ribonuclease (RNase), but was nearly completely degraded by pancreatic deoxyribonuclease (DNase), confirming that the cold acid insoluble product was indeed DNA.

Fig. 3.5. shows that H.cutirubrum growing in defined synthetic medium supplemented with 82.6 µM thymidine, incorporates the latter into DNA linearly. The short lag phase, which was only 5 min (fig. 3.6.), was not detected in this experiment since the first measurement was taken at 15 min.

Table 3.3. Evidence that H.cutirubrum incorporates $[^3H]$-thymidine into DNA

H.cutirubrum was grown in the synthetic medium supplemented with 82.6 µM-thymidine. After 40 hours at 37° $[^3H]$-thymidine (20 µCi/ml) was added and incubation was continued for 1 hour. Samples (1 ml) were withdrawn and added to cold 25% (w/v) NaCl-0.02M-KCN (10 ml). The cells were collected by centrifugation at 45 000g_{max} for 5 min. and were lysed by suspension in distilled water (1 ml). A solution of the appropriate degradative enzyme (5.0 mg/ml) in 50mM-sodium phosphate buffer, pH 6 was added to give final concentrations of 50 µg/ml of the enzyme and 0.5mM-phosphate and the mixture was incubated at 37° for 30 min. DNA was extracted and its radioactivity counted as described in fig. 3.5.

treatment	radioactivity(%)
none (control)	100.0
DNase digestion	6.4
Pronase digestion	98.9
RNase digestion	98.9

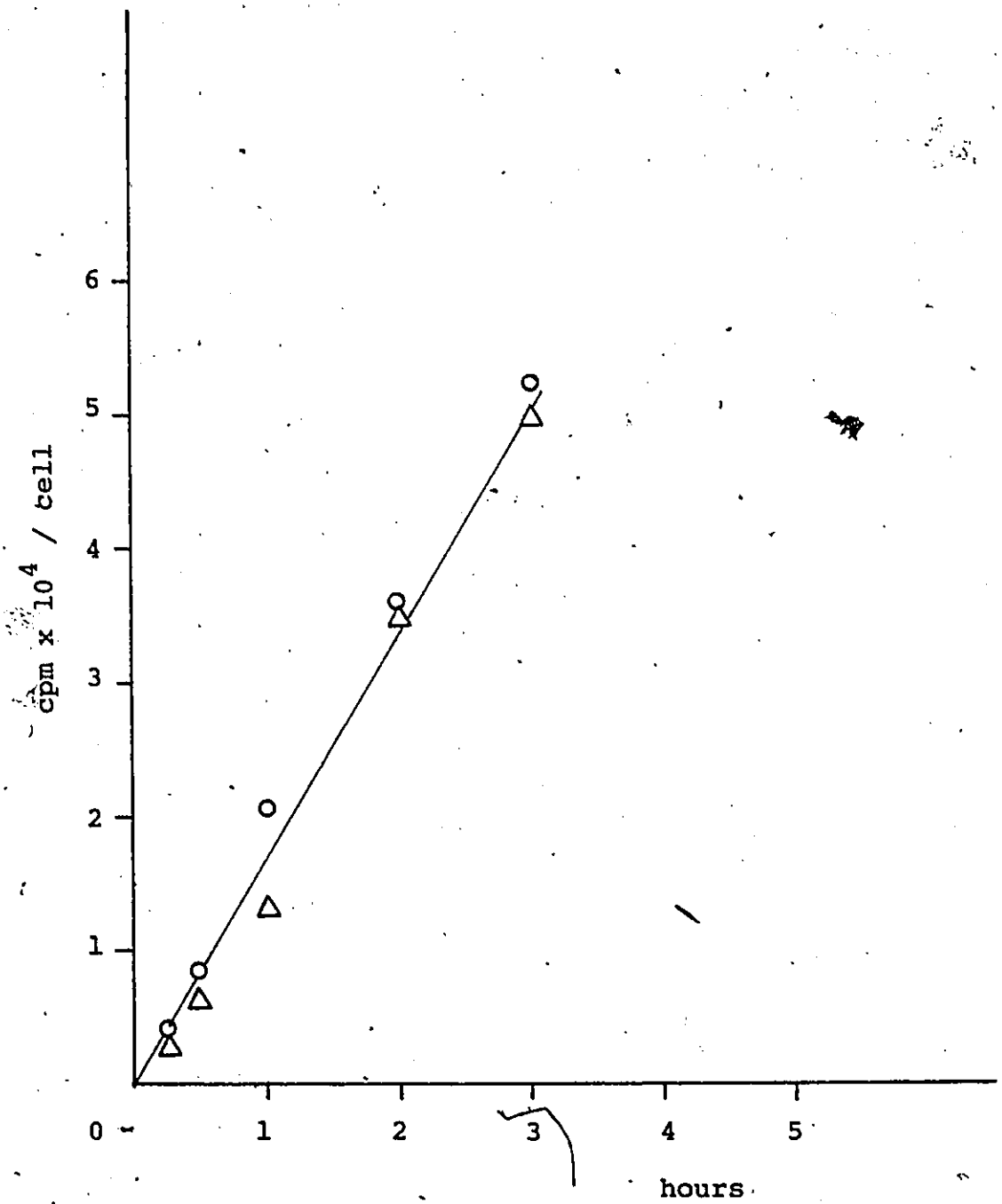
Fig. 3.5. Incorporation of ^{3}H-labelled thymidine into DNA by H.cutirubrum

Expt. 1: H.cutirubrum was grown at 37° in the synthetic medium supplemented with 82.6 µM-thymidine until the cell density was approximately 2 x 10^{8} cells/ml (27 hours). The bacteria were collected by centrifugation at 30° and 28 000g_{max} for 5 min and resuspended in the same volume of prewarmed (37°) synthetic medium, pH 7.1, containing thymidine (82.6 µM). After 30 min at 37°, [Me-^{3}H]-thymidine (sp.act. 18 Ci/mmol) was added to a final level of 20 µCi/ml. Samples (1 ml) were withdrawn at the indicated times and added to ice-cold 5% (w/v)-trichloroacetic acid (5 ml). The precipitates were collected by centrifugation, washed twice by resuspension and centrifugation in a total of 5 ml of ice-cold 5% (w/v) trichloroacetic acid and finally resuspended in 1 ml of the same acid. The suspensions were heated at 85°-90° for 40 min (Hutchison & Munro, 1961) and then centrifuged. The radioactivity of 50 µl of each supernatant was counted as described in section 2.2.4.

Expt. 2: As above, except that (i) thymidine was omitted from the growth medium and (ii) labelled thymidine was added immediately after resuspension of the cells in the fresh medium containing 82.6 µM-thymidine.

Cells grown with thymidine (expt. 1), 0 .

Cells grown without thymidine (expt. 2), Δ .


cpm x 10^4 / cell
6
5
4
3
2
1
0
1
2
3
4
5
hours

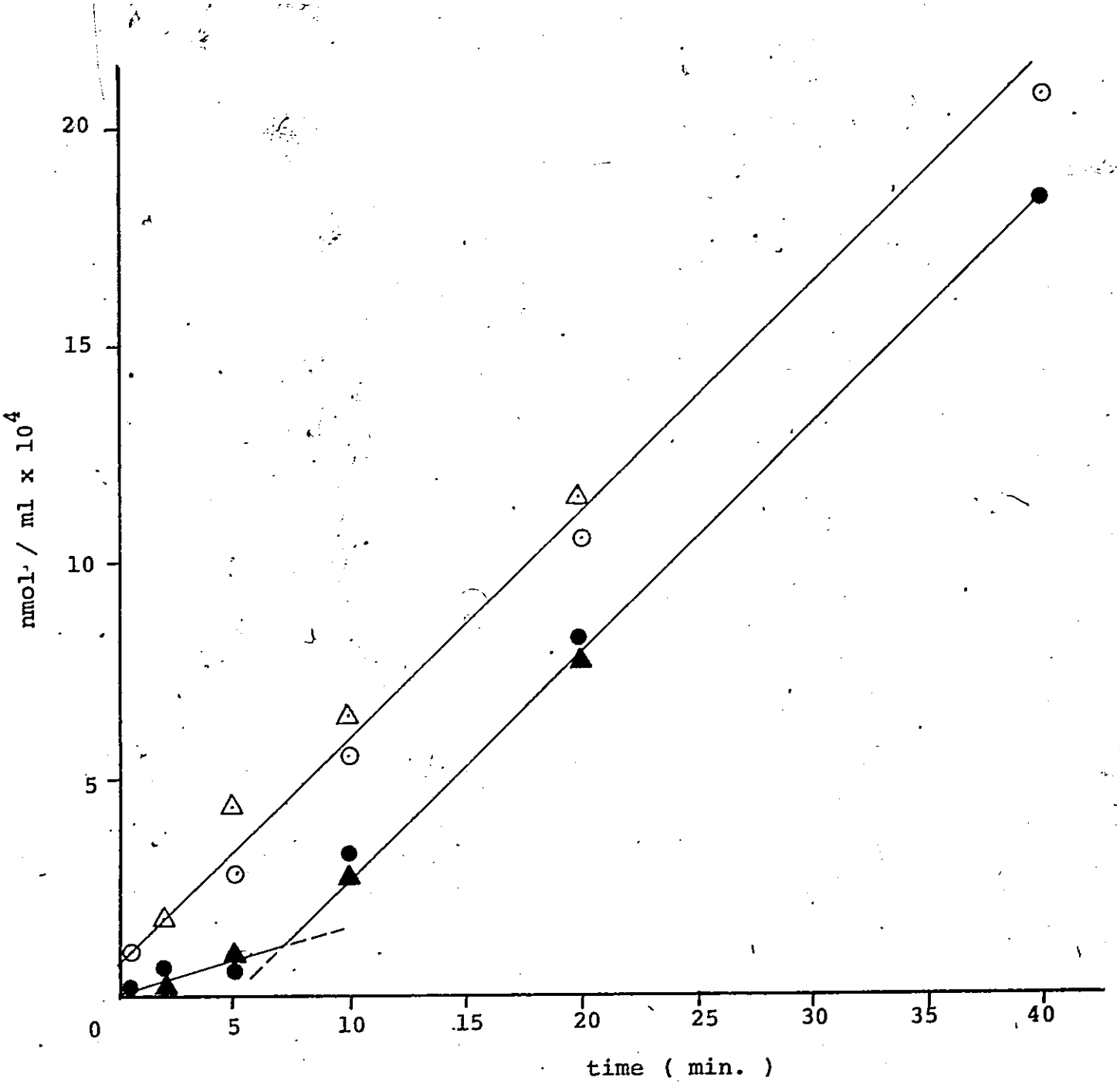
The rate of incorporation was the same with cells grown with thymidine (Δ) and without thymidine (o), suggesting that any necessary permeases or kinases must be constitutive. It should be noted that in experiment no. 2 (o) the radioactive precursor was added immediately to the resuspended bacteria and the 30 min preincubation step omitted, thus eliminating the possibility that enzymes might be induced during the standard 30 min preincubation with unlabelled thymidine (expt. 1,Δ)

The pH of the resuspension medium was always adjusted to 7.1 after autoclaving in order to avoid any shock to the cells. During growth of H.cutirubrum in the synthetic medium the pH of the latter rises from 6.6 to 7.1 or 7.2. It was observed that this rise in pH occured during the first 2 hours of growth and remained constant during the period of exponential growth.

The incorporation of radioactive thymidine into DNA was initially less than its total uptake by the cells, but the rates became identical after 5 min, as shown in fig. 3.6. The shortness of this lag phase, compared with the slow generation time of the organisms in these conditions, shows that the total intracellular pool of intermediates of thymidine and DNA must be small, thus allowing rapid equilibration with the extracellular precursor. The results in fig. 3.6. are for two concentrations of exogenous thymidine, 4.16 µM and 82.6 µM. This 20-fold variation in thymidine concentration, had no effect on either lag phase or the maximum rate of incorporation of

Fig. 3.6. Evidence for the presence of small, fixed pools of thymidine and thymidine nucleotides in H.cutirubrum

The bacteria were grown as described in expt. 1, fig. 3.5, except that the concentration of thymidine in the resuspension medium was either 4.16 µM or 82.6 µM. After addition of the radioactive thymidine, samples were withdrawn at the indicated times and added to ice-cold 25% (w/v) NaCl-0.02M-KCN (5 ml) containing a 20-fold excess of cold thymidine The cells were collected by centrifugation at 45 000g_{max} for 5 min, washed once by resuspension in 25% (w/v) NaCl (10 ml) followed by centrifugation and lysed with ice-cold 5% (w/v) trichloroacetic acid (2 ml). A sample (100 µl) of the mixture was counted to determine the total uptake of radioactivity and the remainder was used to determine the incorporation into DNA as described in fig. 3.5. Total uptake of radioactivity: with 4.16 µM-thymidine, o ; with 82.6 µM-thymidine, Δ . Incorporation into DNA: with 4.16 µM-thymidine, ● ; with 82.6 µM-thymidine, ▲ .


20
15
10
5
0
nmol / ml x 10^4
0
5
10
15
20
25
30
35
40
time (min.)

radioactivity into DNA. Table 3.4. gives the maximum rate of incorporation of radioactivity into DNA under different experimental conditions. The same value was obtained:

(1) with the cells grown previously in the presence or absence of thymidine,

(2) when the concentration of thymidine in the medium was 4.16 µM or 82.6 µM,

and (3) whether or not the cells were resuspended in fresh medium.

Further reduction in the initial exogenous thymidine concentration to 1.26 nM from 4.16 µM, i.e. approximately 3000-fold variation, only caused a 100-fold reduction in the maximum rate. From these results it can be concluded that the system of uptake of thymidine into the cell and DNA becomes saturated at very low concentrations, and that the incorporation of the nucleoside into the precursor pools has little effect on the rate of synthesis of endogenous precursors.

3.2.b. Thymidine phosphorylase

As seen above, exogenous thymidine is incorporated readily into H.cutirubrum DNA and the maximum rate of incorporation is not affected by a 20-fold variation in extracellular concentration. The duration of the process, however, is dependent on the exogenous thymidine concentration (fig. 3.7.).

