EFFECTS OF SOME RADIOMIMETIC CHEMICALS ON *EUGLENA* AND *E. COLI*

SOME EFFECTS OF SOME RADIOMIMETIC CHEMICALS ON *EUGLENA GRACILIS* AND ON *ESCHERICHIA COLI*

By

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TITLE: Some Effects of Some Radiomimetic Chemicals on Euglena gracilis and on Escherichia coli

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SCOPE AND CONTENTS: Studies are described which show that modest doses of 5-nitrofurans, nitrosoguanidine and N-methyl-N-nitroso-p-toluenesulfonamide inhibit the synthesis of proteins and nucleic acids in Euglena. These results are discussed in terms of the "bleaching" and inhibition of growth caused by these compounds. Experiments with N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide show that chloroplast DNA is not specifically labelled. This result is discussed in terms of a proposed molecular mechanism of bleaching.

Studies on the effects of these agents on bacteria have shown that the basis for the bacteriostatic properties of some 5-nitrofurans is more likely inhibition of macromolecule synthesis rather than inhibition of respiration. Experiments are presented which show that nitrosoguanidine induces prophage development in lysogenic bacteria and inhibits the synthesis of macromolecules in E. coli B.

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HISTORICAL INTRODUCTION

I. Some Biological and Chemical Effects of Radiomimetic Agents

Radiomimetic Agents

Muller's observation in 1927 that X-rays could cause mutation in *Drosophila melanogaster* (fruit flies) (1) stimulated a continuing investigation of the molecular consequences of irradiation of biological systems. Irradiation of cells with ionizing radiation is characterized by the production of ions and radicals along the track of the radiation with resulting gross structural changes in the cell. That less energetic treatment can lead to mutation was indicated in 1933 by the finding that ultraviolet light, a non-ionizing radiation, causes mutations in *Drosophila* (2).

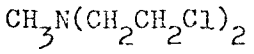
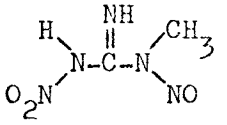
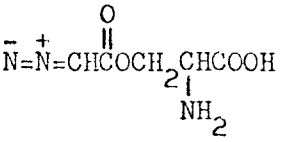
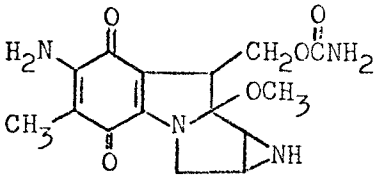
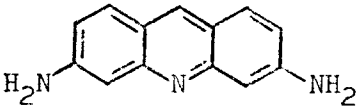
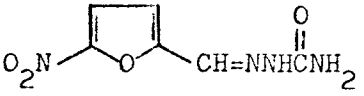
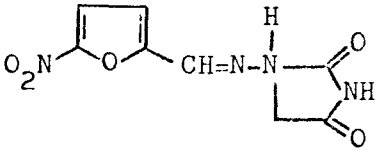
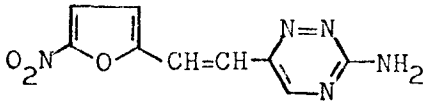
The possibility of producing mutations with chemicals had been extensively explored by numerous workers both before and after the discovery of the mutagenic action of radiation. Thom and Steinberg discovered in 1939 that the exchange of nitrite for nitrate in an acidic growth medium for *Aspergillus niger* resulted in the production of stable mutant strains of the mold (3). While these authors suggested that the effect of nitrite was due to "a reduction in free amino nitrogen of some protein of the mold" it is now apparent that nitrous acid was reacting with DNA. In 1946 Auerbach and Robson reported results of wartime research which established that bis-(2-chloroethyl)-

sulfide (mustard gas) is as effective as radiation in inducing mutations in fruit flies (4). Subsequent work by these and other investigators has revealed that a great variety of chemical mutagens are effective in many organisms. Since many of these chemicals share with radiations the properties of breaking chromosomes and inhibiting cell division, the adjective "radiomimetic" has been applied to them (5,6). The list of such agents continues to grow. For example, Sax and Sax have recently observed that chromosome aberrations, grossly comparable to those induced by ionizing radiation, are produced in onion root tip cells by common beverages including alcohol, coffee, tea and cocoa (7).

A more precise definition of "radiomimetic" agents may be based on the observation that certain single step bacterial mutants which are resistant to radiation are cross resistant to chemicals such as nitrogen mustard. In 1946 Witkin isolated a stable mutant (strain B/r) of Escherichia coli B which is resistant to ultraviolet light and to X-rays (8). Two years later Bryson reported the stepwise isolation of other strains of E. coli resistant not only to nitrogen mustard but also to the lethal effects of ultraviolet light (9). In addition, Bryson found that the radiation-resistant strain, E. coli B/r, was resistant to nitrogen mustard. Subsequent studies by a number of workers have shown that radiation-resistant bacteria are cross-resistant to a variety of chemicals and on this basis each of these compounds may be considered radiomimetic. (Some radiomimetic chemicals are listed in Table I.) The fact that resistance to both radiation and to these chemicals may be acquired in a single mutational event implies that each of these agents

TABLE I

Some Radiomimetic Chemicals

Trivial Name	Structure	Reference
Nitrogen mustards		9
Nitrosoguanidine		10
Azaserine		10 & 11
Mitomycin C		12
Proflavine		13
Nitrofurazone		14
Nitrofurantoin		15
Nitrofurylvinyl triazine (NFT)		15

damages a common "target" or possesses some common features in its action on the bacterial cell.

After a consideration of some of the biological effects of radiation and of radiomimetic chemicals, current views of the chemical events which produce these biological effects will be presented.

Specific Biological Effects

Radiation and radiomimetic chemicals are lethal for a variety of microorganisms and, in fact, lethality is often the major effect. Frequently, modest sub-lethal doses are effective in producing mutations which can be detected by appropriate selective techniques. In many cases, microscopic examination of treated cells reveals broken chromosomes. At the molecular level, the inactivation of transforming DNA *in vitro* by radiation and radiomimetic chemicals indicates that these agents act directly on DNA. It is interesting to note in this respect that some radiomimetic agents inactivate transforming DNA *in vivo* but *in vitro* only after chemical modification. For example, while Mitomycin C does not react directly with DNA, the product formed upon chemical reduction of the quinone portion of Mitomycin C is capable of inactivating transforming DNA *in vitro* (16).

The ability to induce prophage development in lysogenic bacteria is another biological effect of many radiomimetic agents. While infection of a bacterium by a virulent bacteriophage leads to cell death and liberation of newly formed phage particles, infection by a temperate phage produces a lysogenic bacterium which harbours viral DNA in association with the bacterial chromosome. The viral DNA is replicated

each time the bacterial chromosome is replicated. This relatively stable arrangement can be upset by treatment of lysogenic bacteria with ultraviolet radiation or any one of a variety of chemicals. Phage development begins as viral DNA takes over the control of the synthesis of macromolecules in the bacterium, directing the synthesis of new phage DNA, phage-specific messenger RNA and phage proteins. After about 90 minutes the bacterial cell wall is lysed by lysozyme (an enzyme synthesized as a consequence of induction) and the mature virus particles are released (17).

The molecular details of the induction process are not yet clear. It can be said, however, that inducing agents in general have an effect on DNA; on DNA metabolism (18) or cause the formation of abnormal DNA (19).

Chemical Effects of Radiation and Radiomimetic Agents

(i) Ultraviolet Radiation

The molecular consequences of ultraviolet irradiation of DNA and of organisms, particularly bacteria, have been intensively studied during the past few years. Production of intrastand dimers between adjacent pyrimidines in DNA is thought to account for an important fraction of the biological effects of ultraviolet light (see review by Setlow (20)). The relative efficiency of production of dimers is $\hat{T}\hat{T} > \hat{C}\hat{T} > \hat{C}\hat{C}$ (21); however, the proportion of each type of dimer in a given DNA depends upon the base composition of the DNA. An investigation of the products formed upon ultraviolet irradiation of pyrimidine diribonucleoside monophosphates in solution has shown that thymine

dimers (22) and uracil dimers (23) are stable while dimers containing cytosine are not (24). Loss of the 5, 6 double bond of cytosine causes spontaneous deamination to uracil. Thus, cytosine dimers are converted to uracil dimers (via cytosine-uracil dimers) and cytosine-thymine dimers to uracil-thymine dimers. The photochemical dimerization reaction is reversible,

$$- \text{Py Py}^{1} - \rightleftharpoons - \overset{\frown}{\text{Py Py}}^{1} -$$

the equilibrium constant depending upon the wavelength of the ultraviolet light used.

Pyrimidine dimers can be removed from DNA by at least two biological processes. In many organisms (most bacteria and yeast) they can be enzymatically split to monomers in the presence of visible light (photoreactivation). It should be noted that deamination of a cytosine-containing dimer to a uracil-containing dimer, followed by "repair" via photoreactivation leads, upon replication of the DNA, to a transition from a C-G pair to an A-T pair. A different type of repair mechanism, which does not require light (dark repair), involves the excision of an oligonucleotide containing the pyrimidine dimer from the strand of DNA, leaving a portion of the DNA molecule single stranded. The nucleotide sequence of the single-strand provides the information for the enzymatic insertion of the appropriate nucleotides into the broken chain so that the original double helix is restored.

Some ultraviolet-resistant strains of bacteria are resistant not because fewer pyrimidine dimers are formed upon irradiation, but because the resistant strains possess a more efficient repair mechanism

than do sensitive strains. On the other hand, strains B and B/r are able to excise pyrimidine dimers with equal efficiency, while strain B/r shows higher colony survival. While the basis for this difference in colony-forming ability has yet to be elucidated, it has been shown that ultraviolet irradiation of strain B results in the formation of long filaments instead of colonies (25).

At the metabolic level it has been found that ultraviolet irradiation of bacteria selectively inhibits bacterial DNA synthesis (26). This inhibition is permanent and lethal in sensitive strains of bacteria unable to repair the damage while in resistant strains the inhibition is temporary and not lethal (27). The time required for synthesis to resume is approximately the same as the time required for the dimers to be excised from the bacterial DNA (28). Thus, it is thought that DNA synthesis is inhibited as a direct result of the formation in the DNA of pyrimidine dimers which act as blocks to further replication of the DNA. This notion is supported by the finding that ultraviolet irradiation of DNA reduces rapidly its ability to "prime" the cell-free systems in which DNA serves as a template for DNA polymerase (29) and RNA polymerase (30).

While 50-75% of the biological damage resulting from ultraviolet irradiation of bacteria can be attributed to dimer formation, ultraviolet light also affects proteins directly and is known to crosslink proteins to nucleic acids (31). The importance of such DNA-protein crosslinks is not yet clear. However, such lesions could conceivably contribute to the non-reparable fraction of the total ultraviolet damage.