Table 3.4. Effect of growth conditions and extracellular thymidine concentration on incorporation of thymidine by H.cutirubrum

Expts. 1 - 3 were done as described for expt. 1, fig.3.5, using the indicated thymidine concentrations in the growth and resuspension (incorporation) media. In expts. 4 and 5, the cells were not transfered to fresh medium: radioactive thymidine was added directly to the cultures after 40 hours. During growth of the cultures in expt. 4, the thymidine concentration fell from its initial value of 82.6 µM to 4.16 µM at the time the highly radioactive precursor was added. Viable cell counts were determined as described in the experimental section.

expt.	thymidine concn. (µM)		$[^3H]$-thymidine	
	growth med.	incorpn. med.	final sp. act. (µCi/nmol)	incorpn rat (nmol/min/c
1	0	82.6	0.24	1.6×10^{-11}
2	82.6	82.6	0.24	1.9×10^{-11}
3	82.6	4.16	4.8	1.7×10^{-11}
4	82.6	4.16	4.8	1.9×10^{-11}
5	0	1.26×10^{-3}	15.8×10^{-3}	1.5×10^{-13}

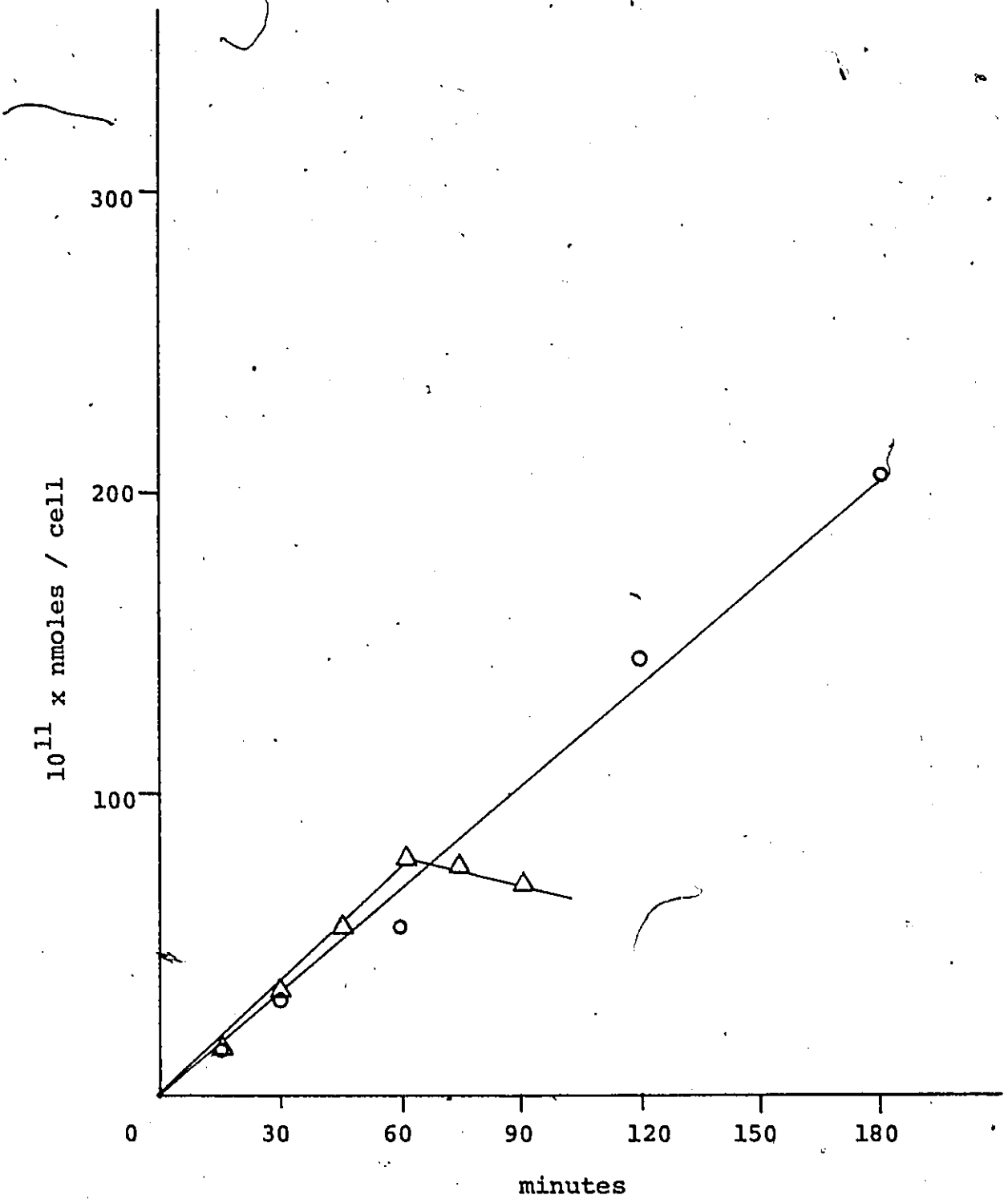
Fig. 3.7. Incorporation of $[^3H]$-thymidine into DNA by H.cutirubrum at 2 exogenous concentrations of thymidine over an extended period

Expt. 1 as described in fig. 3.5.

Expt. 2 The bacteria are grown as described in experiment 1 until the cell concentration was approximately 1 x 10^9 cells/ml (40 hours). At this time $[Me\text{-}^3H]$-thymidine (sp. act. 18 Ci/mmol) was added to a final level of 20 µCi/ml. Samples (1 ml) were withdrawn at indicated times and processed as described in experiment 1.

Expt. 1: o-----o 82.6 µM thymidine

Expt. 2: Δ-----Δ 4.16 µM thymidine


300
200
100
0
30
60
90
120
150
180
minutes
10^{11} x nmoles / cell

The incorporation per cell is linear for at least three hours at 82.6 μM-thymidine but ceases after 1 hour at 4.16 μM. After three hours the incorporation per cell was 207 x 10^{-11} nmoles and after a further five hours was only 422 x 10^{-11} nmoles (i.e. approximately double), indicating that the rate of incorporation was falling off. The results in table 3.5. show that this was due in part to conversion of thymidine to thymine. No other radioactive product was detected during the course of the experiment and the total radioactivity in the medium fell by about 4%, owing to the incorporation into the cells.

The situation here is similar to that in other wild type bacteria (Henderson & Paterson, 1973; Kammen, 1967). In wild type E.coli, thymidine incorporation stops after a very short time owing to its degradation to thymine by an inducible thymidine phosphorylase. The results in table 3.6. confirm that this enzyme is also present in H.cutirubrum. The degradation of thymidine to thymine was dependent on orthophosphate and was therefore due to a phosphorylase (expt. 1). The activity of the enzyme in the whole homogenates was the same for cells grown with or without thymidine. The same was true for the activity found in the extracellular fluid (expt. 2 and 3). This suggests that the enzyme in H.cutirubrum may be constitutive, unlike that in E.coli

The data in experiment 4 show that the intracellular thymidine phosphorylase was readily released from the cells in a

Table 3.5. Formation of thymine from thymidine by H.cutirubru

Synthetic medium (25 ml) containing 82.6 µM-^{3}H thymidine (50 µCi) was inoculated with 2.5 ml of a 48 hour preculture. Incubation was at 37° and samples (1 ml) were withdrawn at the indicated times. The cells were removed by centrifugation at 45 000g_{max} for 5 min. and the supernatants were diluted with 9 ml of aqueous 12mM-thymine-10mM-thymidine. Samples (20 µl) of the dilutions containing approximately 2800 cpm were chromatographed on plastic-backed cellulose t.l.c. sheets with ethyl acetate:water:formic acid, 65:35:5 (v/v/v) by Kammen's (1967) modification of the paper chromatographic method of Fink et al.(1956). Development was for 3 hours in a paper-lined tank previously equilibrated with the solvent system for at least 18 hours at room temperature. The thymine and thymidine spots were identified by comparison with standards and were cut and counted for radioactivity. The R_f's of thymine and thymidine relative to the solvent front were 0.85 and 0.74 respectively. (The results are corrected for a background of 14 cpm).

time (h)	^{3}H distribution (cpm)	
	Thymine	thymidine
0	0	2800
5	1964	780
23	2484	382
40	2530	190
48	2588	154

Table 3.6. Thymidine phosphorylase activity in H.cutirubrum

The bacteria were grown for 48 hours in the synthetic medium (55 ml) either with 82.6 µM-thymidine (expts. 1, 2b, 3b and 4) or without thymidine and were harvested by centrifugation at 45 000g_{max} for 10 min. The cells (about 0.8g, wet weight) were washed once with 25% (w/v) NaCl (3 ml) and then resuspended in 3M-KCl-10mM-Tris-HCl, pH 7.6 (3 ml). The suspension was homogenized with a Bronwill Biosonik II sonicator (30 sec. at setting 60) and the homogenate was separated when necessary into the soluble fraction and cell debris by centrifugation at 45 000g_{max} for 10 min. The cell debris was resuspended in the same buffer (1.5 ml). The assay mixture (1 ml) contained: 10mM-sodium phosphate buffer, pH 7.2; 0.5 ml of the appropriate fraction; 5mM- ^{3}H thymidine (sp. act. 0.38 µCi/mmol) In expt. 1b, 10mM-imidazole buffer, pH 7.2, was used instead of phosphate buffer. Incubation was at 37° and samples (0.3 ml) were withdrawn at the indicated times and added to an ice-cold 10% (w/v) trichloroacetic acid (0.3 ml). The supernatants after centrifugation were extracted twice with ether (1 ml) and 20 µl portions were analysed by t.l.c. as described in table The controls were samples withdrawn at zero time. The volumes in expt. 3a are the total volumes of the fractions.

Table 3.6. continued

experiment	time (min)---	thymidine converted (%) 5	10
1(a) assay with phosphate		13.6	22.7
(a) assay without phosphate		0	0
2(a) growth without thymidine		13.4	24.6
(b) growth with thymidine		13.6	22.7
3(a) extracellular fluid growth without thymidine (55 ml)		1.7	2.6
(b) extracellular fluid growth with thymidine (55 ml)		1.1	2.6
4(a) soluble fraction (3 ml)		10.4	25.5
(b) cell debris suspension (1.5 ml)		not detectable	

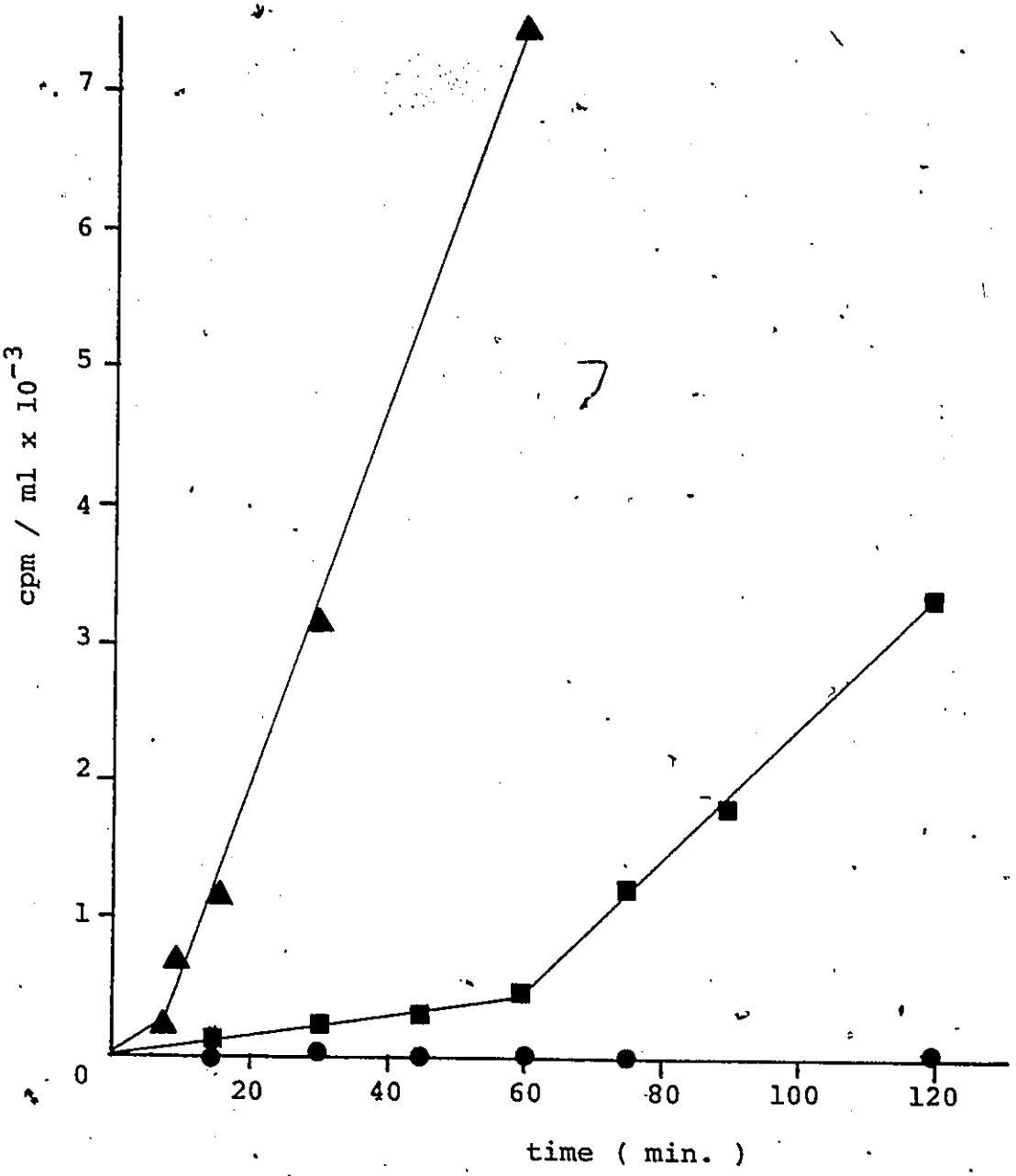
soluble form. If allowance is made for the difference in volume between the extracellular fluid and the soluble extract (expt. 4.a.), about 60% of the total activity was present in the medium. Since the bacteria were harvested in mid exponential phase it is unlikely that this high level of activity in the medium could be due to release from dead cells. It is possible that either H.cutirubrum releases thymidine phosphorylase into the growth medium, or the enzyme is loosely associated with the cells and is easily sheared off during centrifugation. The latter possibility is supported by the fact that no detectable activity was present in the cell debris after sonication of washed cells (expt. 4.b.), so that complete release of enzyme in soluble form readily occurs.

3.2.c. Incorporation of thymine into DNA

The results in fig. 3.8. show that H.cutirubrum NRC 34001 resembles wild type E.coli in its inability to utilize thymine unless supplied with deoxyribose-1-phosphate. $[^3H]$-thymine alone is not incorporated into DNA to any measurable extent. When unlabelled deoxyadenosine was present, rapid incorporation of thymine into DNA began after one hour. The rate of incorporation at this time was of the same order of magnitude (2.1×10^{-11} nmoles/min/cell) as that of thymidine into DNA and is consistent with the formation of thymidine by the reversible reactions catalysed respectively by purine nucleoside

Fig. 3.8. Comparison of the incorporation of thymine and thymidine into DNA by H.cutirubrum

The bacteria were grown and harvested as described in expt. 1, fig. 3.5. They were resuspended in fresh medium (pH 7.1) containing either 82.6 µM-thymidine, 79.2 µM-thymine or 79.2 µM-thymine-39.8 µM-2'-deoxyadenosine. After preincubation at 37° for 30 min, [^{3}H]-thymidine (20 µCi/ml, sp. act. 18 Ci/mmol) was added to the first culture and [^{3}H]-thymine (20 µCi/ml, sp. act. 23 Ci/mmol) to each of the other two cultures. Incorporation of radioactivity into DNA as a function of time was determined as described in fig. 3.5. Radioactivity incorporated in the presence of: [^{3}H]-thymidine, ▲ ; [^{3}H]-thymine, ● ; [^{3}H] -thymine plus non-radioactive 2'-deoxyadenosine, ■ .


cpm / ml x 10^{-3}
0
1
2
3
4
5
6
7
20
40
60
80
100
120
time (min.)

phosphorylase and thymidine phosphorylase:

deoxyadenosine + Pi $\Leftrightarrow$ adenine + deoxyribose-1-P

thymine + deoxyribose-1-P $\Leftrightarrow$ thymidine + Pi

The lag of one hour was in marked contrast to the 5 min delay with thymidine. This lag was not observed in E.coli (Kammen, 1967), where incorporation is detectable within one min after addition of the nucleoside. This suggests that H.cutirubrum must first induce either a purine nucleoside phosphorylase or a deoxyadenosine permease.

3.3. Demonstration of semi-conservative replication

Fig. 3.9. shows the incorporation of $[^3H]$-5-bromo-2'-deoxyuridine into H.cutirubrum DNA. While the cells incorporate this analog readily into their DNA, the maximum rate of incorporation is only 0.38×10^{-11} nmoles/cell/min, about 1/20 the rate of thymidine incorporation. Because H.cutirubrum NRC 34001 incorporates the heavy analog more slowly, and its DNA has a low A+T content (see below), the experiments presented here were done with the cell grown in the presence of the heavy $[^3H]$-5-bromo-2'-deoxyuridine for several generations (section 2.3.1.), and then transfered to light $[^{14}C]$-thymidine, so as to ensure measurable changes in density.