(ii) Alkylating Agents in General

Alkylating agents such as nitrogen mustard are radiomimetic agents which are mutagenic and toxic for a variety of microorganisms. Although DNA, RNA and proteins are alkylated to about the same extent (32), interest has centred on the reaction of these agents with DNA, since modification of a single base in DNA may have very far-reaching consequences. Brookes and Lawley have shown that when native DNA is treated with methyl methanesulfonate the most reactive site is the N-7 position of guanine (80-90%) while methylation takes place to a lesser extent at the N-3 position of adenine (10-20%), the N-1 position of adenine (< 5%) and the N-1 position of cytosine (< 2%) (33). Similar treatment of RNA or denatured DNA leads to alkylation of the N-7 position of guanine (60-70%), the N-1 position of adenine (20-30%), the N-1 position of cytosine (10-15%) and the N-3 position of adenine (< 2%). The differences between RNA and native DNA were attributed to the involvement of the N-1 position of adenine and of cytosine in hydrogen-bond formation in native DNA. It was also shown that at neutral pH alkylated purines are split off from the sugar-phosphate backbone of the nucleic acid, thus suggesting a mechanism for mutation resulting from a deletion in the genetic material. Such deletions could also contribute to the toxicity of alkylating agents.

A second possible mechanism for the mutagenicity of these agents arises from the fact that alkylation of guanine at the 7 position can lead to anomalous base pairing (34). The increased ionization of the proton from the N-1 position of alkylated guanine would permit the

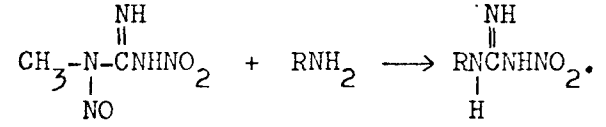
formation of two hydrogen bonds with thymine with the result that a guanine-cytosine base pair could be changed to an adenine-thymine pair in newly replicated DNA.

The difunctional alkylating agents like nitrogen mustard are generally more powerful cytotoxic agents than monofunctional alkylating agents. Treatment of DNA with mustard gas results in about 25% of the alkylation leading to the formation of a diguaninyl product, two guanine residues being linked through the 7-nitrogens by a single molecule of alkylating agent (35). It was suggested that these two guanines may have come from adjacent base pairs in opposite strands of the DNA double helix since the proportion of diguaninyl alkylation products was significantly less for denatured DNA and for RNA. Such interstrand crosslinks would prevent the duplication of DNA and could account for the observed toxicity of these agents.

(iii) "Methylnitroso" Derivatives

(a) N-Methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine)

N-Methyl-N'-nitro-N-nitrosoguanidine was first synthesized by McKay and Wright at the University of Toronto in a wartime search for potential ingredients of flameless propellants (36). They also described the reaction of nitrosoguanidine with primary amines and with dimethylamine to form alkyl nitroguanidines.



Subsequently, Henry found that the methylnitrosamino group serves either directly or indirectly as a methylating agent, since the

principal product of the reaction of nitrosoguanidine with aniline is N-methylaniline (37). The product from treatment of nitrosoguanidine with aqueous potassium hydroxide is diazomethane (38). Diazomethane, however, does not methylate aniline under the conditions used (37). Henry therefore proposed that methylnitrosamine formed from nitrosoguanidine reacts with aniline in the following fashion:

$$CH_3NHNO + C_6H_5NH_2 \longrightarrow CH_3NHN{=}NC_6H_5 + H_2O$$

$$\text{or } C_6H_5NHN{=}NCH_3 + H_2O$$

$$\downarrow$$

$$C_6H_5NHCH_3 + N_2$$

Cancer chemotherapy screening trials reported in 1960 showed that nitrosoguanidine has weak anticancer activity (39). Since then many papers on its biological properties have appeared. Also in 1960, nitrosoguanidine was reported to be a powerful bacterial mutagen (40) and its mutagenicity in a variety of organisms has now been documented (41,42,43). It is noteworthy that it is an effective bleaching agent (see Section II) in Euglena gracilis (44), and is the first mutagen known to cause both chromosomal and nonchromosomal mutations in Clamydomonas reinhardi (45).

A single-step mutant of E. coli S resistant to nitrosoguanidine was found to be cross-resistant to ultraviolet light and to a series of radiomimetic chemicals including nitrogen mustard, azaserine and Mitomycin C (10). For this reason Greenberg added nitrosoguanidine to the list of radiomimetic agents. Like many other radiomimetic agents, this compound causes breaks in Vicia faba root tip chromosomes (46), and in a lysogenic strain of E. coli induces prophage development which

culminates in mass lysis of the bacteria (47). (The data supporting this conclusion are presented in section B of Results and Discussion in this thesis.)

Recent results from Greenberg's laboratory show that a one-hour exposure of E. coli B to nitrosoguanidine inhibits the increase of total RNA, DNA and protein to the same extent (48). The finding that nitrosoguanidine inactivates transforming DNA in vitro indicates that it acts directly on DNA (48).

While the molecular mechanism of action of nitrosoguanidine has not been investigated, there have been suggestions that it acts as a methylating agent (49).

(b) N-Methyl-N-nitroso-p-toluenesulfonamide (MNTS)

Another compound containing the "methylnitroso" grouping is N-methyl-N-nitroso-p-toluenesulfonamide. MNTS has no antileukemia activity (50) and does not produce tumours in rats under conditions where other related compounds do (51). MNTS is also relatively non-mutagenic (52). However, on the basis of cross-resistance studies involving E. coli, Greenberg included MNTS in the group of radiomimetic chemicals (49). McCalla has recently reported that this compound is a very effective bleaching agent for Euglena (53).

Like nitrosoguanidine, MNTS yields diazomethane under alkaline conditions (54) but it is not yet clear whether this property is related to the biological action of MNTS.

(iv) Derivatives of 5-Nitrofuran

In 1944 Dodd and Stillman reported that a number of derivatives

of 5-nitrofuran are bacteriostatic at modest doses and slowly bactericidal at high concentrations (55). Recently pharmaceutical houses in the United States and Japan have patented a host of nitrofuran derivatives. Some of these compounds are clinically useful in the treatment of superficial wounds and of bladder and urinary tract infections. The formulae and trivial names of three nitrofurans of interest are presented in Table I. Two compounds, nitrofurazone and nitrofurantoin are Schiff's bases while NFT has a vinyl group adjacent to the furan ring.

Studies carried out in the late 1940's and early 1950's on the biochemical effects of the Schiff's base nitrofurans were aimed at explaining their bacteriostatic properties. Green found that nitrofurazone inhibits oxygen uptake in Staphylococcus aureus and in Escherichia coli (56). At the enzymic level nitrofurazone inhibits pyruvic oxidase (57) as well as dehydrogenases involved in carbohydrate metabolism (56,58).

More recently it has become apparent that nitrofurans affect nucleic acids. Szybalski first noted that a radiation-resistant mutant of E. coli is many times more resistant to nitrofurazone than is the parental radiation-sensitive strain (59). On the basis of similar observations, Greenberg in 1963 concluded that nitrofurazone is a radiomimetic chemical (14). Other nitrofurans, including nitrofurantoin and NFT, have since been added to this class of compounds (15).

Since other radiomimetic chemicals affect DNA directly, it is to be expected that the nitrofurans should also act on DNA in vivo. The report that nitrofurazone increases the rate of mutation from Lac$^-$

to Lac^+ in E. coli supports this notion (60). However, nitrofurazone does not inactivate transforming DNA in vitro (48), suggesting that metabolic modification of the compound might be required for activity. Terawaki and Greenberg have suggested that DNA replication is necessary for nitrofurazone to be active since they found that nitrofurazone does not inactivate transforming DNA in vitro in the presence of a Bacillus subtilis cell extract capable of "activating" Mitomycin C so that it inhbits transforming DNA in vitro. It is, however, quite possible that factors other than those required to "activate" Mitomycin C are required to "activate" nitrofurazone.

Recent studies on NFT have demonstrated that this nitrofuran derivative specifically inhibits DNA synthesis in E. coli B and induces prophage development in a strain of E. coli lysogenic for λ phage (61). Nitrofurazone and nitrofurantoin, however, do not produce mass lysis of lysogenic strains of E. coli (62,63). Further, the Schiff's base nitrofurans not only inhibit the net increase of DNA but also of RNA and of protein in E. coli B (62,48).

Some of the factors involved in the inhibition of DNA synthesis in E. coli treated with NFT have been elucidated recently (18). NFT appears to inhibit DNA synthesis by reducing the priming activity of DNA while also accelerating the degradation of DNA by increasing the level of deoxyribonuclease activity in treated cells.

It should also be noted that all nitrofurans tested, including the three compounds listed in Table I, bleach Euglena (see Section II), the most potent bleaching agent being NFT (64).

II. Chloroplast Mutagenesis

Molecular Biology of Chloroplasts

A property of some but by no means all of the radiomimetic compounds listed in Table I is the ability to cause mass mutation of a population of cells of the normally green alga Euglena gracilis to permanently "bleached" variants which never regain their chlorophyll. Other chemicals, of a variety of molecular types, as well as certain physical agents also bring about permanent bleaching of Euglena (Table II). Such bleached cells are able to multiply indefinitely if they are provided with a suitable organic carbon source.

Since the "molecular biology" of chloroplasts is relevant to later sections of this thesis, a brief summary of current knowledge will be presented here.

The existence of two systems of heredity - chromosomal and cytoplasmic - was first suggested in 1909 by Correns while working with green-white variegated plants of Mirabilis jalopa (77). In reciprocal crosses, he found that regardless of the source of pollen, flowers on the green sectors of the plant gave only green progeny and flowers on the white sectors gave only white progeny. This result is explained only on the basis of maternal inheritance in which some genetic determinant(s) other than nuclear genes is transmitted to the zygote through the female gamete. Since that time many examples of cytoplasmic inheritance have been described in a variety of organisms (78). One of the most intensively studied examples and the one of immediate interest is plastid heredity.