The results in fig. 3.10. (a,b) show that normal semi-conservative replication occurs in H.cutirubrum, because after

Fig. 3.9. Kinetics of incorporation of [^{3}H]-5 -bromo-2'-deoxyuridine into H.cutirubrum DNA

The experiment is done as described in fig. 3.8., exp. 2, except that 5 -bromo-2'-deoxyuridine is used in the growth medium. The concentration of 5 -bromo-2'-deoxyuridine in the experiment is 3.4 µM. At the start of the experiment [^{3}H]-5 -bromo-2'-deoxyuridine (spec. act. 3 Ci/ml) is added to a final level of 5 µCi/ml. Samples (1 ml) were withdrawn at the indicated times and treated as described in fig. 3.5.

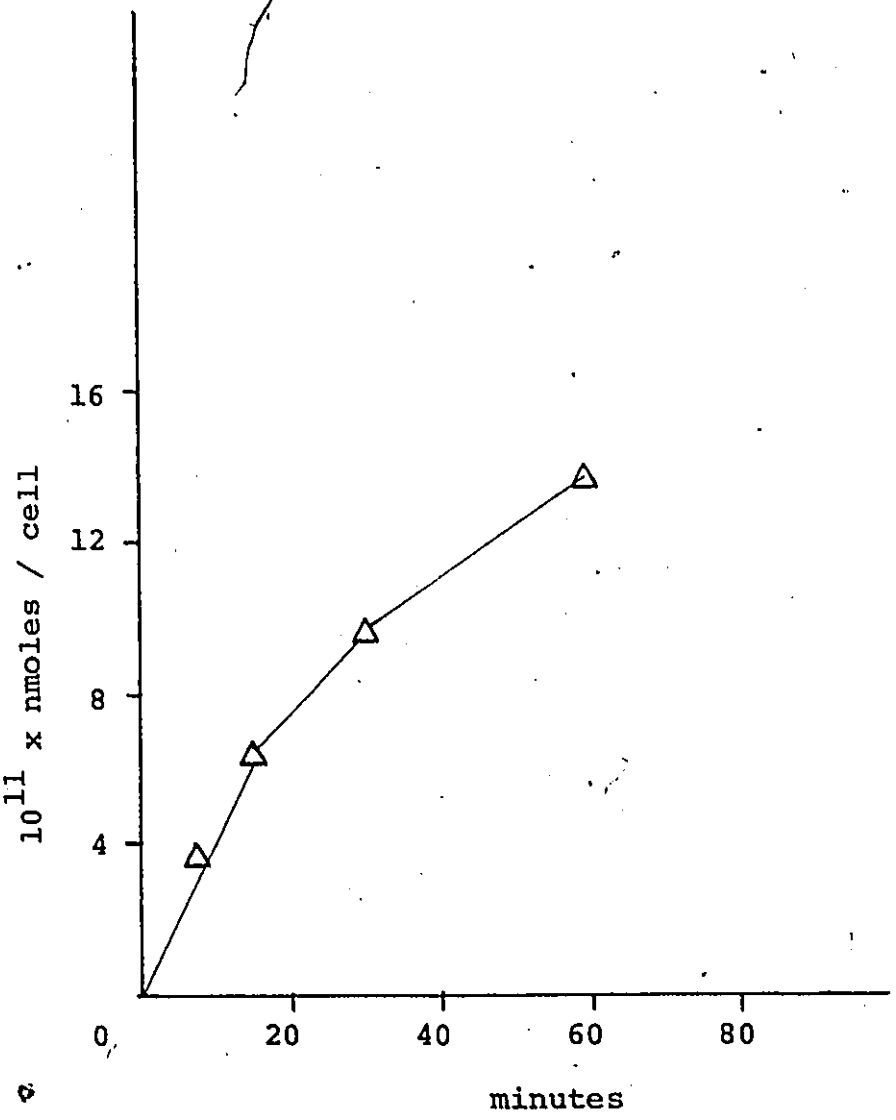
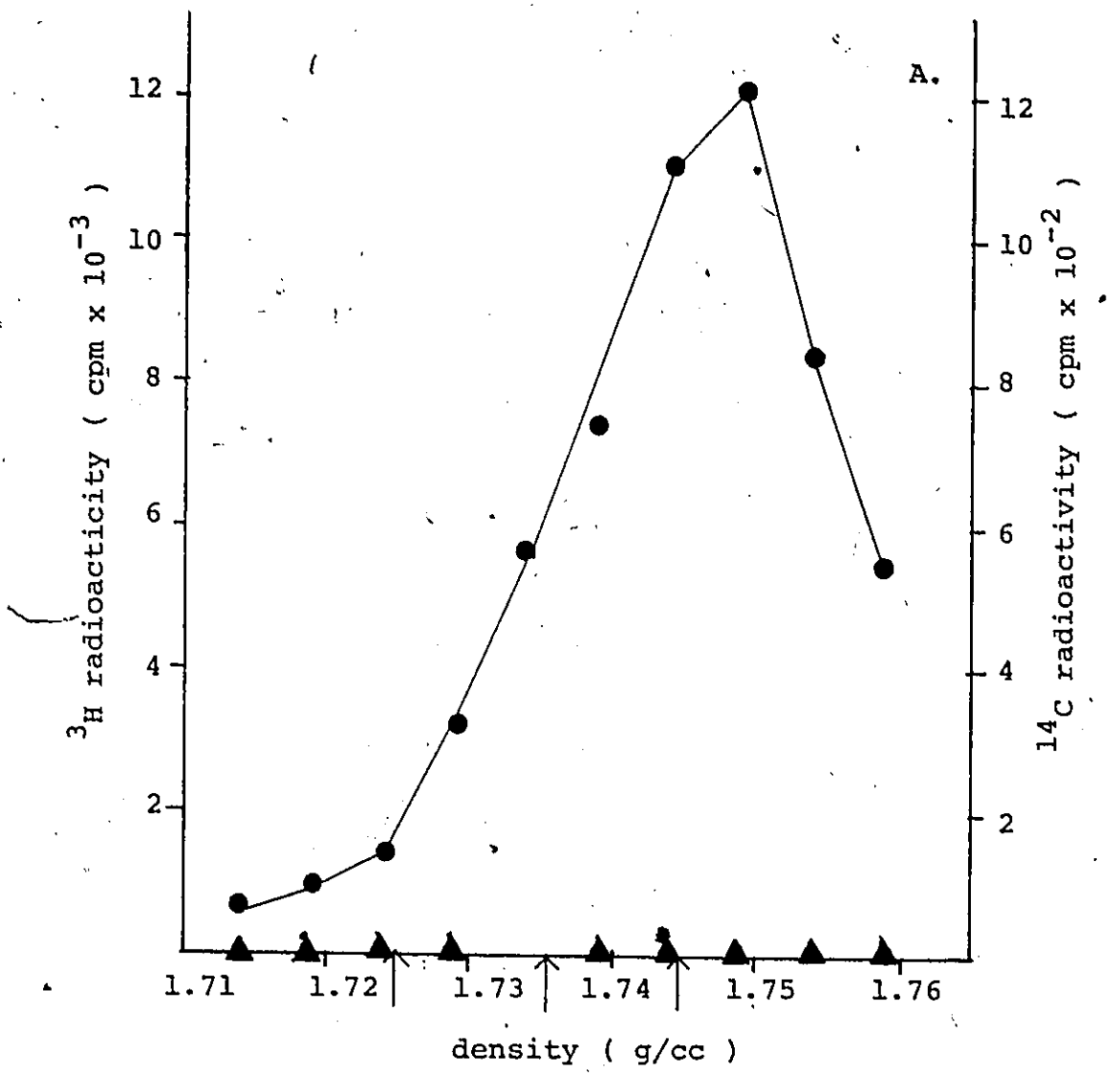

16
12
8
4
0
20
40
60
80
10^{11} x nmoles / cell
minutes

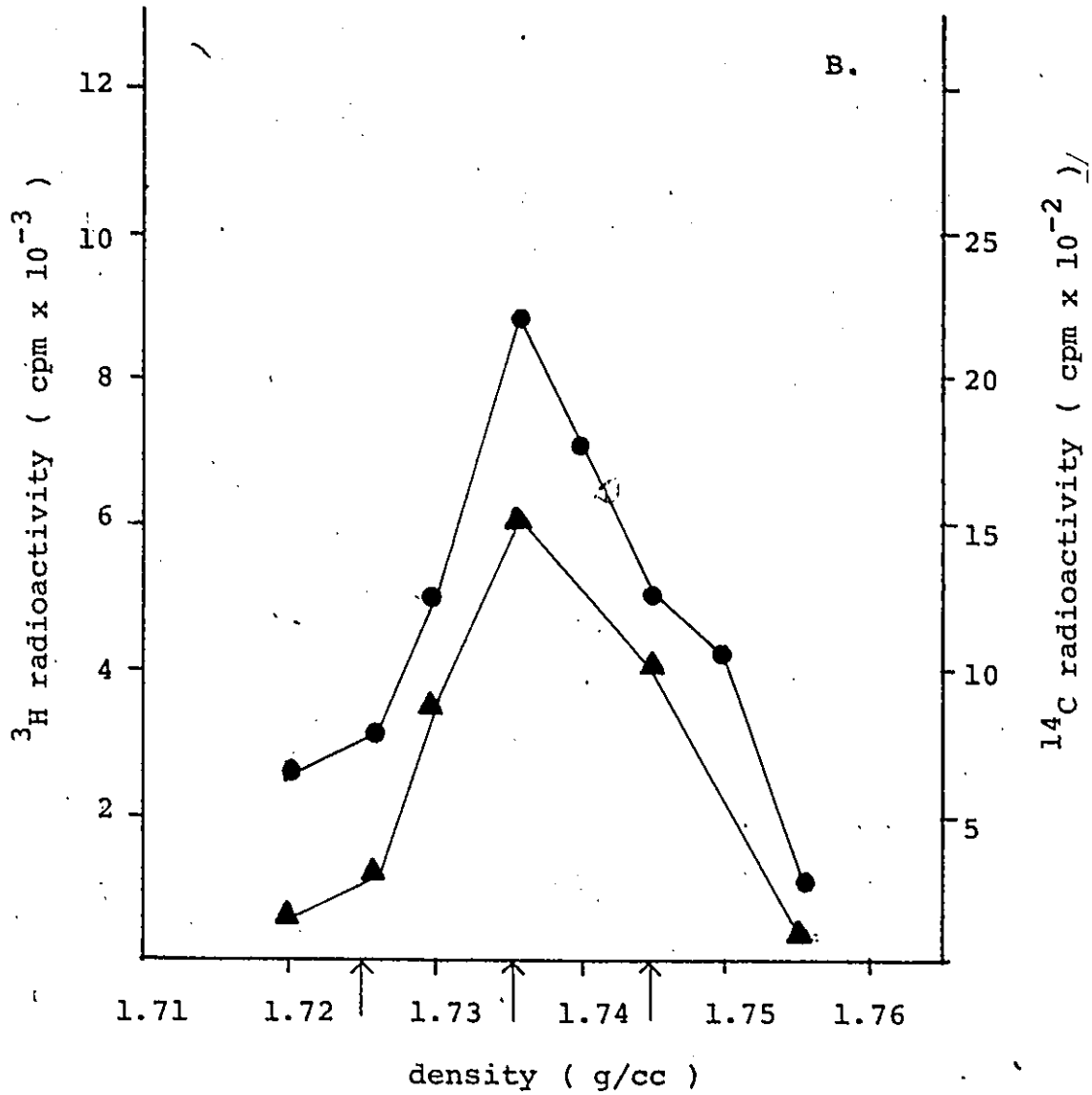
Fig. 3.10. Evidence for semi-conservative replication in H.cutirubrum

The results show the distribution of $[^{3}H]$-5-bromo-2'-deoxyuridine (●) and $[^{14}C]$-thymidine (▲) after transfer of cells to the light thymidine containing medium:

(A) zero time

(B) 1 generation (8 hours).


A.
12
10
8
6
4
2
^{3}H radioacticity (cpm x 10^{-3})
^{14}C radioactivity (cpm x 10^{-2})
1.71
1.72
1.73
1.74
1.75
1.76
density (g/cc)


B.
12
10
8
6
4
2
^{3}H radioactivity (cpm x 10^{-3})
25
20
15
10
5
^{14}C radioactivity (cpm x 10^{-2})
1.71
1.72
1.73
1.74
1.75
1.76
density (g/cc)

one generation (8 hours) all the DNA was in the hybrid form. From this data the following buoyant densities were obtained:

	gm/ml
heavy	1.7480
hybrid	1.7360
light	1.7250

The above value for light DNA agrees well with that obtained for a purified H.cutirubrum DNA prepared by the procedure described in section 2.5.

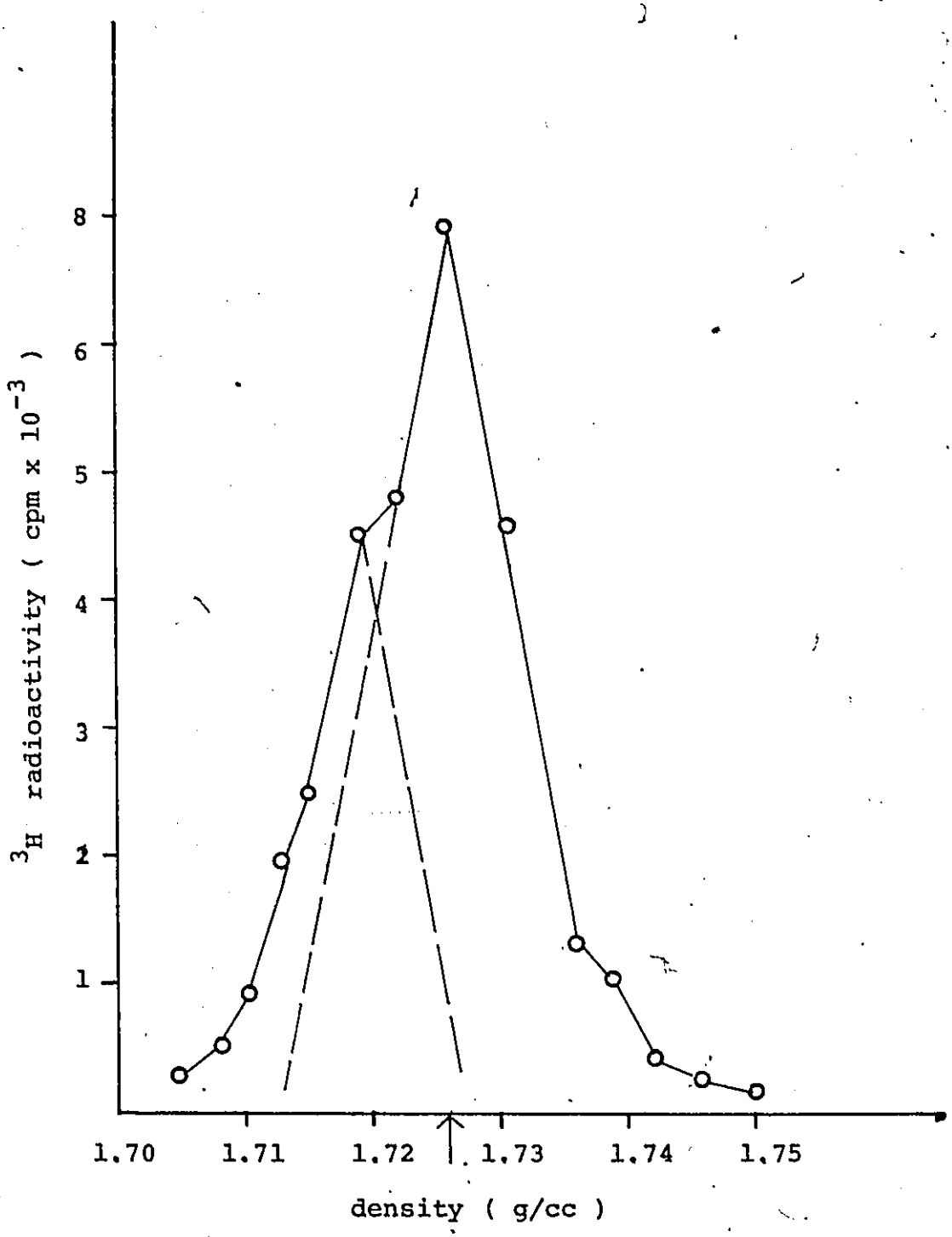
Fig. 3.11. shows the result of a caesium chloride density gradient of the purified H.cutirubrum DNA. The buoyant density of the major component is 1.7260 and corresponds to an A+T content of 33%, as determined by the method of Schildkraut, Marmur & Doty (1962). These results are in good agreement with the value obtained by Moore and McCarthy (1969).