Although genetic studies cannot be carried out with Euglena

TABLE II

Agents which bleach Euglena

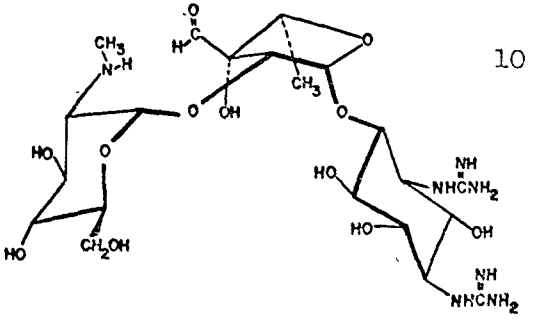
Agent	Reference	Formula	Bleaching Treatment	pH of Medium
Antibiotics of the Streptomycin type				
(a) Streptomycin	65		10 µg/ml	6.8
(b) Kanamycin	66,67	-	30 µg/ml	6.8
(c) Neomycin	66	-	10 µg/ml	6.8
(d) Paromomycin	66	-	1 µg/ml	6.8
(e) Erythromycin	68	-	200 µg/ml	6.8
(f) Spectinomycin	69	-		

TABLE II (CONTINUED)

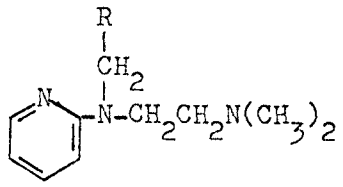
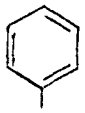
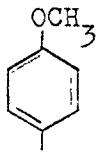
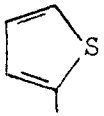
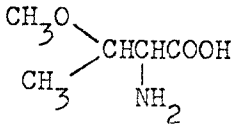
Agent	Reference	Formula	Bleaching Treatment	pH of Medium
Antihistamines				
(a) pyribenzamine	70,66	R = 	300 µg/ml	6.8
(b) pyrilamine	66	R = 	600 µg/ml	6.8
(c) methapyrilene	66	R = 	1000 µg/ml	6.8
O-methylthreonine	71		6 µg/ml	3.5

TABLE II (CONTINUED)

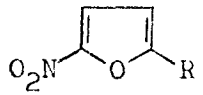
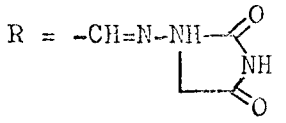
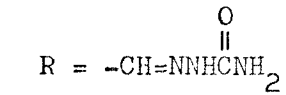
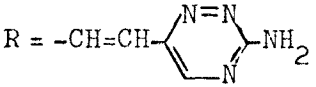
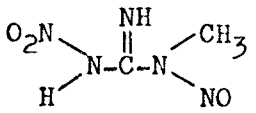
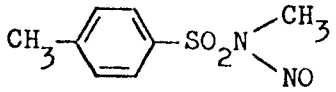
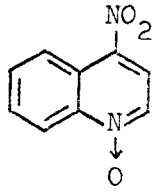
Agent	Reference	Formula	Bleaching Treatment	pH of Medium
Nitrofuran derivatives				
(a) nitrofurantoin	72		16 µg/ml	3.5
(b) nitrofurazone	64		64-128 µg/ml	3.5
(c) NFT	64		2.5 µg/ml	6.8
"Methylnitroso" compounds				
(a) nitrosoguanidine	44		10 µg/ml	6.8
(b) N-methyl-N-nitroso-p-toluenesulfonamide	53		2.5 µg/ml	6.8

TABLE II (CONTINUED)

Agent	Reference	Formula	Bleaching Treatment	pH of Medium
Miscellaneous Chemical Agent				
4-Nitroquinoline-N-oxide	73			3.5
Physical Agents				
(a) growth at elevated temperature	74	-	34-35°	
(b) ultraviolet light	75	-	150 ergs/mm^2	
(c) hydrostatic pressure	76	-	1000 atm. for 20 min.	

which is an asexual organism, Gibor and Granick have elegantly demonstrated that cytoplasmic factors are involved in the bleaching of Euglena (79). Using an ultraviolet microbeam they were able to irradiate the cytoplasm alone while shielding the nucleus, or to irradiate the nucleus while most of the cytoplasm was shielded. Only irradiation of the cytoplasm caused irreversible loss of the ability to form mature chloroplasts.

During the past few years, considerable indirect evidence has accumulated indicating that the physical basis of the chloroplast's heritable system is DNA (75,80,81). In 1963 a number of laboratories reported more direct evidence for chloroplast DNA. Density gradient ultracentrifugation of the DNA from a variety of green organisms demonstrated a "satellite" DNA band, associated with the chloroplasts, at slightly different buoyant density than the principal band of nuclear DNA (82,83,84). For example, the buoyant density of the satellite DNA of Euglena is 1.685 g/cc. (corresponding to an adenine plus thymine content (A + T) of about 75%) as compared to 1.707 g/cc. (A + T = 50%) for the principal DNA (85,86,87). The buoyant density of the principal DNA from spinach is 1.695 g/cc. while the satellite DNA associated with chloroplasts has a density of 1.719 g/cc. (82).

In order to eliminate the possibility of contamination of chloroplast preparations by nuclear DNA, Gibor and Izawa isolated chloroplasts from enucleated fragments of Acetabularia and found that the chloroplasts contained approximately 1 x 10^{-16} gram of DNA per plastid (88). Estimates of the DNA content of spinach and Euglena plastids are also of the order of 10^{-16} to 10^{-15} gram of DNA per

plastid (89,87). This amount of DNA is similar to that found in vaccinia virus and in T-even bacteriophages (90) and would be sufficient to code for many types of proteins.

According to current concepts concerning the synthesis of macromolecules it would be expected that the nucleotide sequences in the chloroplast DNA would code for the nucleotide sequence of plastid RNA, thus providing a template for the RNA moiety of chloroplast ribosomes and perhaps for transfer RNA as well. In addition, other segments of the plastid DNA might specify the amino acid sequence of some or all of the chloroplast proteins.

Brawerman has reported that a specific type of ribosomes differing in both sedimentation constant and base composition from the cytoplasmic ribosomes is associated with Euglena chloroplasts (91). The RNA of chloroplast ribosomes, like chloroplast DNA, contains a lower proportion of guanine and cytosine than does the RNA of microsomal particles. This fact is consistent with the notion that chloroplast DNA codes for the RNA of chloroplast ribosomes. Evidence that the DNA of the plastids serves as a template for RNA synthesis has been obtained by Kirk (92) who showed that the incorporation of labelled precursors of RNA by a cell-free chloroplast preparation was reduced by Actinomycin D, a drug which has been shown to inhibit specifically DNA-dependent-RNA synthesis in animal and bacterial cells (93).

Additional experiments have provided indirect evidence that DNA participates in the synthesis of chloroplast RNA. When Euglena is grown in the dark, chloroplast development is arrested at an early

stage in which the typical lamellae and chlorophyll are lacking (94). Such cells are yellow (etiolated) but retain the ability to form chloroplasts when illuminated. Illumination results in the synthesis of chloroplast RNA and protein before mature green chloroplasts are formed (95). Based on the assumption that Actinomycin D inhibits DNA-dependent-RNA synthesis in such cells, it has been demonstrated that the synthesis of the RNA required for "greening" depends on the participation of DNA (96,97). Although it remains to be shown that chloroplast DNA codes for chloroplast proteins, labelled amino acids are incorporated into proteins by chloroplast preparations of both Euglena (98) and spinach (99).

The fate of the plastid in bleached strains of Euglena remains an open question. Gibor and Granick have examined normal and bleached Euglena gracilis strain bacillaris and have found that the bleached cells all possess structures which they interpret to be damaged proplastids (100). On the basis of fluorescence microscopy before and after incubation of the cells with δ-aminolevulinic acid, these workers have described four types of proplastids and have arranged these types according to the apparent degree of damage to the proplastid system. Electron microscopic studies have also indicated the presence of proplastids in bleached strains of Euglena (101). On the other hand, studies on the nucleic acids of Euglena gracilis strain Z have shown that dark-grown cells, which can form chloroplasts in the presence of light, contain chloroplast DNA (86), while bleached strains, unable to form chloroplasts, completely lack chloroplast DNA (86,102). The question of the fate of the plastid could be examined more critically

by coupling an investigation of the deoxyribonucleic acids of a bleached strain of Euglena with electron microscopy of the same bleached strain.

A discussion of the deoxyribonucleic acids of Euglena would not be complete without mentioning the recent characterization of a second type of extranuclear DNA which has been shown to be associated with the mitochondria (103).

Mode of Action of Agents which Bleach Euglena

Because of the diversity of agents capable of bleaching Euglena, it is difficult to formulate a single comprehensive hypothesis to explain the phenomenon. The task is made more difficult by the very limited amount of data relating to the mechanism(s) by which these agents affect Euglena. In general, the more plentiful information pertaining to the mode of action of these agents in bacteria has been used as a basis for speculation on the mode of action in other organisms. Accordingly, the data pertaining to Euglena will be presented and supplemented with related data pertaining to bacteria.

(i) Ultraviolet light

The effects of ultraviolet light on bacteria have been discussed previously. Studies on the inactivation of Euglena chloroplast formation by ultraviolet light have been carried out by Schiff, Epstein and coworkers who showed that the action spectrum for bleaching has peaks at 260 mμ and 280 mμ suggesting that a nucleoprotein is the probable site of action of ultraviolet radiation in Euglena (75). They inferred from the multiplicity of inactivation sites that these targets are cytoplasmic. Since chloroplast DNA has a relatively high

thymine content and ultraviolet irradiation of bacteria produces thymine dimers, it seems possible that ultraviolet light may act selectively upon chloroplast DNA in Euglena, although direct evidence on this point is lacking. By analogy with E. coli, such dimers could block the synthesis of chloroplast DNA thus preventing the duplication of chloroplasts. The ultraviolet-induced damage to Euglena is completely photoreactivable by visible light (104).

(ii) Streptomycin

Streptomycin is an antibiotic with bactericidal properties. The presence of two basic guanidine groups in the molecule confers the ability to bind strongly to nucleic acids (105). Streptomycin has an early inhibitory effect upon bacterial protein synthesis and subsequently upon RNA and DNA synthesis as well (106,107). Gorini and his coworkers have demonstrated that streptomycin binds to ribosomes thereby causing a "misreading" of the messenger RNA code (108). The result of such misreading would be to flood the cell with fraudulent protein which could ultimately lead to cell death. However, Cohen and coworkers have recently shown that streptomycin is lethal for a thymine-arginine- and uracil-requiring strain of E. coli when protein synthesis is blocked by a lack of arginine (109). They found that streptomycin causes a stimulation of RNA synthesis in the absence of protein synthesis and that the inception of stimulation coincides closely with the onset of lethality. Clearly, the primary lethal effect of streptomycin in bacteria remains to be established.

In 1964 Scher and Collinge suggested that streptomycin-induced misreading of the genetic code could account for the observed bleaching

of Euglena if the ribosomes associated with the chloroplasts are more sensitive than cytoplasmic ribosomes (110). However, Scher has recently reported that spectinomycin, an antibiotic closely related to streptomycin, which inhibits protein synthesis in bacteria without causing errors in the translation of messenger RNA nevertheless bleaches Euglena. There are no data bearing directly on the mechanism of streptomycin bleaching of Euglena.

(iii) Other agents

Data relating to the mechanism of bleaching by other bleaching agents is almost totally lacking and, in fact, in many cases little is known of how these agents affect bacteria. Based upon the known effects of ultraviolet light and nitrofuran derivatives on bacteria, McCalla has suggested that ultraviolet light and radiomimetic bleaching agents may bleach Euglena by selectively damaging chloroplast DNA thus preventing its replication and in turn preventing the replication of the chloroplasts. It is not necessary, however, to postulate that all agents bleach Euglena by the same mechanism but only that there be a selective effect on a process vital to the existence of the chloroplasts.

MATERIALS AND METHODS

A. Organisms and Growth Conditions

The bacterial strains used for studies of respiration and of macromolecule synthesis, Escherichia coli strains B and B/r, were obtained from Dr. E. Witkin and grown with shaking at 37°C in the following medium (slightly modified from Roberts, et al. (111));

NH_4Cl	2 g	per litre of medium
$Na_2HPO_4 \cdot 7H_2O$	11.3 g	
KH_2PO_4	3 g	
NaCl	3 g	
$MgSO_4 \cdot 7H_2O$	100 mg	
Na_2SO_4	60 mg	

After sterilization, one tenth the final volume of sterile 10% glucose solution was added to provide a carbon and energy source. For studies of viable cell count and of macromolecule synthesis using radioactive precursors, this medium was supplemented with 100 µg/ml each of adenine and DL-leucine.