A minor component with buoyant density 1.7190 gm/ml and A+T content of 40%, is also present and comprises approximately 30% of the total DNA. The proportion of satellite present in the total DNA was calculated, using the extrapolation shown in fig. 11. These results indicate that H.cutirubrum used in these studies, contain more than twice the amount of satellite DNA reported by Moore and McCarthy (1969). There are two possible explanations for this:

(1) H.cutirubrum in our laboratory has been kept under different conditions. Our stocks have always been maintained on

Fig. 3.11. Equilibrium caesium chloride gradient of purified $[^3H]$-DNA from H.cutirubrum

The DNA is prepared as described in section 2.5. The cells are grown in Gochnauer and Kushner's medium in the presence of $[Me\text{-}^3H]$-thymidine at a final concentration of 2.5 μCi/ml. Equilibrium caesium chloride density gradient was performed as described in section 2.3.1. The results show the distribution of ^3H in the gradient. 20 fractions were collected in the density range 1.69 - 1.77 g/cc.


^3H radioactivity (cpm x 10^{-3})
8
6
5
4
3
2
1
1.70
1.71
1.72
1.73
1.74
1.75
density (g/cc)

2% (w/v) agar slants of Gochnauer and Kushner's complex medium, while they used a high salt medium containing 0.5% tryptone, 0.5% yeast extract, 2.0% $MgSO_4 \cdot 7H_2O$, 25% NaCl, 0.2% $CaCl_2$, and 0.001 $FeCl_2$.

(2) The sensitivity of our experiments may not be as great as that of Moore and McCarthy (1969), since they employed the analytical ultracentrifuge, while our studies employed the preparative ultracentrifuge.

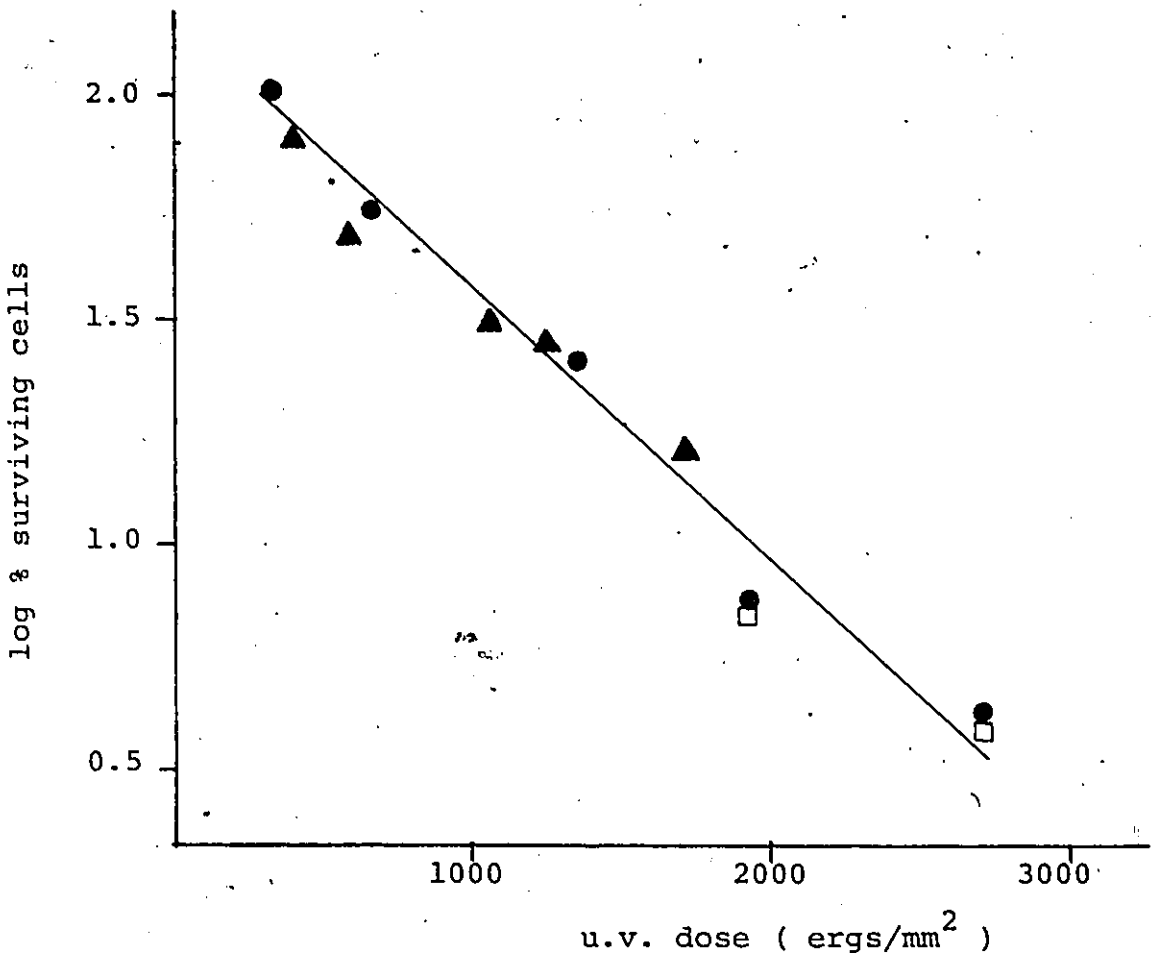
3.4. Repair in H.cutirubrum

3.4.a. Effect of ultraviolet irradiation on growth

The survival curves for H.cutirubrum, irradiated with u.v. light in either synthetic medium (Grey & Fitt, 1976), or complex medium of Gochnauer and Kushner (1969) are shown in fig. 3.12. The survival in these growth media was about the same as observed by Hescox and Carlberg (1972) in another complex medium. In these experiments the viable counts were done on a 2% agar solution in complex medium. The same results were obtained when the viable counts were done on a 2% agar in synthetic medium. The growth medium did not affect the sensitivity of the bacteria to u.v. irradiation nor its recovery. All other experiments were done in the synthetic medium, and viable count determinations were done on complex medium agar plates.

Fig. 3.12. Survival curves for H.cutirubrum irradiated with u.v. light

H.cutirubrum growing in either the synthetic medium (●) or the complex (▲) medium was irradiated with 11 erg/mm^2/sec and the number of surviving viable cells determined as described in section 2.2.2. (□) indicate the results obtained when viable counts are plated on synthetic medium.


log % surviving cells
2.0
1.5
1.0
0.5
1000
2000
3000
u.v. dose (ergs/mm2)

In table 3.7. the viability of samples of a culture irradiated with 4500 erg/mm^2 of u.v. light and then either photoreactivated or kept in the dark, is compared with that of an untreated control sample of the same culture. To test for dark repair, the Petri dish containing irradiated cells was covered with aluminium foil, placed on ice and swirled exactly as described for photoreactivation (section 2.4.2.), but without illumination. After 50 min photoreactivation, the original number of viable cells was completely restored, whereas no restoration of viability occurred in the dark during the same time.

Fig. 3.13. compares the growth, over an extended period, of u.v. irradiated cultures of H.cutirubrum that had been photoreactivated or kept in the dark, with that of a control culture. If the lag phase is taken into account, the culture that was irradiated with 4500 erg/mm^2 and photoreactivated for 50 min, grew as well as did the control. The lag phase is caused by the need to cool the culture on ice during photoreactivation, to prevent damage from the heat of the fluorescent lamp. In contrast, the cultures kept in the dark following irradiation with either 1800 erg/mm^2 or 4500 erg/mm^2 showed little or no growth during the same period.

3.4.b. Incorporation of acid soluble precursors into irradiated cells

The results in fig. 3.13. and table 3.7. confirm that

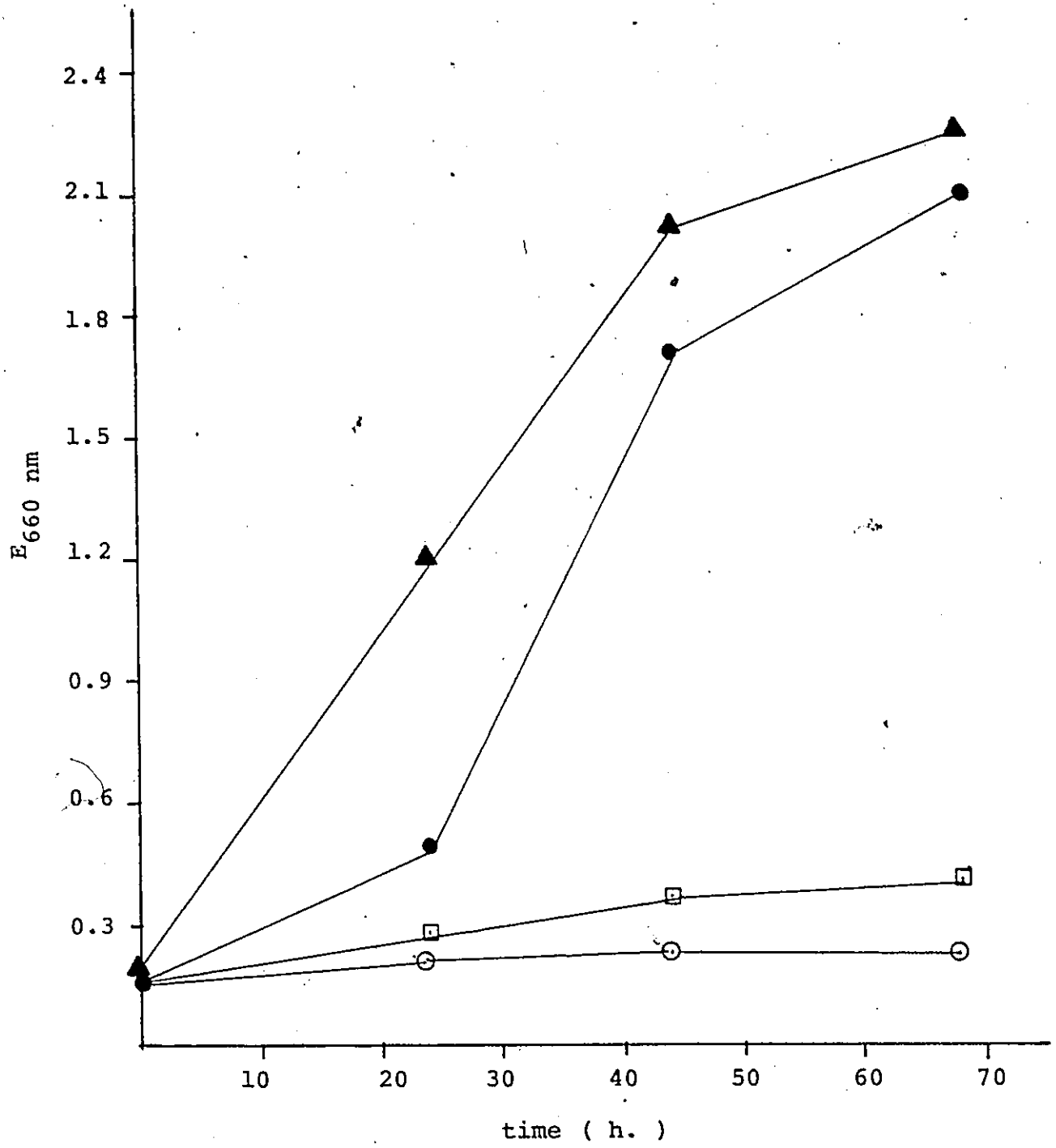
Table 3.7. Photoreactivation and the absence of dark repair in ultra-violet-irradiated H.cutirubrum

	No. of viable cells / ml	
time (min)	visible light	dark
0	4×10^5	2×10^5
10	6×10^6	3×10^5
50	1.8×10^9	2×10^5

The original control culture contained 2×10^9 cells/ml. In this experiment viable cell counts were determined on the synthetic medium (Grey & Fitt, 1976) supplemented with 2% (w/v) agar.

Fig. 3.13. Effect of u.v. irradiation with and without photoreactivation on the growth of H.cutirubrum

Portions (15 ml) of a culture of H.cutirubrum growing in the synthetic medium were irradiated with 1800 erg/mm^2 or 4500 erg/mm^2. A culture subjected to the higher dose was photoreactivated for 50 min. Samples (5 ml) of the treated cultures and of the original untreated control culture were then transferred to flasks containing fresh synthetic medium (50 ml) previously warmed to 37°. The flasks containing the non-photoreactivated samples were covered with aluminium foil. Incubation was continued at 37° in the standard conditions and growth was determined by turbidimetry at 660nm. Control culture, ▲ ; culture irradiated with 4500 erg/mm^2 and photoreactivated, ● ; cultures irradiated with 1800 erg/mm^2, □ , and 4500 erg/mm^2, o , and kept in the dark.

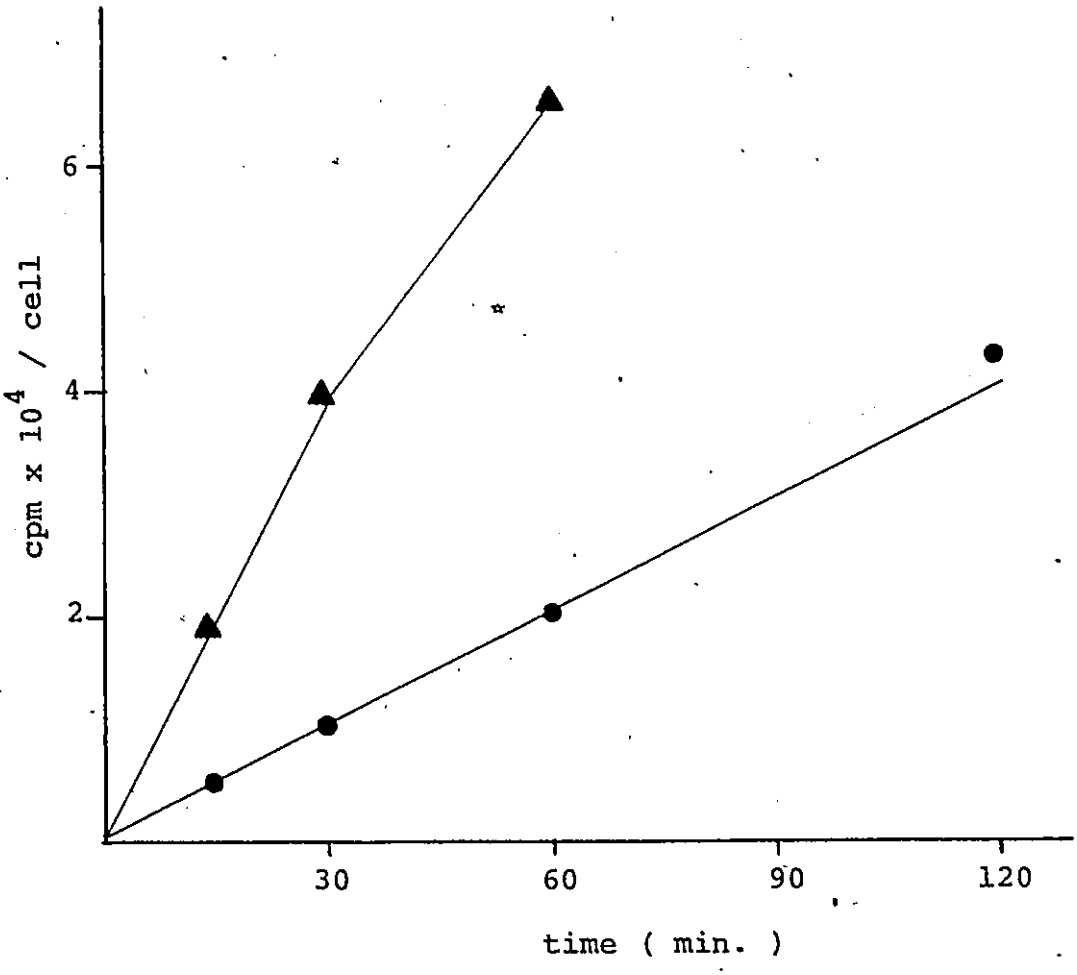

2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
$E_{660\ nm}$
10
20
30
40
50
60
70
time (h.)