For investigations of prophage induction both the lysogenic strain, E. coli C600 (λ), and the indicator strain, E. coli C600, were obtained from Dr. I. Takahashi and grown with shaking in λ broth (112) at 37°.

The green phytoflagellate, Euglena gracilis strain Z originally obtained from Dr. S. H. Hutner was grown in defined medium (pH 6.8)

(113) at 26°. Normally the carbon source was supplied by adding enough sterile glucose solution to the medium to give a final concentration of one gram of glucose per 100 ml. When chloroplasts were to be isolated, the cells were grown autotrophically. Air enriched to 5% CO_2 was bubbled through the culture after passing through a sterile cotton filter. Continuous illumination was provided by a bank of cool white fluorescent tubes which yielded approximately 200 foot-candles light intensity.

Stock cultures of all organisms were maintained on tryptic soy agar and transferred periodically.

B. Chemicals

All chemicals for media and other solutions were reagent grade or the most highly purified available commercially.

Nitrofurantoin, N-(5-nitro-2-furfurylidine)-1-aminohydantoin, and nitrofurazone, 5-nitro-2-furaldehyde semicarbazone, were kindly supplied by Norwich Pharmical, Norwich, N.Y. 3-Amino-6-[2-(5-nitro-2-furyl)vinyl]-1,2,4-triazine (NFT) was synthesized according to the procedure of Miura, et al. (114).

N-Methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) and N-methyl-N-nitroso-p-toluenesulfonamide (MNTS) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

Radioactive chemicals were purchased from the sources listed below:

DL-leucine-1-C^{14} (18 mc/mM); Volk Radiochemical Co., Skokie, Ill.

L-leucine-1-C^{14} (8.0 mc/mM); Volk Radiochemical Co., Skokie, Ill.

adenine-8-C^{14} (5.0 mc/mM); New England Nuclear Corp., Boston, Mass. (8.0 and 10 mc/mM); Volk Radiochemical Co.

N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide (6.1 mc/mM); New England Nuclear Corp.

"Optical grade" CsCl used in density gradient centrifugation (115) was purchased from Harshaw Chemical Co., Cleveland, Ohio.

C. Bacterial Respiration Studies

Conventional manometric techniques were used (116) with a Bronwill, model UV, Warburg apparatus (Bronwill Scientific Inc., Rochester, N.Y.). The centre well of each Warburg vessel contained 10% KOH and a filter paper wick. The main compartment contained bacteria suspended in buffer containing 1% glucose as the substrate for respiration. The flasks were attached to manometers and agitated at 37°. After a constant rate of oxygen uptake had been established, an equal volume of buffer containing the appropriate concentration of drug was tipped from the side arm into the main compartment. The bacteria were treated with nitrofurazone, nitrofurantoin or NFT in various concentrations.

D. Prophage Induction by Nitrosoguanidine

Growth and lysis experiments were carried out under similar conditions with 10 ml of bacterial culture in 250 ml Erlenmeyer flasks with attached ½ in. diameter colorimeter tubes. Cultures were prepared by mixing equal volumes of a freshly prepared solution of nitrosoguanidine in λ broth at twice the desired final concentration and a

cell suspension of twice the desired optical density. Both the solution and the suspension were prewarmed to 37° before mixing and the resulting culture was incubated at 37° with shaking. Turbidity was measured in a Bausch and Lomb "Spectronic 20" colorimeter at 660 mμ.

In order to show that the decrease in OD_{660} of the culture was accompanied by the release of free bacteriophage particles, a one-step growth experiment was carried out. Early log phase cells of E. coli C600 (λ) were harvested by centrifugation at approximately 3000 x g for 5 minutes in order to separate the bacterial cells from free bacteriophage particles. The pellet was resuspended in fresh medium (37°) containing the appropriate concentration of nitrosoguanidine, and the resulting suspension incubated at 37° with shaking. At various intervals, samples were plated for plaque counts by overlaying solid medium with top agar (0.6% agar in λ broth at 45°) containing the indicator strain, E. coli C600, and the diluted sample. Plates were incubated overnight at 37° before counting the plaques.

E. Viable Cell Count

In order to determine the number of viable bacterial cells in cultures similar to those described in section F, dilutions were made in defined medium lacking glucose but containing 100 μg/ml leucine and adenine. One tenth ml of the final dilution was spread on solid agar in a petri dish. Colonies were counted after incubation overnight at 37°.

F. Macromolecule Synthesis in E. coli B

The incorporation of C^{14}-leucine and of C^{14}-adenine into acid

precipitable material was used as an index of protein and nucleic acid syntheses, respectively. Radioactivity in RNA and DNA was determined separately by taking advantage of the fact that dilute NaOH hydrolyses RNA but not DNA (117). Similar techniques have been used by Dubin, et al. (107) to study the effects of streptomycin on E. coli.

The medium was supplemented with 100 µg/ml each of non-radioactive adenine and leucine so that incorporation of labelled material continued for at least one hour. The experimental details are outlined in Table III.

Experiments commenced with the addition of one volume of log phase culture to one volume of prewarmed fresh medium containing the appropriate concentration of nitrosoguanidine and either adenine-8-C^{14} or leucine-1-C^{14}. When leucine incorporation was measured, the final volume of 5 ml contained 2 µc of DL-leucine-1-C^{14}. When incorporation of adenine was measured, 2 µc of adenine-8-C^{14} were present in a volume of 10 ml. The final OD_{660} was approximately 0.2. The cultures were shaken at 37°.

At intervals 1.0 ml samples were pipetted from each culture into 1 ml of cold 10% trichloroacetic acid (TCA) solution. A second 1.0 ml sample from the cultures containing radioactive adenine was pipetted into 2 ml of 1 N NaOH solution and incubated at 37° overnight. Following neutralization of the alkaline samples, TCA was added to a final concentration of 5% to precipitate the proteins and nucleic acids. After allowing the samples to stand at 0-4° for at least one hour, the precipitates were collected on membrane filters (type RAWP 02500; Millipore Filter Corp., Bedford, Mass.), washed with ice-cold 5% TCA

TABLE III

Experimental Details of Incorporation Studies with *E. coli* B

Flask number	1	2	3		4	
Volume of fresh medium (ml)	2.5	2.5	5		5	
Labelled precursor (in 10 λ)	leucine-1-C^{14}	leucine-1-C^{14}	adenine-8-C^{14}		adenine-8-C^{14}	
Agent (in 10 λ)	-	+	-		+	
Volume of parent culture (ml)	2.5	2.5	5		5	
Samples (1.0 ml)	↓	↓	↙	↘	↙	↘
Treatment of sample	10% TCA (1 ml)	10% TCA (1 ml)	10% TCA (1 ml)	1 N NaOH (2 ml)	10% TCA (1 ml)	1 N NaOH (2 ml)
Index for synthesis of	Protein	Protein	Total Nucleic Acids	DNA	Total Nucleic Acids	DNA

solution containing 50 µg/ml each of non-radioactive leucine and adenine, and then counted as described in section I.

G. Macromolecule Synthesis in Euglena

(i) Incorporation experiments

Techniques similar to those outlined in section F were used to detect early changes in the rates of macromolecule synthesis in Euglena cultures treated with nitrofurantoin, NFT, nitrosoguanidine or MNTS.

In these experiments it was unecessary to add non-radioactive leucine and adenine to the medium since the amount of radioactive material supplied (2 µc; 3γ/ml) was not limiting.

Log phase cultures containing about 6×10^5 cells per ml ($OD_{750} \simeq 0.6$) were used. Controls were diluted with an equal volume of fresh medium containing 2 µc of the appropriate radioactive precursor while treated cultures were diluted with fresh medium containing the appropriate concentration of agent as well as the labelled precursor. Final volume in the adenine incorporation experiments was 10 ml and in the leucine incorporation experiments 5 ml.

At one hour intervals, one 1.0 ml sample was removed from each of the flasks containing radioactive leucine and two 1.0 ml samples from each of the flasks containing radioactive adenine. After a low speed centrifugation to collect the cells, each sample was extracted once with 3-4 ml of ice-cold methanol. Following another low speed centrifugation, the leucine-labelled samples and one of each pair of adenine-labelled samples were extracted overnight at 4° with 5% TCA

solution containing 50 µg/ml each of non-radioactive leucine and adenine. The residue from each of the other adenine-labelled samples was treated with 1 ml of 0.5 N NaOH solution overnight at 37°. Using phenolphthalein as indicator, the alkali was neutralized with a few drops of 6 N HCl solution. The precipitation of macromolecules was effected by the addition of an equal volume of 10% TCA solution and subsequent chilling of the suspension.

The TCA-insoluble precipitate from each chilled sample was collected on a membrane filter (type RAWP 02500, Millipore Filter Co., Bedford, Mass.) and washed with cold 5% TCA solution containing non-radioactive leucine and adenine. Radioactivity was determined as described in section I.

For short-term experiments in which samples were taken at 15 minute intervals, only one sample was removed from the cultures exposed to radioactive adenine. These samples were extracted with methanol and 5% TCA solution as described above. Since MNTS is sparingly soluble in water it was dissolved in 95% ethanol prior to addition to the medium, with the restriction that the concentration of ethanol in the culture not exceed 0.1 M (118). An equal amount of ethanol was added to the control cultures. A preliminary experiment showed that 0.1 M ethanol does not inhibit the incorporation of either adenine or leucine.

(ii) Control experiments

(a) Leucine

Euglena cells grown for 4 hours in the presence of radioactive leucine were collected and extracted, first with cold methanol and

then with cold 5% TCA solution. The residue was hydrolysed overnight in 6 N HCl in a sealed tube at 110°. The hydrolysate was evaporated to dryness and the residue dissolved in water. Portions of the resulting solution as well as a series of reference amino acids were subjected to descending paper chromatography using n-butanol-acetic acid-water (4:1:1.8; v:v:v) as the solvent. After scanning the chromatogram to locate the radioactivity (section I), the amino acids were visualized by spraying the chromatogram with a 0.2% solution of ninhydrin in acetone.

(b) Adenine

In routine incorporation experiments described in part (i) of this section, one of the two samples removed from the cultures exposed to radioactive adenine was hydrolysed in NaOH solution. The macromolecules were precipitated by the addition of TCA to a final concentration of 5% and the precipitate filtered and counted. The filtrates which contained the products from hydrolysis of RNA were pooled and continuously extracted with ether for 2 days in order to remove the TCA. The aqueous solution was then evaporated to dryness in a rotary evaporator, and the residue hydrolysed in 88% formic acid in a sealed tube at 175° for 30 minutes (119) in order to convert the nucleotides to free bases. The residue resulting from concentration of this hydrolysate was dissolved in a small volume of 1 N HCl and subjected to descending paper chromatography using the isopropanol-HCl solvent (isopropanol, 170 ml; conc. HCl, 41 ml; water to make 250 ml) described by Wyatt (120). Radioactivity was determined by scanning the chromatogram (section I), and the purines and pyrimidines located with

the aid of an ultraviolet lamp.