H.cutirubrum has a highly efficient photoreactivating system, and prove that there is no restoration of the viability of the u.v. irradiated cells kept in the dark. The bacteria are clearly unable to perform dark repair of their DNA. Dark repair of DNA is a multi-step process, as described in the introduction (section 1.3.), and it is possible that the failure to reactivate the cells in the dark could be due to the inability of the bacteria to perform any or all of these steps. The stage of repair of primary interest in these studies is the incorporation of acid soluble precursors for the polymerisation step.

It was conceivable that the absence of dark repair was not due to the inability of the cells to incorporate small precursors into damaged DNA, but to the lack of the enzymes involved in any of the other steps, e.g. the u.v. endonuclease. To eliminate such a possibility , which would invalidate experiments based on the assumption that the incorporation of thymidine into DNA in H.cutirubrum was associated solely with replication, the rate of incorporation of thymidine into DNA by u.v. irradiated cells and unirradiated cells were compared. The results are shown in fig. 3.14. In these experiments cells irradiated with a total of 1320 erg/mm^2 (this corresponds to 25% survival) were used to ensure that some measurable synthesis of DNA would occur in the irradiated but nonphotoreactivated culture. It should be noted that the results are expressed in terms of incorporation observed per viable cell and not per ml

Fig. 3.14. Effect of u.v. irradiation on $[^3H]$ -thymidine incorporation by H.cutirubrum

The culture and irradiation of H.cutirubrum were carried out as described in sec. 2.4.1. The u.v. dose was 1320 erg/mm^2. Incorporation of $[^3H]$ -thymidine (4.16 µM, spec. act. 4.8 Ci/mol) into DNA was measured as described in fig. 3.5. During the incorporation experiment, the flask containing the irradiated cells was covered with aluminium foil. The viable cell counts were 4 x 10^9 cells/ml in the control culture and 1 x 10^9 cells/ml in the irradiated culture. Control culture, ▲ ; irradiated culture, ● .


6
4
2
cpm x 10^4 / cell
30
60
90
120
time (min.)

of culture or number of original cells in the sample. This was done so as to obtain a true measure of DNA synthesis in the viable cells and to avoid the artefactual apparent inhibition of DNA biosynthesis caused by the decrease in the number of viable cells (Smith & O'Leary, 1968). The results in fig. 3. 14. show that the rate of incorporation per viable cell in the irradiated culture was linear for the time period studied, and was less than in the control, the maximum values being 3.9×10^{-6} cpm/min and 12×10^{-6} cpm/min respectively. Thus irradiation did not cause a marked increase in thymidine incorporation as would be expected if repair biosynthesis of u.v. damaged DNA ocurred in the non-viable cells (Huang et al., 1972). In fact, irradiation caused an actual inhibition of DNA biosynthesis in the surviving cells.

The absence of any stimulation of thymidine incorporation was not due to a decrease in the specific activity of the radioactive thymidine, caused by release of unlabelled thymidine or its readily dephosphorylated nucleotides from the non-viable cells, for the following reasons:

(1) Incorporation in the irradiated culture was linear throughout the entire experiment, therefore release of intracellular material would have to be instantaneous. The fact that cells irradiated with 1320 erg/mm^2 can be photoreactivated completely, suggests that they do not disintegrate rapidly.

(2) The intracellular pools concerned are small and fixed,

as shown above (see fig. 3.6. section 3.2.a.).

(3) The results in table 3.8. show that irradiated cells do not release significantly more acid soluble thymidine or thymidine derivatives into the extracellular fluid, so that the specific activity is not altered. This experiment was designed to test the permeability of the cells following irradiation, and it can be seen that this was not changed during the time span of these experiments. The temperature was maintained at 0^{o} to prevent DNA synthesis or possible excision of thymine dimers.

3.4.c. Repair during normal growth

Hanawalt and Cooper (1971) have shown that during repair biosynthesis, radioactive precursors incorporated into DNA are associated with parental rather than hybrid daughter molecules. The results in fig. 3.15 show that the newly incorporated $[^{14}C]$-thymidine is associated with hybrid DNA and not parental DNA. In this experiment cells are grown first in the presence of heavy $[^{3}H]$-5-bromo-2'-deoxyuridine for several generations, then transferred to fresh medium containing the light $[^{14}C]$-thymidine (section 2.3.1.). After 0.125 (1 hour) and 0.188 (1½ hour) generations, all the $[^{14}C]$-thymidine was found with hybrid DNA (ρ = 1.7340) and not parental DNA (ρ = 1.7475) Thus, during normal replication, no significant repair biosynthesis of DNA was occurring.

Table 3.8. Release of acid-soluble thymidine and thymidine derivatives by u.v.-irradiated H.cutirubrum

H.cutirubrum was grown at 37° in the synthetic medium (20 ml) supplemented with 82.6 µM-thymidine to a cell density of 7×10^9 cells/ml. The internal pools were then labelled with [^{3}H]-thymidine (20 µCi/ml) for 30 min. at 37°. The cells were collected by centrifugation and resuspended in fresh synthetic medium (20 ml) containing unlabelled thymidine (4.16µM). Portions (5 ml) of the culture were irradiated with 1440 erg/mm^2 (expt. 1) and 3600 erg/mm^2 (expt. 2) of u.v. light, respectively and the remainder was the non-irradiated control. All three cultures were shaken (250 rpm) in the dark at 0° on a small gyratory shaker. Samples (1 ml) were withdrawn at the indicated times and added to 10 ml of 25% (w/v) NaCl-0.02M-KCN. The supernatant obtained after centrifugation at 45 000g_{max} for 5 min. was counted to measure the release of radioactivity into the extracellular fluid. The cell pellet was lysed in ice-cold 5% (w/v) trichloroacetic acid (3 ml) and the radioactivity of the acid-soluble and the acid-insoluble fractions was determined. The results show the distribution of radioactivity in the 1 ml samples.

Table 3.8. continued

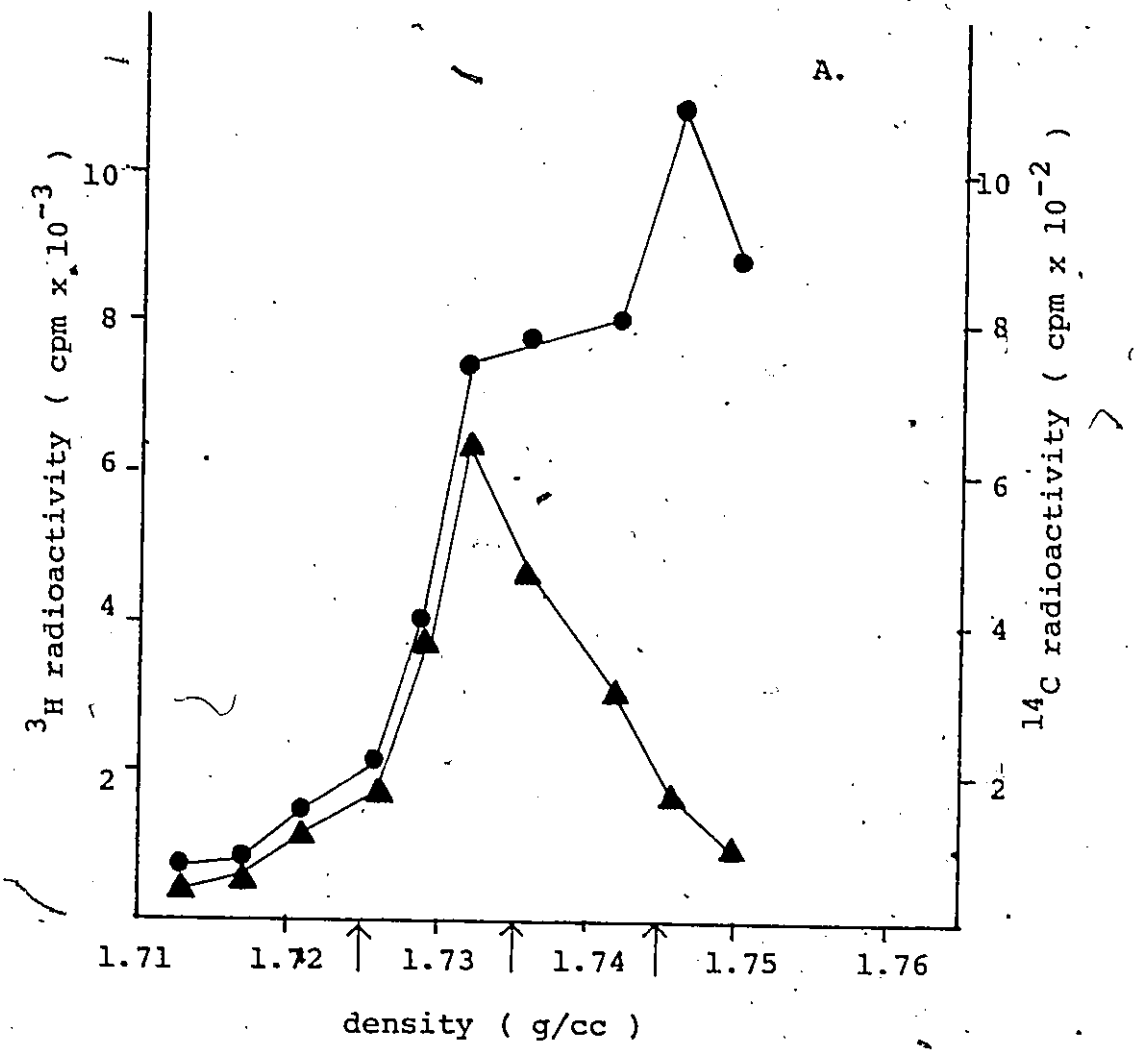
time (min)	radioactivity (cpm / ml x 10^{-4}) extracellular	acid-soluble	acid-insoluble	total
control				
0	3.7 (4.0)	58.2	31.5	93.4
10	4.4 (5.7)	40.0	32.1	76.6
25	3.5 (4.5)	50.4	22.9	76.8
expt. 1				
0	4.4 (5.7)	46.4	25.1	76.1
10	3.9 (5.2)	39.7	30.8	74.4
25	4.8 (5.2)	51.4	35.1	91.3
40	4.1 (4.5)	53.9	33.8	91.8
expt. 2				
0	4.6 (5.5)	43.8	34.3	82.7
10	5.0 (5.3)	60.9	28.0	93.9
25	4.8 (5.8)	51.2	26.3	82.3

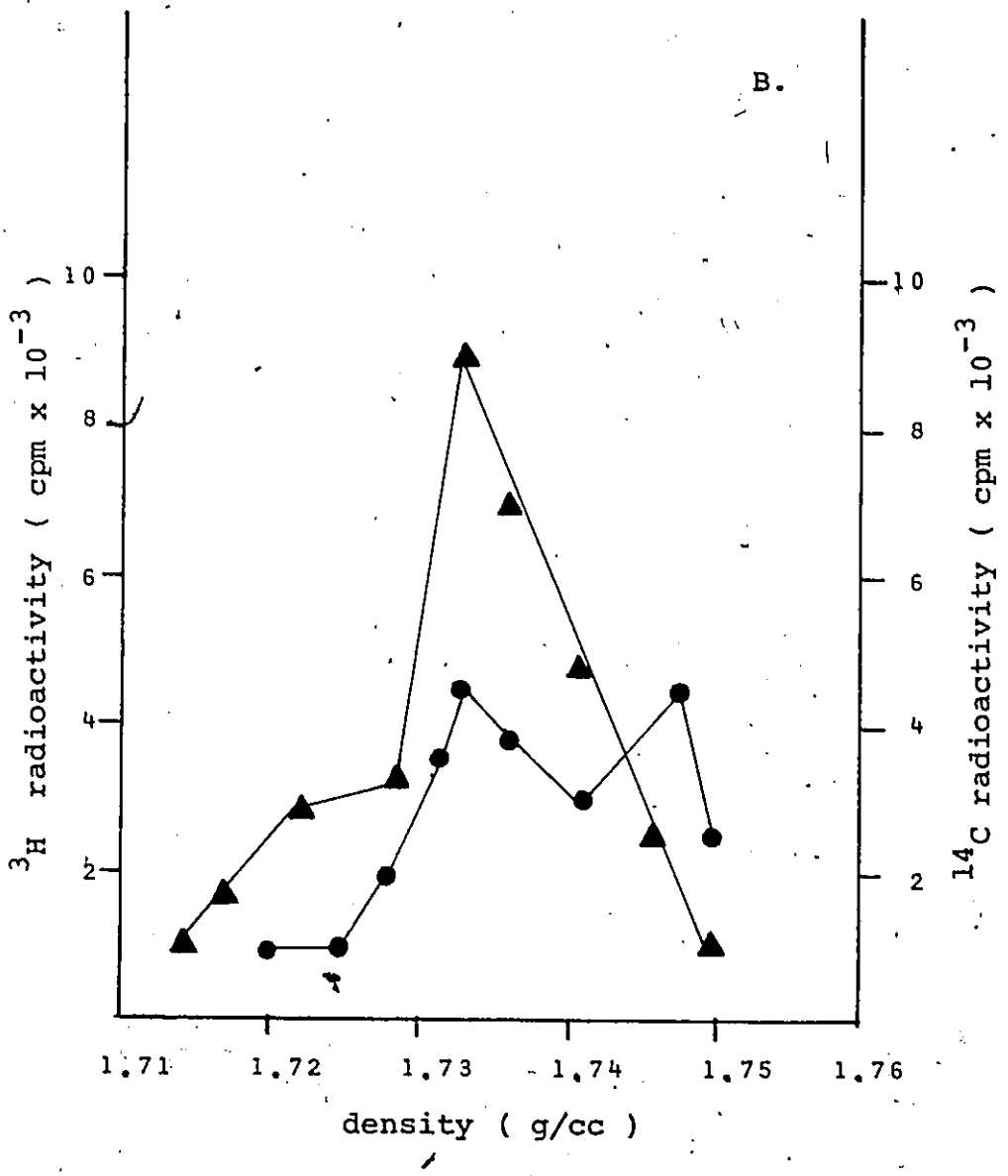
The values in brackets refer to the % of total radioactivity present in the extracellular fluid.

Fig. 3.15. Evidence for the lack of dark repair of DNA during normal growth of H.cutirubrum

The results show the distribution of $[^{3}H]$-5 - bromo-2'-deoxyuridine (●) and $[^{14}C]$ thymidine (▲) after: (a) 60 min, 0.125 generation, (b) 90 min, 0.188 generation in ^{14}C light thymidine containing medium.

The experiment was done as described in sec. 2.3.1. except that samples were withdrawn at 60 min and 90 min.


A.
^{3}H radioactivity (cpm x 10^{-3})
^{14}C radioactivity (cpm x 10^{-2})
10
8
6
4
2
1.71
1.72
1.73
1.74
1.75
1.76
density (g/cc)


B.
^{3}H radioactivity (cpm x 10^{-3})
^{14}C radioactivity (cpm x 10^{-3})
10
8
6
4
2
1.71
1.72
1.73
1.74
1.75
1.76
density (g/cc)

3.5. Incorporation into the intracellular nucleotide pools and DNA

3.5.a. 37° and 4.16 µM thymidine

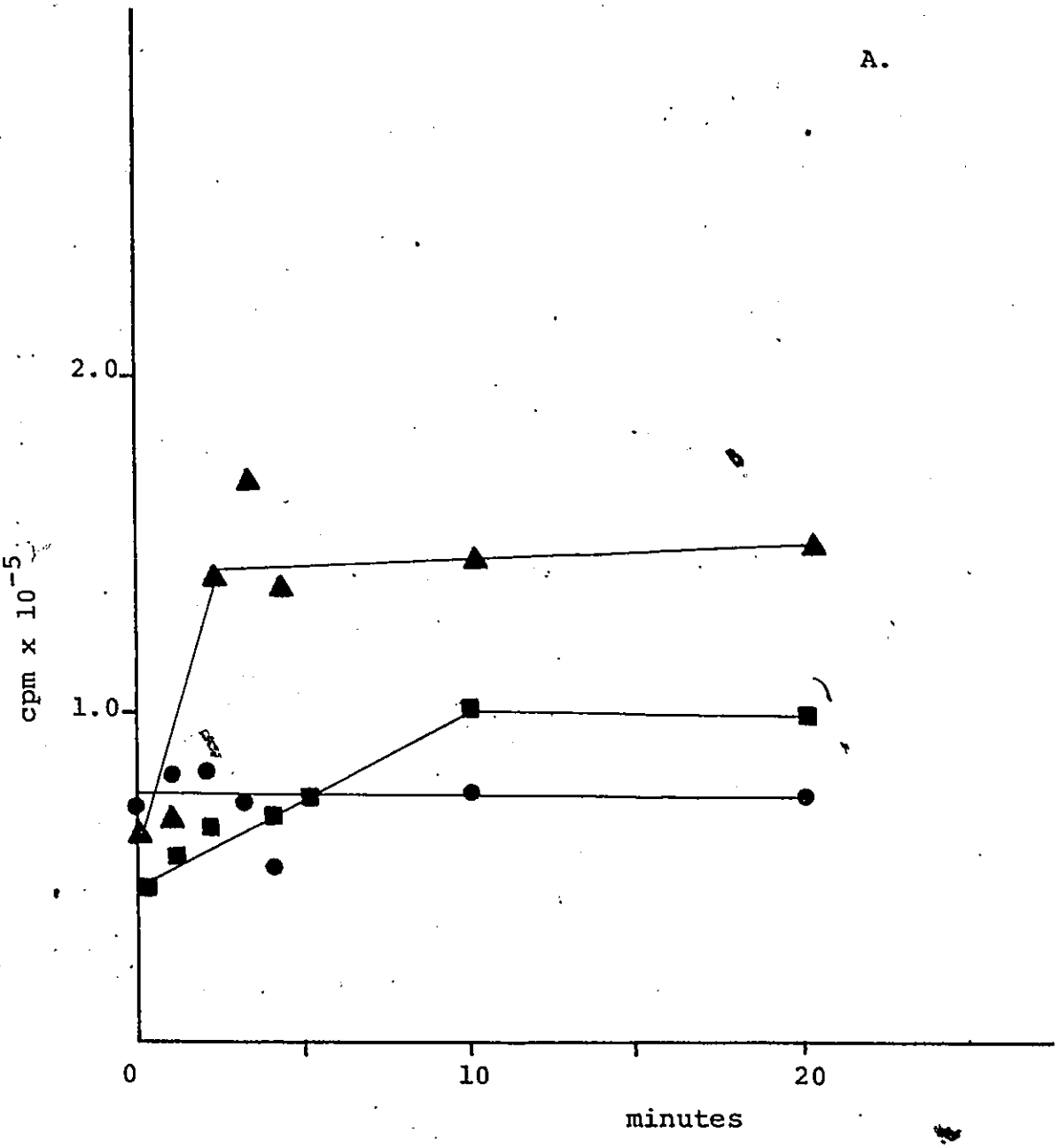
Fig. 3.16 shows the incorporation of extracellular [^{3}H]-thymidine into the intracellular dTMP, dTDP, dTTP pools and DNA of H.cutirubrum, followed during the first 20 min after addition of radioactive precursor to the culture medium. In this experiment the cells were grown in the presence of 82.6 µM thymidine for 42 hours, and the radioactive precursor was added immediately The maximum rate of incorporation into DNA was reached within one min, while maximum labelling of the thymidine nucleotides occured immediately for dTMP, and 10 min for dTDP, and 3 min for dTTP. Since maximum labelling of the immediate precursor should be attained at the same time or shortly before the maximum rate of entry of radioactivity into DNA, it was difficult to decide from this data what the precursor would be, since dTMP was labelled much too quickly, and dTDP and dTTP too late.

It should be emphasized that, both in these and other experiments, no other acid soluble radioactive compounds other than thymidine (and thymine), thymidine mono-, di-, and triphosphates were detected on the chromatograms, and the separations were sharp (see section 2.2.3.b.). Any other possible unknown precursor would have to have an R_f identical with that of one of the other radioactive components of the mixture.

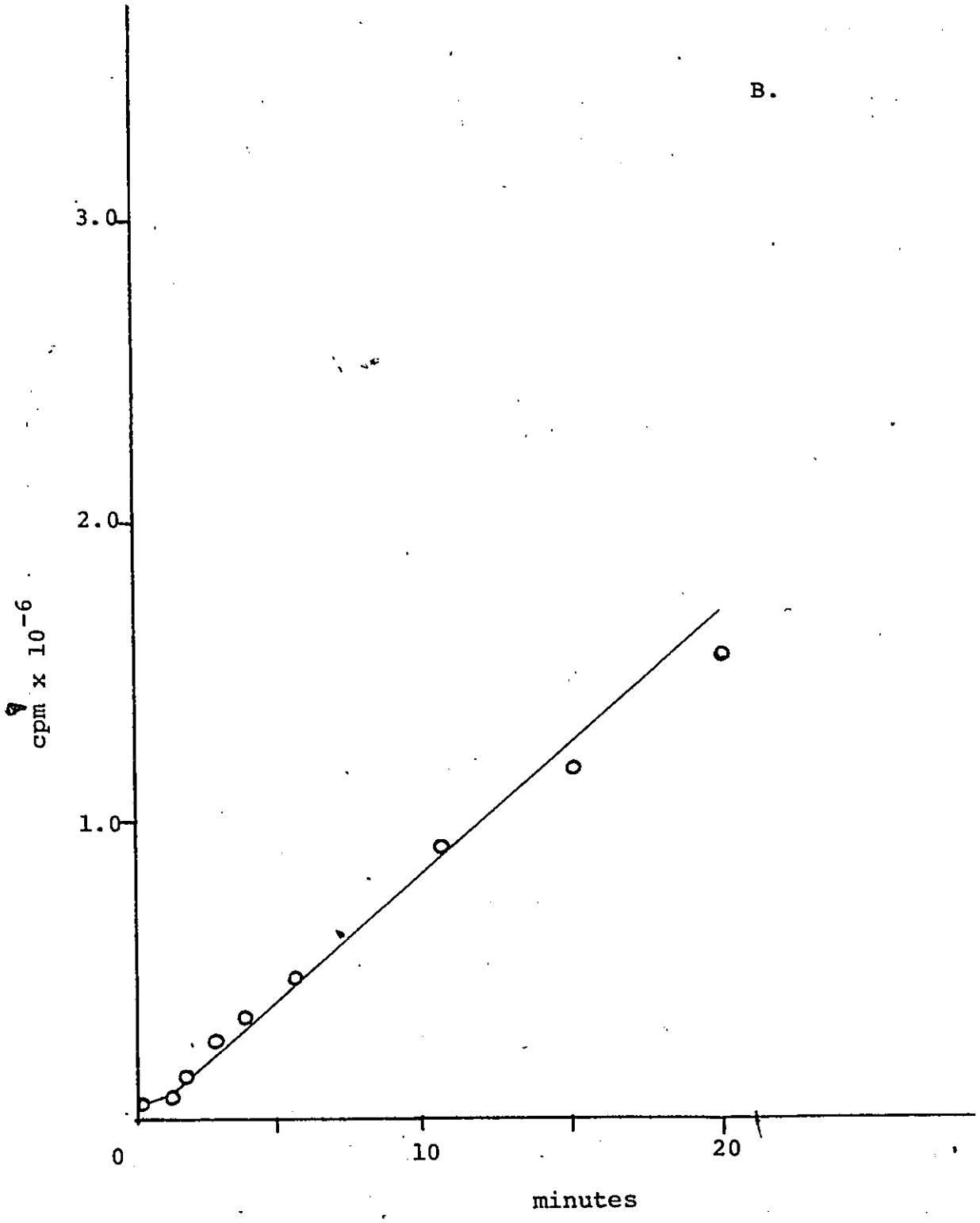
Fig. 3.16. Incorporation of [^{3}H]-thymidine into intracellular nucleotide pools and DNA of H.cutirubrum at 37°

H.cutirubrum was grown at 37° in the synthetic medium with 82.6 µM-thymidine. When the cell density was 1 x 10^9 cells/ml (approximately 40 hours), the radioactive precursor [Me-^{3}H]-thymidine was added as described in fig. 3.7., exp. 2. At this time the exogenous thymidine concentration is 4.16 µM. Samples (1 ml) were withdrawn at the indicated times and added to ice-cold 5% (w/v) trichloroacetic acid (5ml). The precipitate was collected by centrifugation and incorporation into DNA was determined as previously described in fig. 3.5. An aliquot (1 ml) of the supernatant was extracted twice with ether (2 ml); 40 - 60 µl were then applied to a PEI-cellulose t.l.c. sheet and chromatographed as described in the experimental section. The nucleotide spots were identified by comparison with standards, cut out and counted for radioactivity. Incorporation of ^{3}H into dTMP, ● ; dTDP, ■ ; dTTP, ▲ ; is shown in fig. 16.a., and into DNA in fig. 16.b.

A.



B.



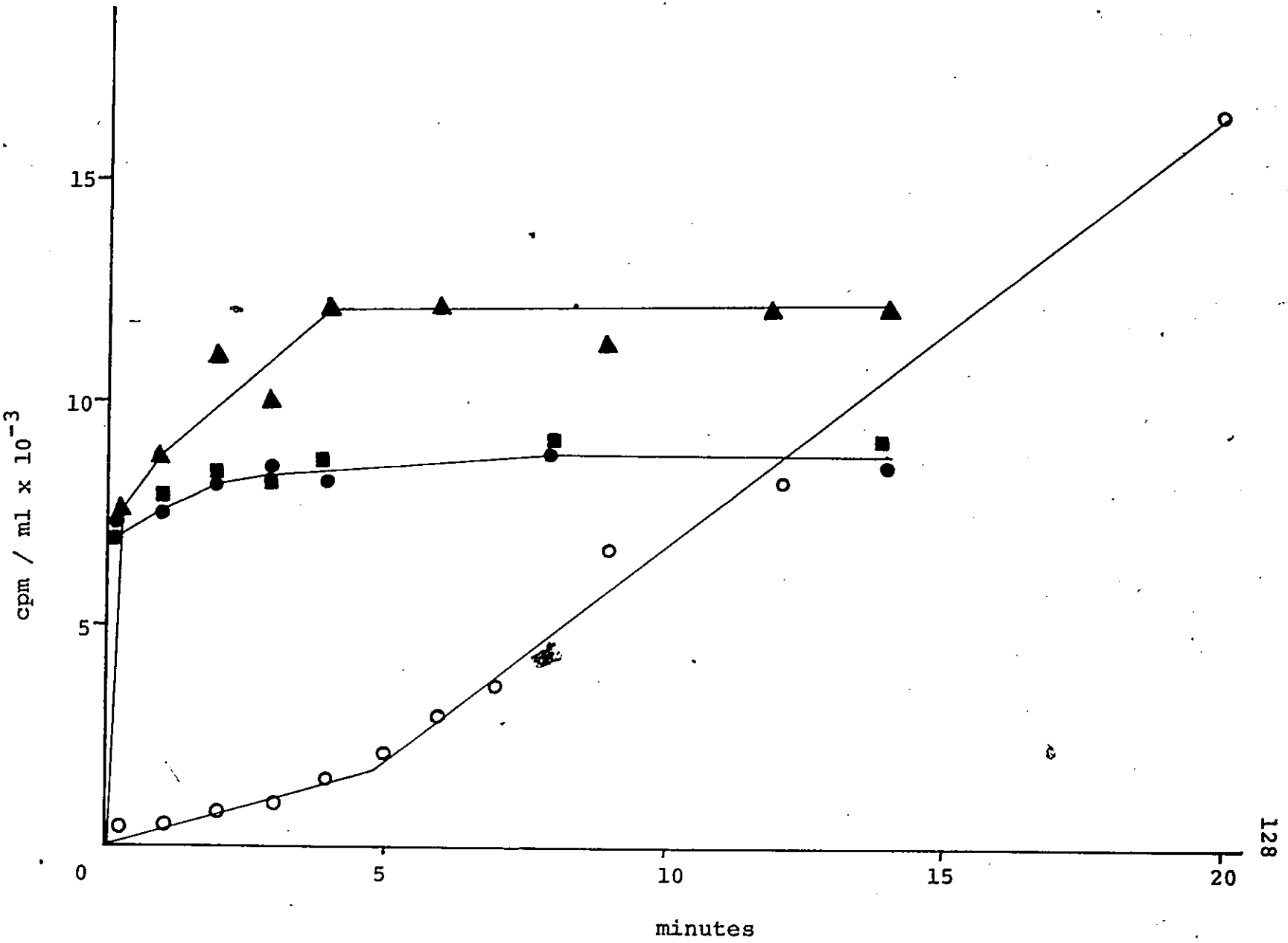
One major criticism of this experiment was the presence of a large excess of thymine during the experiment, since the cells were not spun down and not resuspended in fresh medium. The cells were grown in the presence of thymidine at 82.6 µM, and at the start of the incorporation experiments only 5%(4.16 µM) remained (see table 3.5., section 3.2.2.). To avoid any complicatons that could arise because of the presence of thymine, the experiments were repeated with the cells resuspended in fresh medium and at the original concentration of 82.6 µM thymidine.

3.5.b. 37^{o} and 82.6 µM thymidine

The results in fig. 3.17. describe an incorporation experiment done at 37^{o} and with the extracellular thymidine concentration 82.6 µM instead of 4.16 µM. In this experiment the cells were grown in the presence of 82.6µM thymidine. At 27 hours the cells were harvested and resuspended in prewarmed 37^{o} synthetic medium, pH 7.1, containing 82.6 µM thymidine. After 30 min $[Me\text{-}^{3}H]$-thymidine was added, and its incorporation into the intracellular dTMP, dTDP, dTTP pools and DNA, was followed for the first 20 min. The resuspension in fresh medium eliminated the presence of thymine in the incorporation experiment. It can be seen that the maximum rate of incorporation of radioactivity into DNA was reached at approximately 5 min after the addition of radioactive thymidine, while maxi-

Fig. 3.17. Incorporation of ^{3}H-labelled thymidine into the nucleotide pools and DNA of H.cutirubrum growing at 37°

H.cutirubrum was grown at 37° in the synthetic medium supplemented with 82.6 µM-thymidine and transferred to fresh medium containing [Me-^{3}H]-thymidine as described in fig. 3.5., expt.1. After addition of the radioactive precursor, samples (1 ml) were withdrawn at the indicated times and added to ice-cold 5% (w/v) trichloroacetic acid (5 ml). The incorporation of [^{3}H]-thymidine into DNA and nucleotide pools is done as described in fig. 3.16. Incorporation of ^{3}H into: dTMP, ● ; dTDP, ■ ; dTTP, ▲ ; DNA, o .