In order to show that the DNA was labelled, another type of experiment was performed. A small culture of cells which had been exposed to radioactive adenine for 4 hours was diluted with a large portion of non-radioactive cells. The nucleic acids were extracted from these cells by the modification of Marmur's method (121) described by Ray (102) (presented in detail in section H), and precipitated by the addition of 2 volumes of 95% ethanol to the aqueous solution. The precipitate was collected and dissolved in 3.0 ml buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris; pH 8). The resulting solution was added to 3.85 g of CsCl in a 5 ml capacity cellulose nitrate tube and stirred to produce a solution which was then overlaid with mineral oil and spun at 37,000 r.p.m. (120,000 x g) for 48 hours in an SW39 rotor in a Spinco model L preparative ultracentrifuge at about 20°. Following centrifugation the bottom of the tube was punctured using a dropwise fractionator constructed according to a modification of Szybalski's design (122), and 10-drop fractions collected and diluted with 3.5 ml of the pH 8 buffer. The solution in each test tube was thoroughly mixed with the aid of a Vortex Jr. mixer. A 0.4 ml sample was taken from each tube and placed on a planchet for determination of radioactivity as described in section I. The OD_{260} of each solution was measured on the remaining portion.

Some of the solutions were analysed further by adding non-radioactive carrier DNA (1 mg to each sample) and NaOH to a final concentration of 0.5 N. After overnight incubation at 37° the solution was neutralized and TCA solution added to a concentration of 5%. After

thorough cooling the precipitates were filtered off on Millipore filters and radioactivity determined (section I).

This experiment was carried out in duplicate. A third experiment differed only in the inclusion of nitrosoguanidine in the original culture exposed to radioactive adenine.

H. Studies with N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide

(i) Preliminary work

One hundred ml cultures of Euglena were treated with 3 to 4 µg/ml of C^{14}-labelled MNTS for 1, 4 or 10 hours after which two one-ml samples were removed; one for the determination of the total incorporation into acid-precipitable material and the other for determination of the incorporation into the alkali-stable portion of the acid-precipitable material as described in section G(i). The cells from the remainder of the culture were collected, extracted with methanol and treated with 5 ml of 0.5 N NaOH overnight at 37°. The hydrolysate was neutralized, diluted with an equal volume of 10% TCA solution, chilled thoroughly, and centrifuged at low speed. The supernatant, which contained the nucleotides, was desalted in the following manner. The solution was applied to a column (2.2 cm diameter x 15 cm) of charcoal (Darco G-60; Matheson, Coleman and Bell, East Rutherford, N.J. - washed twice with boiling 2 N acetic acid and finally with the eluant described below) which was then washed to neutrality with distilled water. The nucleotides were eluted with eluant of the following composition (123):

95% ethanol	50	v/v/v
distilled water	43	
28% ammonia	1.8	

Fractions with significant absorbance at 260 mμ were combined and evaporated to dryness in a rotary evaporator. The nucleotides were hydrolysed to the free bases in 88% formic acid in a sealed tube at 175° (119). The hydrolysate was then passed through a Millipore filter (0.22 μ pore size) to remove any particulate matter. After the final concentration in a rotary evaporator, portions of the solution were chromatographed in the following solvents (33):

solvent 1.	methanol	7	v/v/v
	concentrated HCl	2	
	distilled water	1	
solvent 2.	n-butanol	85	v/v/v
	concentrated ammonia	2	
	distilled water	12	

The developed chromatograms were visualized with the aid of an ultraviolet light. Radioactivity was determined as described in section I(d).

(ii) Reaction with Euglena Deoxyribonucleic acids in vivo

(a) Isolation of Chloroplasts

Euglena cells grown on CO_2 as the sole source of carbon were treated with 3 μg/ml (75 μc/l) of C^{14}-labelled MNTS for 75 minutes. Chloroplasts were isolated from 750 ml of the culture by the floatation procedure described by Eisenstadt and Brawerman (124). The cells, after collection by centrifugation, were suspended in buffer (pH 7.6) containing 10% sucrose and disrupted by passing through a French Pressure cell operated at 2000 p.s.i. The resulting suspension was

then centrifuged at 500 x g for 10 minutes and the pellet resuspended in buffer. After the suspension was allowed to stand for 10 minutes, membranous material was removed by filtration through several layers of cheesecloth. The pellet from another low-speed centrifugation was resuspended in one volume of buffer and mixed with 2 volumes of 75% sucrose solution. The resulting suspension (30 ml) was centrifuged at 23,000 x g in an SW25 rotor in a Spinco Model L preparative ultracentrifuge for 30 minutes. The tube was sliced (Tube Slicer, Beckman Instruments Inc., Spinco Division, Palo Alto, California) just below the floating layer of chloroplasts which was then suspended in 15 ml of buffer with the aid of a Potter-Elvejham homogenizer. The chloroplasts were recovered by centrifugation at 3,000 x g for 5 minutes. The floatation step was repeated. The recovered chloroplasts were washed three times with 0.01 M tris buffer (pH 7.0) (87). All these operations were carried out in the cold.

(b) Isolation of Nucleic Acids

Nucleic acids were isolated from Euglena chloroplasts prepared as described above and from whole cells which were collected from 75 ml of culture treated with C^{14}-labelled MNTS for 60 minutes as outlined at the beginning of this section. The modification of Marmur's procedure (120) described by Ray (102) was employed. After collection by low-speed centrifugation at 4°, the whole cells were washed twice at 4° with buffer (pH 8) containing 0.1 M NaCl, 0.001 M EDTA and 0.001 M tris. The pellet of washed cells was resuspended in 95% ethanol and allowed to stand for 10 minutes at 4°. After the material was sedimented by low-speed centrifugation, the ethanol

extraction was repeated. The pellet from another low-speed centrifugation was suspended in 0.1 M NaCl, 0.01 M EDTA, 0.01 M tris buffer (pH 8) containing 1% sodium dodecyl sulphate and the mixture allowed to stand for 30 minutes at room temperature. One-half volume of 3 M sodium perchlorate solution was added and the mixture was deproteinized by shaking, using a Vortex Jr. Mixer, with an equal volume of chloroform-octanol (9:1, v/v) for three 5 to 10 second periods. The resulting emulsion was separated into 3 layers by low-speed centrifugation for 5 minutes. The upper (aqueous) phase containing the nucleic acids was decanted and dialysed overnight against saline-citrate buffer (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

In the isolation of nucleic acids from a purified chloroplast preparation, a similar procedure was used except that the ethanol extractions were omitted and a 1% solution of sodium dodecyl sulphate in buffer was added directly to the purified chloroplast preparation.

The pH of each sample was adjusted to 7.0 and ribonuclease (0.2% in 0.15 M NaCl, pH 5.0 heated at 80° for 10 minutes to inactivate any contaminating DNase) added to a final concentration of 50 µg/ml. The mixture was incubated at 37° for 30 minutes. After deproteinization by shaking the mixture with an equal volume of chloroform-octanol (9:1, v/v), the aqueous solution was dialysed against several changes of saline-citrate buffer for 48 hours.

(c) Determination of Relative Specific Activity

The radioactivity of a portion of each sample was determined in a naphthalene-dioxane scintillation fluid miscible with water (section I (ic)). The absorbance at 260 mµ of each sample was used as

a measure of the concentration of nucleic acids. For these measurements a Hitachi Perkin-Elmer Model 139 ultraviolet-visible spectrophotometer was employed. The relative specific activity of each sample was taken as the ratio of disintegrations per minute per unit volume of sample to the absorbance at 260 mμ.

I. Determination of Radioactivity

(i) General Procedures

(a) Precipitates on Membrane Filters

In the incorporation experiments described in sections F and G (ia) each wet membrane filter was glued ("Clean grip rubber cement", Carter's Ink Co., Montreal, Canada) to an aluminum planchet, dried and counted at infinite thinness using a Nuclear-Chicago D-47 gas flow detector fitted with a "Micromil" window. Counting efficiency was approximately 20%.

Each membrane filter from the short time incorporation experiments described in section G (ib) was dried and then submerged in approximately 4 ml of scintillation fluid A (listed below) and counted in a Nuclear-Chicago Mark I scintillation counter. The counting efficiency as determined by the channels ratio method (125) was approximately 75%, the value for each sample being used to calculate the number of disintegrations per minute in that sample.

(b) Fractions from CsCl Density Gradient Centrifugations

Samples from the cesium chloride density gradient centrifugations of nucleic acids described in section G (iib) were pipetted directly onto aluminum planchets (four quadrant ringed) dried and counted in a low-background Nuclear-Chicago counter.

(c) Aqueous Solutions

The radioactivity of aqueous solutions was determined by adding to 5 ml of scintillation fluid B a known volume of sample which was then counted using the "channels-ratio method" (125) to determine the number of disintegrations per minute.

(d) Radiochromatograms

The chromatograms resulting from the experiments described in section G (ii) were scanned using a Packard model 7201 Radiochromatogram Scanner.

The chromatograms resulting from the experiments with C^{14}-labelled MNTS (described in section H (i)) were cut into 1 cm strips which were submerged in scintillation fluid A and counted. Efficiency as determined by the "channels-ratio method" (125) was approximately 70%.

(ii) Scintillation Fluids (126)

A.
4 gm/l	PPO
50 mg/l	POPOP

Solvent is reagent grade toluene

B.
7 gm/l	PPO
0.3 gm/l	POPOP
100 gm/l	naphthalene

Solvent is reagent grade dioxane

PPO - 2,5-diphenyloxazole

POPOP - 2,2-p-phenylenebis(5-phenyloxazole)

purchased from Nuclear-Chicago Corp., Des Plaines, Ill.

(iii) Counting Errors

All samples counted with a gas-flow detector or with a scintillation detector were counted to a total of at least 2000 counts which represents a maximum counting error of 2.3%.

RESULTS AND DISCUSSION

A. Action of Nitrofuran Derivatives: Bacterial Respiration and Growth Rate Studies

In early work directed towards explaining the bacteriostatic properties of nitrofurans, particularly nitrofurazone, little attention was paid to the question of the relative importance of observed effects. Inhibition of bacterial respiration has been reported along with an inhibition of enzymes involved in carbohydrate metabolism (56). Since the relationship between inhibition of respiration and inhibition of growth had not been critically examined even with Escherichia coli, a detailed study of the effects of nitrofurans on respiration and growth was carried out.

The results of a typical respiration experiment are shown in Figure 1. The rate of oxygen consumption is obtained from the slope of the curve for the period following addition of the agent.

The averages of triplicate experiments are recorded in Table IV. Per cent of control values are calculated from the ratio of the rate of oxygen consumption in the treated culture to the rate in the control culture. The difference between this value and 100 per cent is the per cent inhibition.