15
10
5
0
5
10
15
20
cpm / ml x 10^{-3}
minutes
128

mum labelling of dTMP and dTDP occurred after 0.5 - 1 min, and dTTP after 4 min. These results clearly suggest that only dTTP could be the intermediate concerned.

3.5.c. Growth of H.cutirubrum at lower temperatures

In order to determine the rate of disappearance of radioactivity from prelabelled pools and its simultaneous appearance into DNA after transfer of the cells to non-radioactive medium, it was necessary to work at a lower temperature (section 3.5.e.). Fig. 3.18. shows the growth curves of H.cutirubrum in synthetic medium done at the three different temperatures 37^{o}, 22^{o}, and 20^{o}. The generation times calculated from these curves are given in table 3.9. Since at 22^{o} the growth rate was halved, this temperature was chosen for the subsequent experiment. At this temperature, the maximum rate of thymidine incorporation was also approximately halved. A further reduction in temperature from 22^{o} to 20^{o} caused a further doubling of the generation time.

3.5.d. 22^{o} and 82.6 µM thymidine

Fig. 3.19. describes an experiment similar to that in section 3.5.b., but done at 22^{o}. At this lower temperature, the rate of incorporation of $[^{3}H]$-thymidine into DNA reached a maximum at 10 min and the pools of dTMP and dTDP at 6 - 8

Fig. 3.18. Growth curves of H.cutirubrum in synthetic medium of Grey & Fitt (1976) at 37° (□), 22° (Δ), 20° (o)

———————— O.D.$_{660nm}$

---------- viable cell count

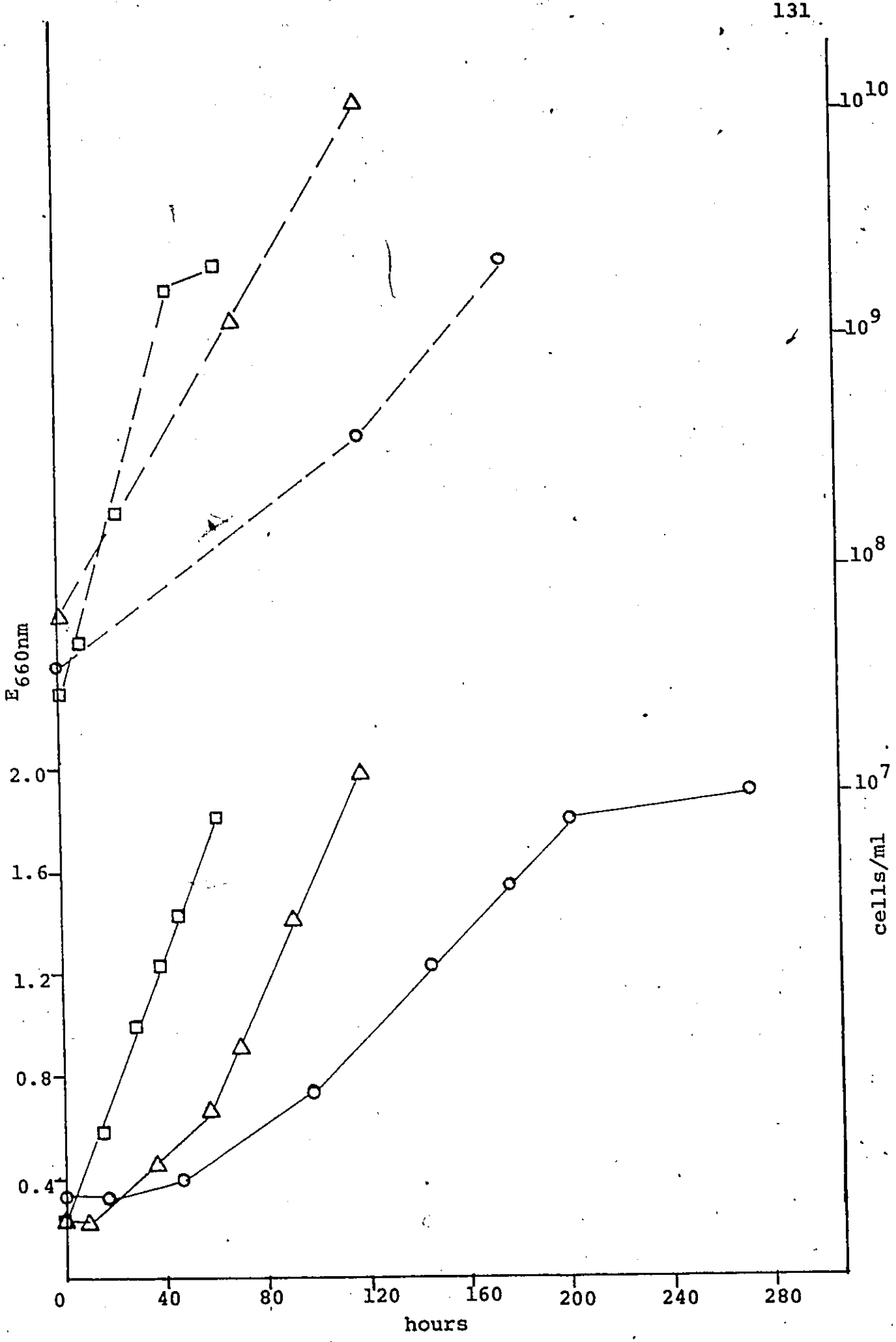

E_{660nm}
2.0
1.6
1.2
0.8
0.4
0
40
80
120
160
200
240
280
hours
10^{10}
10^{9}
10^{8}
10^{7}
cells/ml

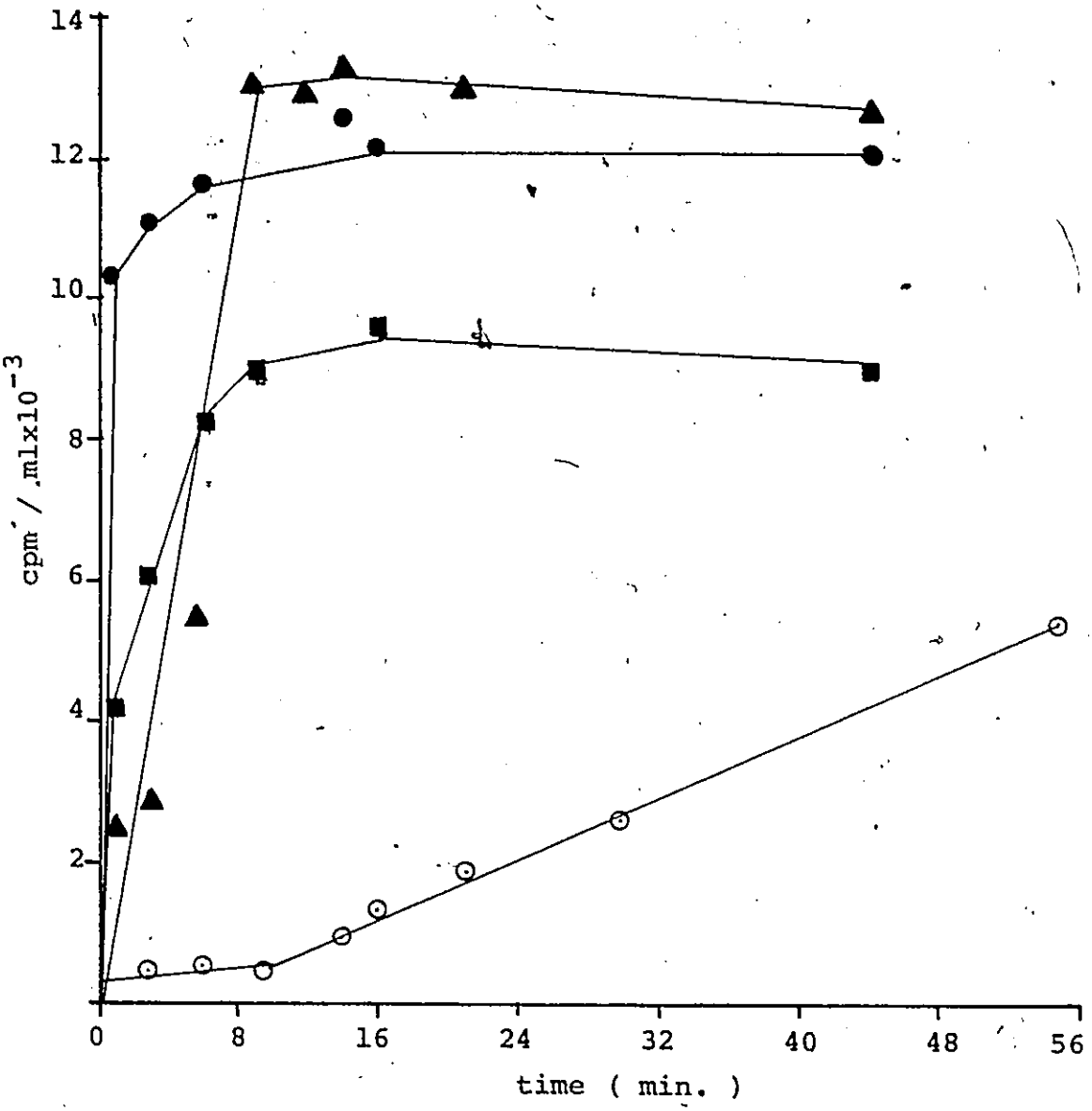
Table 3.9. Effect of temperature on the generation time of H.cutirubrum and on its rate of incorporation of $[^3H]$-thymidine into DNA

Generation times were calculated from growth curves obtained by turbidimetry and viable cell counts (fig. 3.18.). The incorporation of ^3H-labelled thymidine into DNA was measured as described in fig. 3.5.

growth temperature (^oC)	generation time (h)	$[^3H]$-thymidine incorporation (nmol/cell/min)
20	33	-
22	16	0.7×10^{-11}
37	8	1.7×10^{-11}

Fig. 3.19. Incorporation of ^{3}H-labelled thymidine into the nucleotide pools and DNA of H.cutirubrum growing at 22°

The experiment was done as described in fig. 3.17, except that incubation was at 22°. Incorporation of ^{3}H into: dTMP, ● ; dTDP, ■ ; dTTP, ▲ ; DNA, O .


14
12
10
8
6
4
2
cpm / ml x10^{-3}
0
8
16
24
32
40
48
56
time (min.)

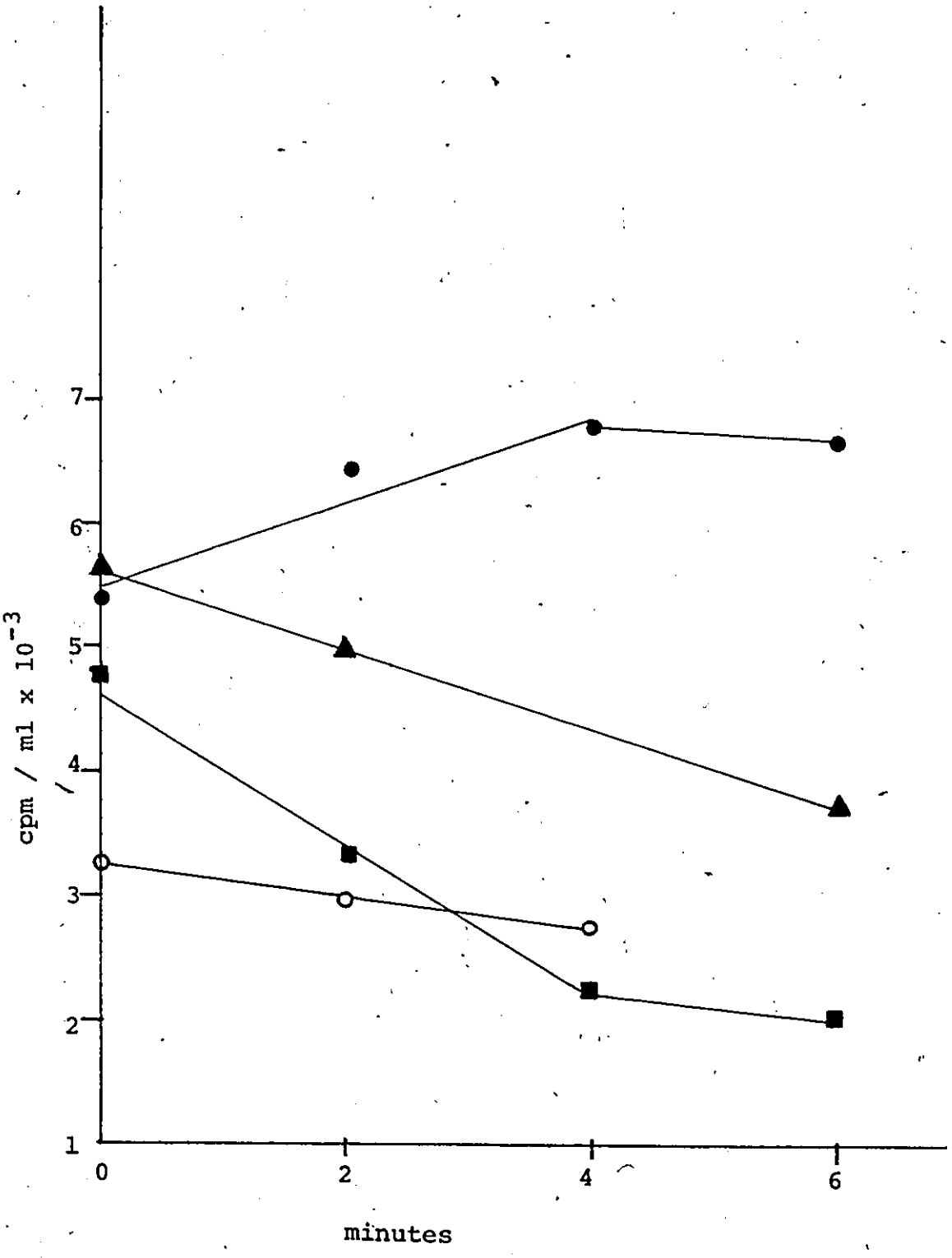
min, and dTTP at 9 min. Thus at lower temperatures, the maxima for dTMP and dTDP were not as clearly defined as at 37°. The results point however to dTTP being the immediate precursor.

3.5.e. Fall of radioactivity at 22°

The disappearance of radioactivity from prelabelled pools and its simultaneous appearance in DNA after transfer of the cells to non-radioactive medium, is shown in fig. 3.20. This experiment is similar to that described by Fridland (1973) and was performed at 22°. This temperature was chosen because the total time required for harvesting and resuspension of the cells in non-radioactive medium was 10 min. If the experiment were done at 37°, the rapid decline in the radioactivity in the pools could not have been measured. It can be seen in fig. 3.20. that rapid incorporation of radioactivity continued for about 4 min after transfer of cells to the medium containing non-radioactive thymidine: the rate of this reaction was similar to that observed in the radioactive medium prior to centrifugation of the cells. The rate of loss of radioactivity from the nucleotide pools and its simultaneous uptake into DNA was calculated from the first 4 min after the transfer into the non-radioactive medium, and these results are given in table 3.10. Only the loss of radioactivity from the dTTP pool compares with the rate of uptake into DNA. The radioactivity of

Fig. 3.20. Loss of radioactivity from prelabelled nucleotide pools and its incorporation into DNA in H.cutirubrum during growth in non-radioactive medium

H.cutirubrum was grown at 22° and labelled with Me-^{3}H -thymidine as for the experiments in fig. 3.19. After 45 min, a sample (10 ml) of the culture was centrifuged at 22° and 27 000g_{max} for 2 min. The bacteria were then resuspended in fresh preincubated medium (pH 7.1) containing non-radioactive thymidine (82.6 µM). The total time required for harvesting and resuspension of the cells was 10 min. Samples (1 ml) were withdrawn immediately after mixing and at the indicated times during subsequent incubation at 22°. The radioactivity present in the nucleotide pools and DNA was measured as described in fig. 3.5. Radioactivity in: dTMP, ○ ; dTDP, ■ ; dTTP, ▲ ; DNA, ● .


7
6
5
4
3
2
1
cpm / ml x 10^{-3}
0
2
4
6
minutes

the dTMP pool declined rapidly during the processing of the culture, and during the period of measurement a steady fall of 250 dpm/min/cell continued. In contrast, the fall in radioactivity of the dTDP and dTTP pools was rapid, while linear incorporation into DNA still continued. The loss in radioactivity from the dTDP pool was approximately three times the rate of incorporation into DNA.

Since elimination of radioactivity should be slowest from the pool closest to the final product and fastest from the one furthest from it during the non-radioactive "chase", these results are consistent with the radioactivity passing through the nucleotide pools and into DNA in the sequence:

thymidine $\longrightarrow$ dTMP $\longrightarrow$ dTDP $\longrightarrow$ dTTP $\longrightarrow$ DNA

The close correspondence in the rates of loss of label from dTTP, and its entry into DNA makes it unlikely that any other precursor could be involved apart from some transient enzyme bound intermediate.

The sequence shown above implies that free thymidine is present in the cells. The results in fig. 3.21. show that H.cutirubrum growing at 22° in the synthetic medium supplemented with 82.6 µM thymidine does indeed contain a small pool of the deoxynucleoside, whose activity reaches its maximum value within 2 - 5 min after addition of the $[^3H]$-thymidine to the culture. Thymine is also found in the cells but only after

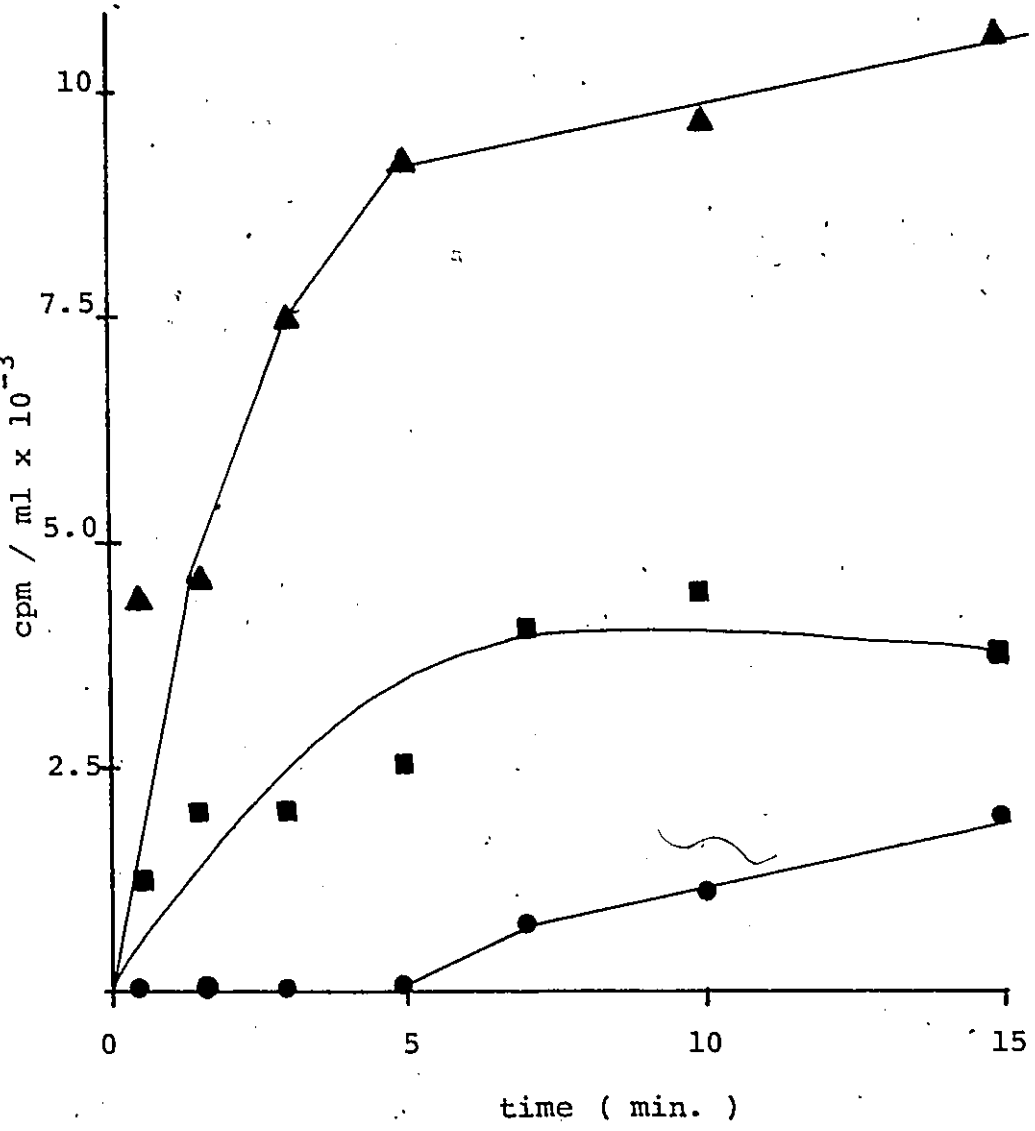
Table 3.10. Rate of loss of radioactivity from prelabelled nucleotide pools and its appearance in DNA during growth in non-radioactive medium

The rates of loss of radioactivity from the prelabelled pools and of its entry into DNA during the first 4 min. after transfer of the cells to the non-radioactive medium were calculated from the results in fig. 3.20.

compound	d.p.m./min/ml
dTMP	250
dTDP	2165
dTTP	825
DNA	750

Fig. 3.21. Evidence for the entry of free thymidine into H.cutirubrum growing in its presence

H. cutirubrum was grown at 22° in the presence of 82.6µM thymidine and transferred to fresh medium containing ^{3}H-labelled thymidine as in the experiment described in fig. 3.17. After the addition of the radioactivity (20µCi/ml) samples were withdrawn at the indicated times and mixed with ice-cold 25% (w/v) NaCl-0.02M-KCN (10 ml). The cells were collected by centrifugation at 4° and 45 000g_{max} for 2 min, washed once with 25%(w/v) NaCl (10 ml) and lysed in ice-cold 5%(w/v) trichloroaceti acid (1ml). The supernatant was extracted twice with ether (2 ml) and portions (10µl) were chromatographed (i) on PEI-cellulose t.l.c. sheets to measure the radioactivity into dTMP (see fig. 3.16) and (ii) on cellulose t.l.c. sheets (sec. 2.2.3.a.) to determine the incorporation into intracellular thymine and thymidine. Radioactivity in : thymine, ● ; thymidine, ■ ; dTMP, ▲ .


10
7.5
5.0
2.5
cpm / ml x 10^-3
0
5
10
15
time (min.)

about 5 min; this is consistent with the evidence that the thymidine phosphorylase responsible for the formation of the thymine from thymidine is either in the extracellular fluid or very closely associated with the cell. The thymine would thus be released and then enter the bacterium instead of being produced within the cell.

4. CONCLUSIONS

4.1. Dark repair and photoreactivation

The data presented here show clearly that H.cutirubrum NRC 34001 is not capable of performing dark repair of DNA following u.v. irradiation. The reasons for the lack of dark repair need further investigation, but it is possible that the lack of dark repair is due to the absence of a u.v. endonuclease. Preliminary results have detected the presence of nascent short chains, Okazaki pieces, in H.cutirubrum (Grey & Fitt, unpublished results), indicating that there must be ligases present to join these pieces. Also an in vitro replication system for DNA, capable of utilising dTTP as substrate, was developed (Barua & Fitt, unpublished results), implying that the organism must have a DNA polymerase. Unless a specific repair polymerase was required, this suggests that the missing enzyme in H.cutirubrum is most likely a u.v. endonuclease. Preliminary data have indicated this, but it requires further confirmation.

This organism appears to rely solely on photoreactivation for its repair of damaged DNA. Because photoreactivation is specific for the repair of pyrimidine dimers, this result implies that only this type of lesion could be corrected by the bacteria. The efficient photoreactivating system found in this organism suggests that it may offer unique advantages for a study of this mechanism. In its natural habitat, H.cutirubrum

is exposed to intense sunlight and it is possible that selection may have occured for a cell able to use the energy of incident light to repair damage caused by the latter. As pointed out by Hescox and Carlberg (1972), there has been no definitive study of repair of u.v. damaged DNA in bacteria exposed to u.v. irradiation in their natural environment. The photoreactivating enzymes for E.coli, K12 and B strain (bacteria hardly exposed to u.v. light in their natural environment), have only been recently purified (Sutherland & Chamberlain, 1973) in spite of interest in the mechanism, because of the small number of molecules per cell.

The nature of the chromophore involved in photoreactivation, and the mechanism of its utilization is not known. In H.cutirubrum the pigment has been implicated as a possible chromophore (Hescox & Carlberg, 1972). It is known that the purple membrane of H.halobium is capable of converting absorbed light energy into a proton gradient which in turn is utilized for ATP synthesis (Oesterhelt & Stoeckenius, 1973). This purple membrane has also been identified in H.cutirubrum grown anaerobically and in the presence of glycerol (Gochnauer et al., 1972). Further studies on the role of the pigment in photoreactivation are needed to elucidate its possible role as a chromophore in this process. A comparison of the efficiency of photoreactivation under different growth conditions, and

also of different halophiles, and other carotenoid pigmented bacteria, would also be interesting.

H.cutirubrum replicates by the semi-conservative mechanism of Meselson and Stahl and no repair replication is detectable during growth. The absence of dark repair synthesis during growth and also following u.v. irradiation indicates that H.cutirubrum offers a uniquely favorable system in which to analyse the detailed mechanism of DNA replication. The wild type bacteria generally used for the study of DNA synthesis, namely E.coli and B.subtilis, have the complications of repair synthesis. The absence of dark repair has also been reported in mammalian mitochondrial DNA (Clayton et al., 1974). This system has also been used to study replication (Berk & Clayton, 1973), but the extent to which this resembles the replication of nuclear DNA is uncertain. Nuclear and chloroplast DNA of Chlamydomonas reinhardti (Swinton & Hanawalt, 1973) are also deficient in DNA dark repair synthesis, but this system is not simple relative to the prokaryote since in the latter only one chromosome is involved.

4.2. The precursors of DNA replication

The results in section 3.5., clearly indicate that dTTP is the precursor of DNA biosynthesis Since repair biosynthesis does not take place in this organism, it can be concluded that dTTP and by extension the other deoxynucleoside triphosphates

are the precursors of DNA replication *in vivo*. This has been suggested before and generally believed to be the case, but no conclusive evidence has so far been put forward in support of this. This conclusion is in agreement with the fact that all three DNA polymerases isolated so far, and capable of utilising deoxynucleoside triphosphates , are believed to play a role in replication.

The data (section 3.5.e.) also suggest that *H.cutirubrum* synthesizes dTTP by an endogenous pathway similar to that of *E.coli*, namely:

thymidine⟶dTMP⟶dTDP⟶dTTP

Studies in our laboratory (Peterkin & Fitt, unpublished results), have shown that all three kinases (thymidine, thymidylate and nucleoside-diphosphate kinases) are present in extracts of *H.cutirubrum*.

4.3. Metabolism of exogenous thymine and thymidine and thymidylic acid

The overall picture for the metabolism of thymine, thymidine and thymidylic acid resembles that of the widely used bacteria *E.coli*. Exogenous thymine is incorporated into DNA only in the presence of deoxyadenosine (sec. 3.2.c.). Our data suggests, however, that thymine and deoxyadenosine is not as readily utilised as thymidine, since in the former incorporation takes place after a 1 hour lag phase. The incorporation of

thymidine occurs after only a 5 min lag. This is not the case for E.coli where incorporation is detectable within 1 min of addition of the deoxynucleoside.

Thymidine is rapidly incorporated into DNA, but the length of the incorporation is dependant on the exogenous thymidine concentration. This is because of the presence of thymidine phosphorylase that degrades thymidine.

$$\text{thymidine} \xrightarrow{Pi} \text{thymine} + \text{deoxyribose 1-phosphate}$$

The utilisation of thymidine for DNA by H.cutirubrum, and its breakdown to thymine occurs by reactions similar to that of E.coli as evident by the finding of similar enzymes. However the data here seems to suggest that the H.cutirubrum thymidine phosphorylase differs from that of E.coli in two ways: (a) It is constitutive. The E.coli enzyme is inducible in wild type cells. (b) It is readily released from cells. The E.coli enzyme is shock-releasable, suggesting that it is more tightly bound (Hochstadt, 1974).

The other enzymes involved in thymidine metabolism, namely thymidine kinase and thymidine permease, appear to be constitutive. H.cutirubrum thymidine kinase is also capable of utilising the substituted thymidine analog 5-bromo-deoxyuridine, though not as efficiently.

Nucleotides are first dephosphorylated prior to entry into the cells (see table 3.1.).

4.4. Synthetic medium

A synthetic medium is described in which H.cutirubrum grows as well as in the complex medium of Gochnauer and Kushner (1969). In this medium only L-amino acids are used and it is suggested that this eliminates the need for NH_4Cl, since as pointed out by Onishi and Gibbons (1965), the inorganic ammonium ion requirement in these organisms is related to the utilization of amino acids. The medium also does not contain nucleotides; it was shown that these are readily dephosphorylated prior to entry into the cell. The addition of nucleosides to the growth medium was not stimulatory, but increasing the Pi concentration gave better growth than that obtained in the medium of Onishi et al. (1965).

4.5. Concluding remarks

The organism offers a good simple system for studying the mechanism of DNA replication. However, because of the high intracellular ionic strength in H.cutirubrum the detailed enzymology (e.g. size of enzyme, cofactor requirements) of replication may differ from that of non-halophiles but the mechanism of action should be the same. This was found to be the case with other enzymes of halophilic nucleic acid enzymology. DNA dependant RNA polymerase isolated from this organism (Louis & Fitt, 1972) has a molecular weight of 36,000 less than a tenth that of E.coli polymerase but functionally

resembling normal bacterial polymerase. A polynucleotide phosphorylase isolated from this organism (Peterkin & Fitt, 1971) is also functionally typical of other polynucleotide phosphorylases. The studies on RNA (Peterkin, Barua & Fitt, unpublished results) have also led to the isolation of the nucleoid or folded chromosome from H.cutirubrum. This folded chromosome is a potentially good template for DNA synthesis (section 1.2.g.), and together with the in vitro system developed for the synthesis of DNA in this bacterium (Barua & Fitt, unpublished results), should be potentially useful in studying the mechanism of DNA replication.

H.cutirubrum would also provide a useful tool for the study of the mechanism of photoreactivation. Not only is the photoreactivation process more efficient, but the role of the carotenoid pigment in the process needs further investigation. The organism could be useful for a study of the interaction of the chromophore, the photoreactivating enzyme, and the thymine dimer.

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