The effects of nitrofurantoin, nitrofurazone and NFT on the growth rate of E. coli B and B/r, as measured by the change of a culture in absorbance at 750 mμ, are shown in Table V. For each

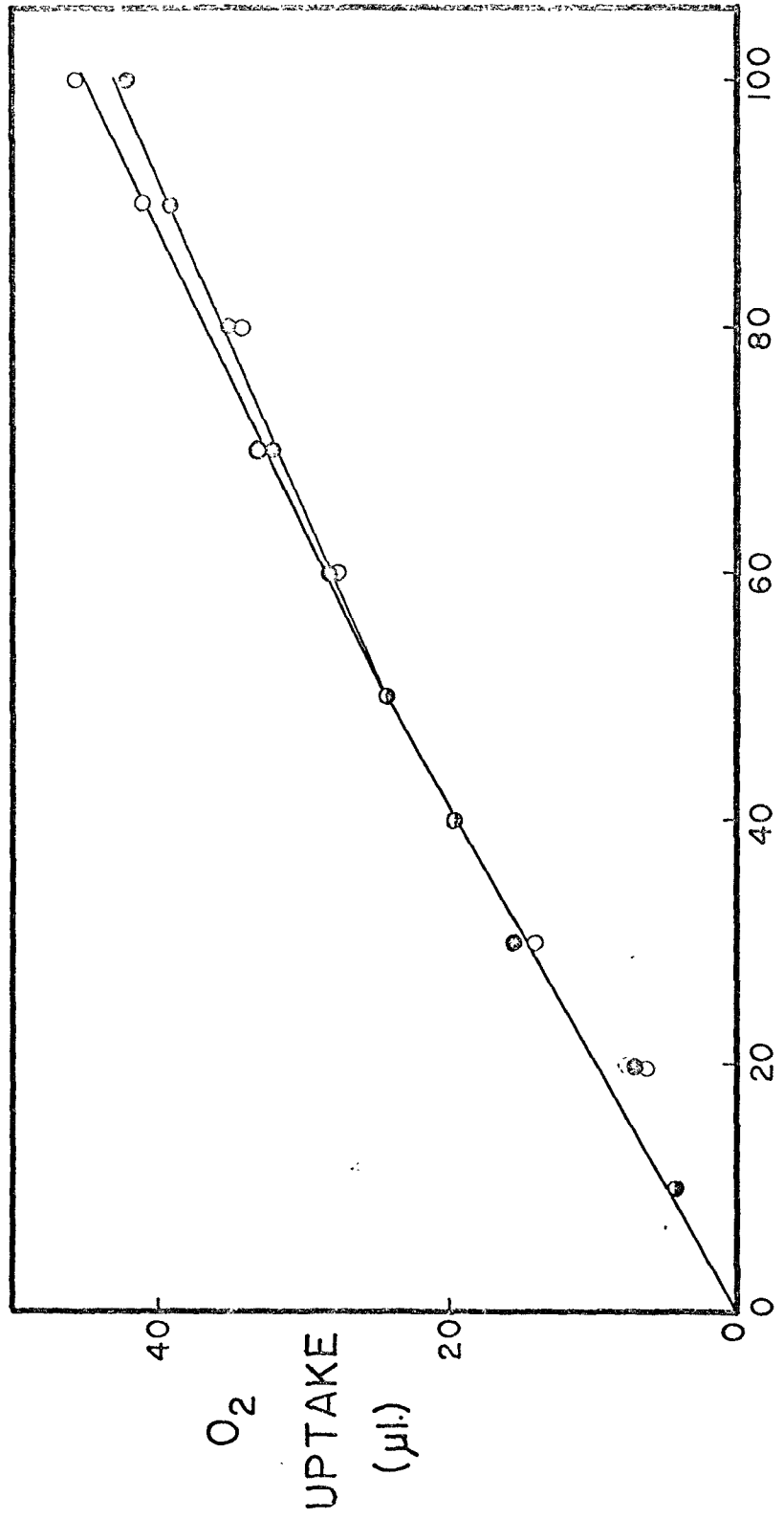


Figure 1. Inhibition by nitrofurazone of oxygen uptake by *E. coli* B. After 50 minutes nitrofurazone was added to the cell suspension. Control, open circles: 10 µg/ml nitrofurazone, closed circles. Results were normalized to coincide at the 50-minute point.

treatment the value given is the per cent of the control rate of increase during the first hour after addition of the chemical.

The data in Tables IV and V reveal that strains B and B/r behave similarly towards all these agents in so far as short-term growth and respiration are concerned. This is in marked contrast to the difference in colony-forming ability (15). McCalla has noted that the minimum concentration of either nitrofurazone or nitrofurantoin required to inhibit colony formation of strain B/r is 17 times greater than that required to inhibit colony formation of strain B. The ratio for NFT is four. Thus, it is clear that the factors which prevent death of strain B/r cells do not reduce the early effects on growth rate and respiration in this strain.

A comparison of the results in Tables IV and V (see Table VI) indicates that growth is more strongly inhibited than respiration by comparable concentrations of nitrofurans. Thus, while 0.3 μg/ml NFT reduces the rate of growth by approximately 30%, no effect on respiration is observed even at 2.5 μg/ml. A concentration of 5 μg/ml of nitrofurazone inhibits growth approximately 40% while the same concentration reduces the rate of respiration less than 10%. Similarly, the rate of growth is reduced by approximately 45% by 5 μg/ml of nitrofurantoin while the rate of oxygen uptake is decreased by approximately 20%. Furthermore, with higher concentrations of any of these agents, growth is brought almost to a halt under conditions where the rate of respiration is still over 60% of the control. It is clear, therefore, that while nitrofuran derivatives have an inhibitory effect on respiration in E. coli, this effect is of

Table IV

Effect of Nitrofuran Derivatives on Uptake of Oxygen by *E. coli* B and by *E. coli* B/r

Agent	Concentration (ug/ml)	*E. coli* B: Rate of O_2 uptake (ul/min)	*E. coli* B: % of control	*E. coli* B/r: Rate of O_2 uptake (ul/min)	*E. coli* B/r: % of control
Nitrofurantoin	0	0.65	100	0.45	100
	5	0.51	78	0.39	87
	10	0.51	78	0.35	78
	20	0.41	63	0.27	60
Nitrofurazone	0	0.42	100	0.39	100
	5	0.39	93	0.40	100
	10	0.38	90	0.38	97
	20	0.37	88	0.35	90
NFT	0	0.69	100	0.41	100
	1.25	0.64	93	0.42	100
	2.5	0.69	100	0.42	100
	0	0.47	100	0.40	100
	5	0.34	72	0.36	90
	10	0.35	74	0.28	70
	20	0.29	62	0.28	70

NOTE: The different physiological conditions of the bacteria from one experiment to another could account for the different rates of oxygen consumption by the controls.

Table V

Effect of Nitrofuran Derivatives on Growth of *E. coli* B and of *E. coli* B/r

Agent	Concentration (µg/ml)	Per Cent of Control Rate of Increase	
		E. coli B	*E. coli* B/r
Nitrofurantoin	5	54	62
	10	<10	31
	20	<10	10
Nitrofurazone	5	62	58
	10	30	29
	20	<10	<10
NFT	0.3	65	75
	1.0	15	38

secondary importance in bacteriostasis.

It is interesting to compare these data with previously published data on the effects of nitrofurantoin (62) and NFT (18) on macromolecule synthesis in E. coli B (see Table VI). A one-hour treatment with 5 ug/ml of nitrofurantoin reduced the net increase of both DNA and RNA by approximately 50% which is close to the 45% reduction in the rate of growth by the same concentration of nitrofurantoin. NFT, which selectively inhibits DNA synthesis in E. coli B, shows a similar pattern: treatment with 0.3 ug/ml reduces the net increase of DNA by 36% and the rate of growth by 35%. At 1 ug/ml of NFT the net increase of DNA is 84% inhibited and the growth rate 85% inhibited. A similar correlation between inhibition of growth and inhibition of macromolecule synthesis in E. coli B has recently been noted by Greenberg (48).

Table VI

Summary of Data for *E. coli* B

Compound	Concentration (µg/ml)	Per Cent of Control				
		Growth	O_2 Uptake	DNA*	RNA*	Protein*
Nitrofurantoin	5	54	78	~50†	~50†	-
	10	10	78	17†	12†	-
	20	10	63	-	-	-
Nitrofurazone	5	62	93	-	-	-
	10	30	90	-	-	-
	20	10	88	-	-	-
NFT	0.3	65	-	64‡	98‡	86‡
	1.0	15	-	16‡	70‡	84‡
	1.25	-	93	-	-	-
	5.0	-	72	-	-	-

* Values for 60 minutes exposure to agent.

† From reference 62.

‡ From reference 18.

B. Induction of Prophage Development in Lysogenic Bacteria by Nitrosoguanidine

In order to explore further the biological properties of nitrosoguanidine, its effects on a lysogenic strain of bacteria were investigated. The results of typical growth experiments are shown in Figure 2. lysis is indicated by the abrupt decrease in optical density after about 90 minutes.

Figure 2 shows that treatment of E. coli C600(λ) with 12 to 24 μg/ml nitrosoguanidine results in mass lysis of the bacteria. Treatment with 6 μg/ml results in partial lysis, while the highest concentration tested, 36 μg/ml, suppressed growth almost completely.

The results of a one-step growth experiment, shown in Figure 3, demonstrate that lysis of the cells is accompanied by release of free bacteriophage particles. After a latent period of about 60 minutes, the number of free phage particles in the culture rises to a value which indicates an average burst size of approximately 65 virus particles per original bacterium.

During the early stages of this work, the extent of lysis obtained in successive experiments with 18 μg/ml nitrosoguanidine varied considerably. This variation was traced to differences in sensitivity of the cells at various stages of the growth cycle. Late log phase cells are not nearly so effectively lysed by nitrosoguanidine treatment as are earlier log phase cells (Figure 4). In this respect, the action of nitrosoguanidine resembles that of ultraviolet light and is quite different from the action of NFT which, as is also shown in Figure 4, induces lysis of both early and late log phase cells. It

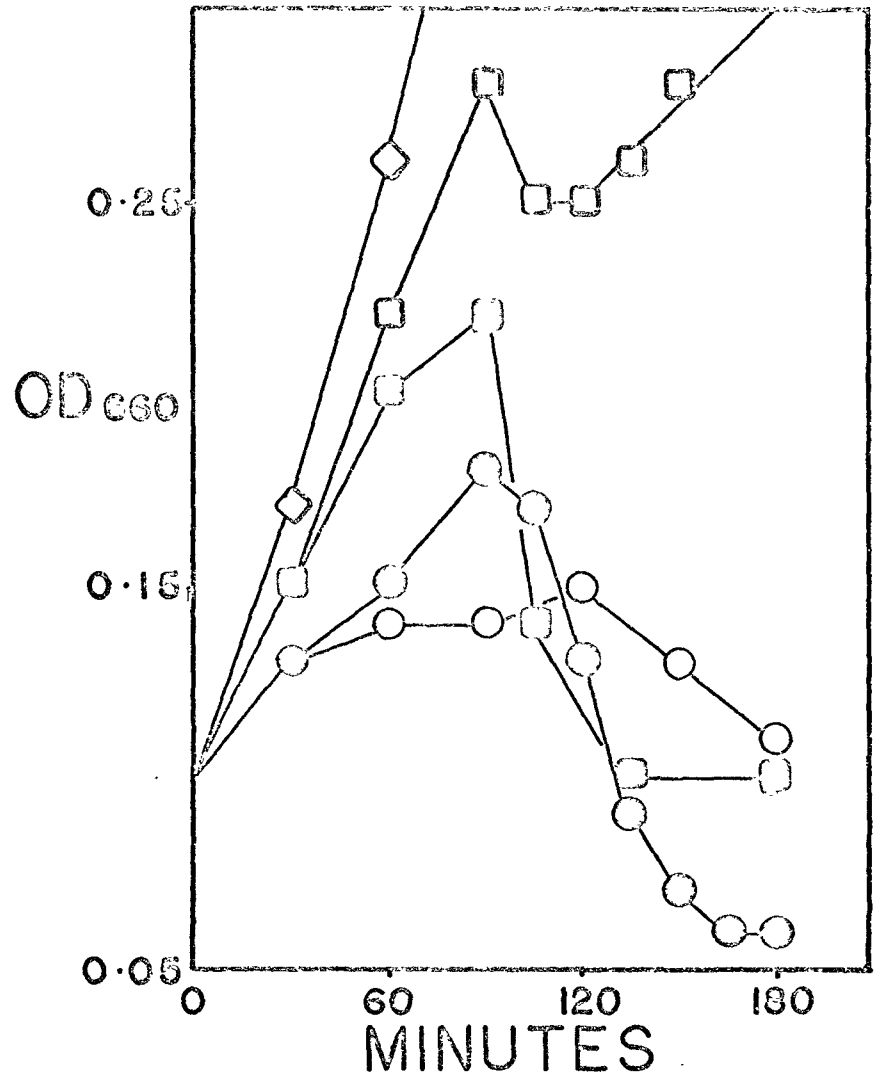


Figure 2. Effect of nitrosoguanidine on early log phase E. coli C600 (λ) cells. Early log phase cells ($OD_{660} \simeq 0.2$) were diluted with an equal volume of fresh medium (37°) containing the appropriate concentration of nitrosoguanidine, and incubated at 37° with shaking. Open diamonds, control; open squares, 6 µg/ml; closed squares, 12 µg/ml; closed circles, 24 µg/ml; open circles, 36 µg/ml nitrosoguanidine.

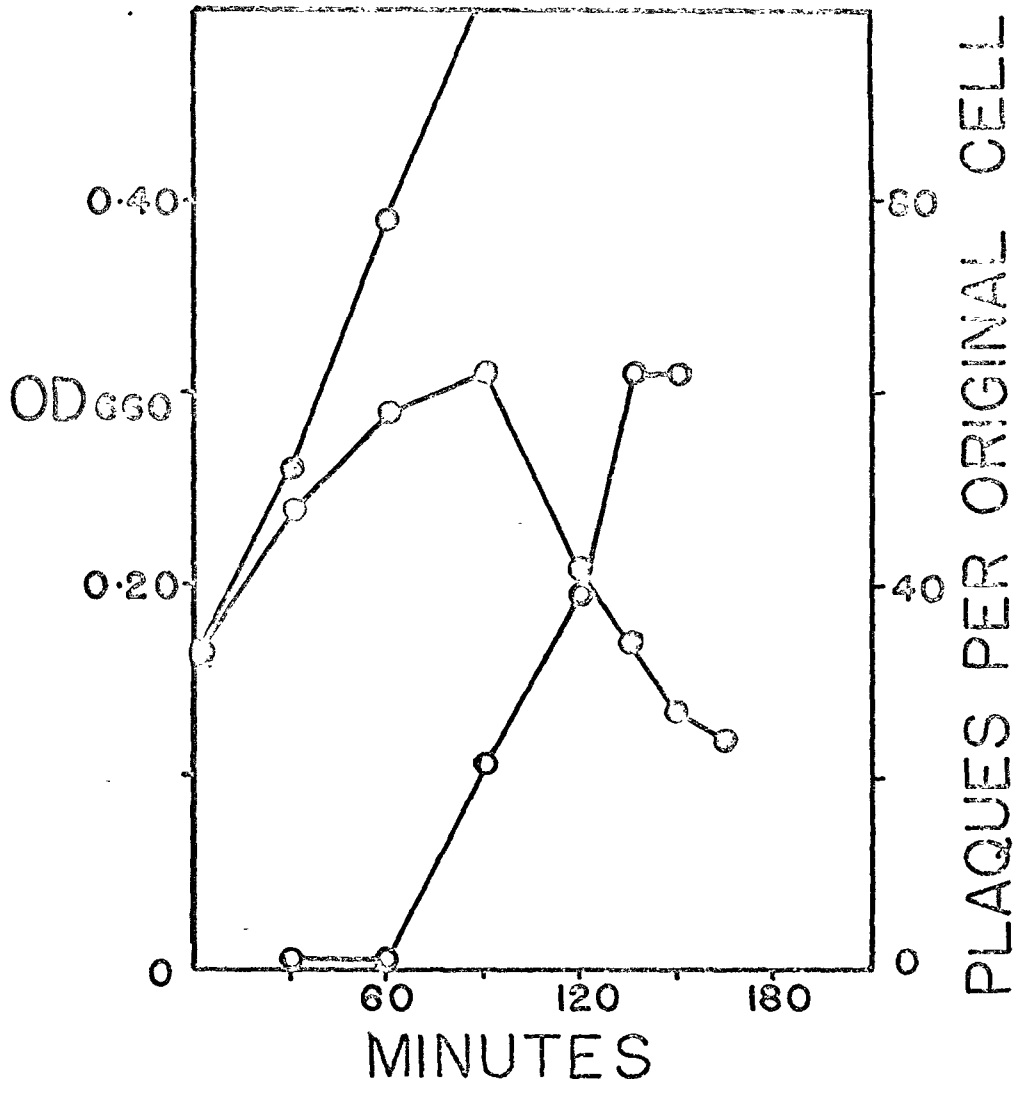


Figure 3. One-step growth experiment. Early log phase *E. coli* C600 (λ) cells were harvested, resuspended in fresh broth (37°) containing the indicated concentration of nitrosoguanidine, and incubated at 37° with shaking. After 30 min. a sample was removed, diluted and incubated at 37° with shaking. At various intervals, samples were plated for plaque counts. Open circles, one-step growth experiment with 18 µg/ml nitrosoguanidine (right-hand ordinate scale). Optical density measurements: half-closed circles, control; closed circles, 18 µg/ml nitrosoguanidine.

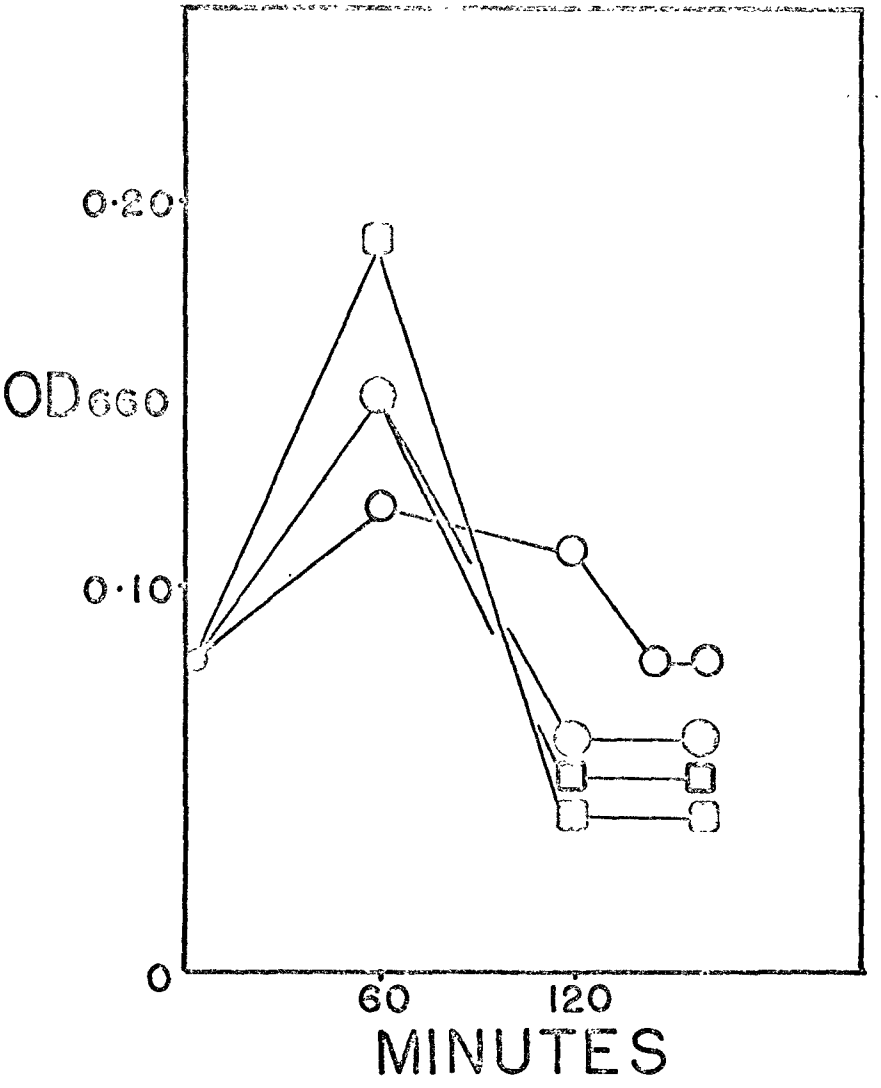


Figure 4. Action of nitrosoguanidine (18 ug/ml) and NFT (0.3 ug/ml) on E. coli C600 (λ) cells taken from early log phase cultures (ELP) ($OD_{660} \simeq 0.2$) and from late log phase cultures (LLP) ($OD_{660} \simeq 0.7$). Closed squares, NFT, ELP; open squares, NFT, LLP; closed circles, nitrosoguanidine, ELP; open circles, nitrosoguanidine, LLP.

seems likely that the conditions used by Adelberg, et al. (127) who have described optimal conditions for the production of mutations in E. coli K12(λ) by nitrosoguanidine, were unsuitable for prophage induction and bacterial lysis.

Other agents which induce prophage development inhibit DNA synthesis selectively [ultraviolet light (128), Mitomycin C (129), NFT (18) and nitrogen mustard (130)]. It is of considerable interest, therefore, that Terawaki and Greenberg have shown that nitrosoguanidine has no such selectivity, inhibiting the net increase of protein, RNA and DNA almost equally (48). The results of the next section of this thesis confirm and extend Terawaki and Greenberg's observation that nitrosoguanidine is not a specific inhibitor of DNA synthesis.

It is noteworthy that an independent study of the effects of various anticancer agents on inducible systems of Bacillus megaterium has shown that 10 to 15 μg/ml of nitrosoguanidine induces prophage development in Bacillus megaterium strain 899 which is lysogenic for phage 1 (140).

C. Effects of Nitrosoguanidine on Macromolecule Synthesis in E. coli B

As noted previously, agents such as NFT, ultraviolet light and Mitomycin C which induce prophage development in lysogenic bacteria also selectively inhibit DNA synthesis in E. coli B. The parallel between the inhibition of macromolecule synthesis and of bacterial growth by derivatives of 5-nitrofuran has been mentioned in a preceeding section. Since nitrosoguanidine can inhibit bacterial growth and induce prophage development in lysogenic strains of bacteria, it became of interest to investigate the effect of nitrosoguanidine on the synthesis of macro-

molecules in E. coli B.

Radioactive precursors of proteins and of nucleic acids were used to detect early effects of nitrosoguanidine on macromolecule synthesis. Protein synthesis was measured as the incorporation of leucine-1-C^{14} into acid-precipitable material, while the incorporation of adenine-8-C^{14} into acid-precipitable material was used to estimate the amount of nucleic acid synthesis. Advantage was taken of the fact that dilute sodium hydroxide solution hydrolyses RNA but not DNA (117) to separate the incorporated adenine into alkali-stable and alkali-labile components attributable to DNA and RNA respectively. While this experimental approach is technically more convenient than the measurement of the amount of each type of macromolecule by standard colorimetric methods, other advantages accrue as well. For example, analytical data represent the net change in the total amount of material and reflect both synthesis and metabolic breakdown, while incorporation data more nearly reflect the synthesis of macromolecules. In addition, the errors in analytical data can be expected to be greater than those of incorporation data because of the difficulty, in the former case, of detecting small differences in the relatively large amount of macromolecule present at the beginning of the experiment.

In order to ascertain whether incorporation experiments lead to the same conclusions as experiments involving standard analytical techniques based on colour reactions, the effect of NFT on the incorporation of labelled precursors of macromolecules was determined in E. coli B. The data of Table VII show that NFT selectively inhibits the incorporation of adenine-8-C^{14} into alkali-stable counts, consistent

Table VII

Comparison of Analytical and Isotopic Incorporation Techniques:

Effect of NFT on Macromolecule Synthesis in *E. coli* B

Concentration (ug/ml)	Time (min.)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
0.6	15	85	100	66
	30	88	99	35
	45	80	80	40
	60	82 (86)*	78 (100)	40 (55)

* Values in parentheses are those obtained by Kato, et al. (18) using analytical procedures.

with the findings of Kato, et al. who found that NFT selectively inhibits the net increase of DNA (18). These workers report analytical data obtained after 60 minutes exposure to NFT, which show that a concentration of 0.6 μg/ml NFT reduces the increase in total protein to 86% of control, has no effect on the level of RNA but reduces the increase in total DNA to 55% of control. It is apparent, therefore, that these two procedures lead to the same general conclusion.

Data from typical incorporation experiments are shown in Figures 5 and 6. These data have been tabulated using the "per cent of control" value for the treated sample at each sampling time.

The data of Table VIII show that while nitrosoguanidine does inhibit bacterial DNA synthesis, it also causes a comparable reduction in the synthesis of RNA and of protein. Although the inhibition of DNA synthesis is not specific, as is the case with NFT, there does appear to be some slight selectivity, notable at high concentrations, in that in these experiments the synthesis of DNA is always inhibited to the greatest extent. The effect of nitrosoguanidine on macromolecule synthesis can, however, best be described as a nonselective general inhibition.

The data of Table IX and the curves of Figure 6 show the effect of nitrosoguanidine on macromolecule synthesis in E. coli B at times that are short in comparison with the generation time of 45 minutes. It can be seen that macromolecule synthesis is reduced appreciably within 5 to 10 minutes after exposure to the chemical. An initial effect on protein synthesis, apparent at 20 μg/ml, is quickly followed by a general inhibition of macromolecule synthesis.

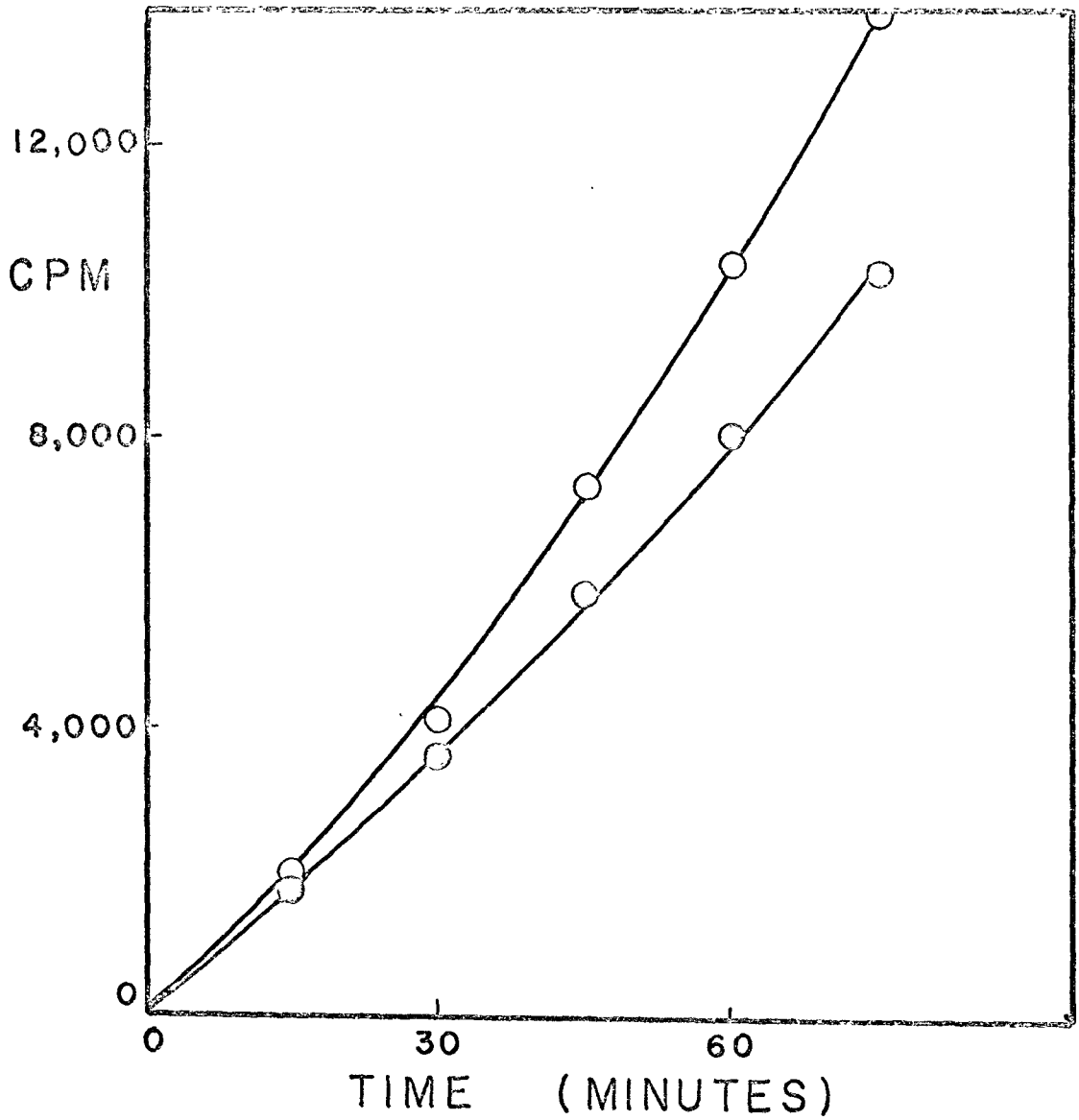


Figure 5a. Effect of 6 ug/ml nitrosoguanidine on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material of E. coli B. Control, open circles; treated, closed circles.

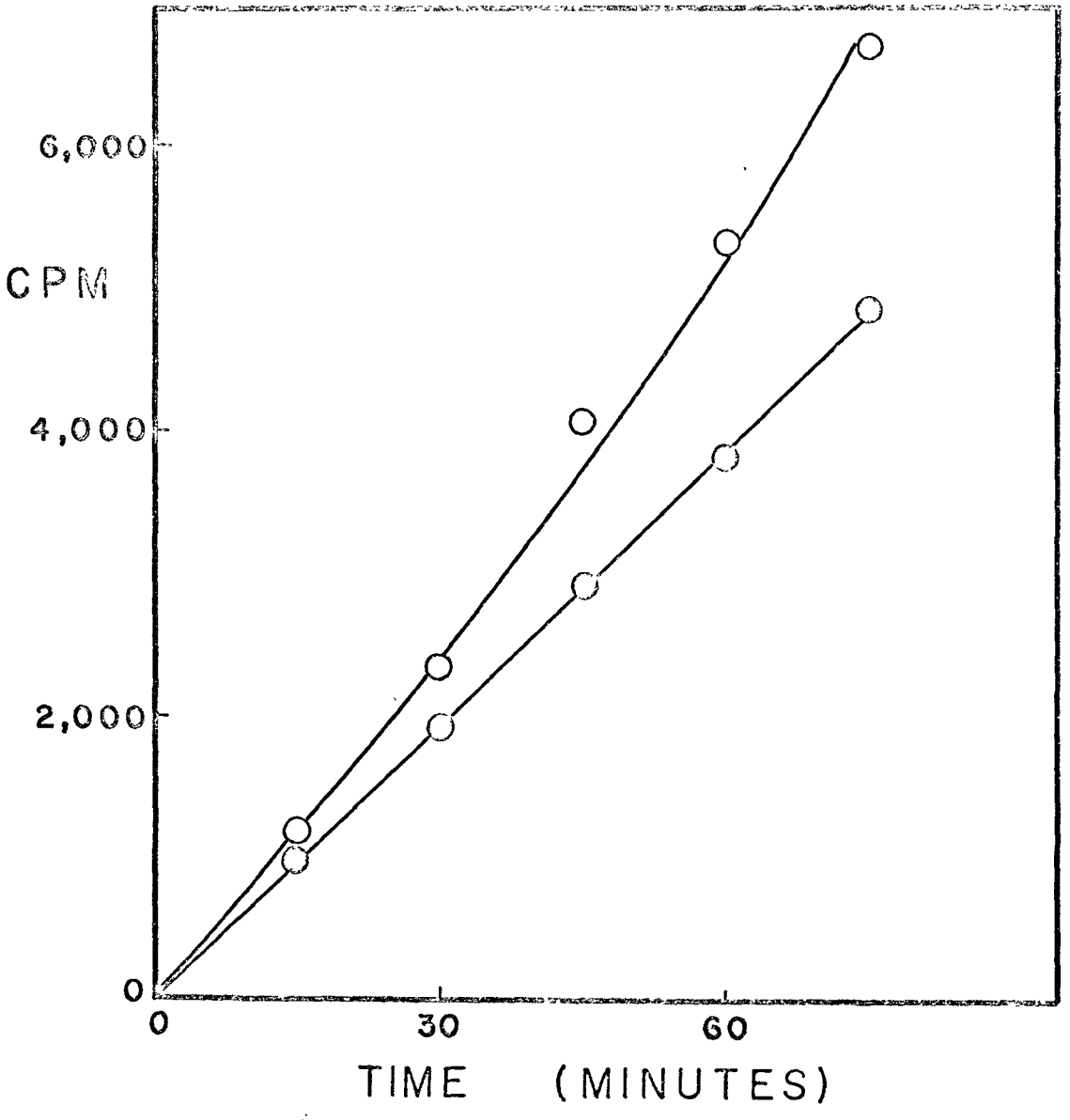


Figure 5b. Effect of 6 µg/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-labile portion of the TCA-insoluble material of E. coli B. Control, open circles; treated, closed circles.

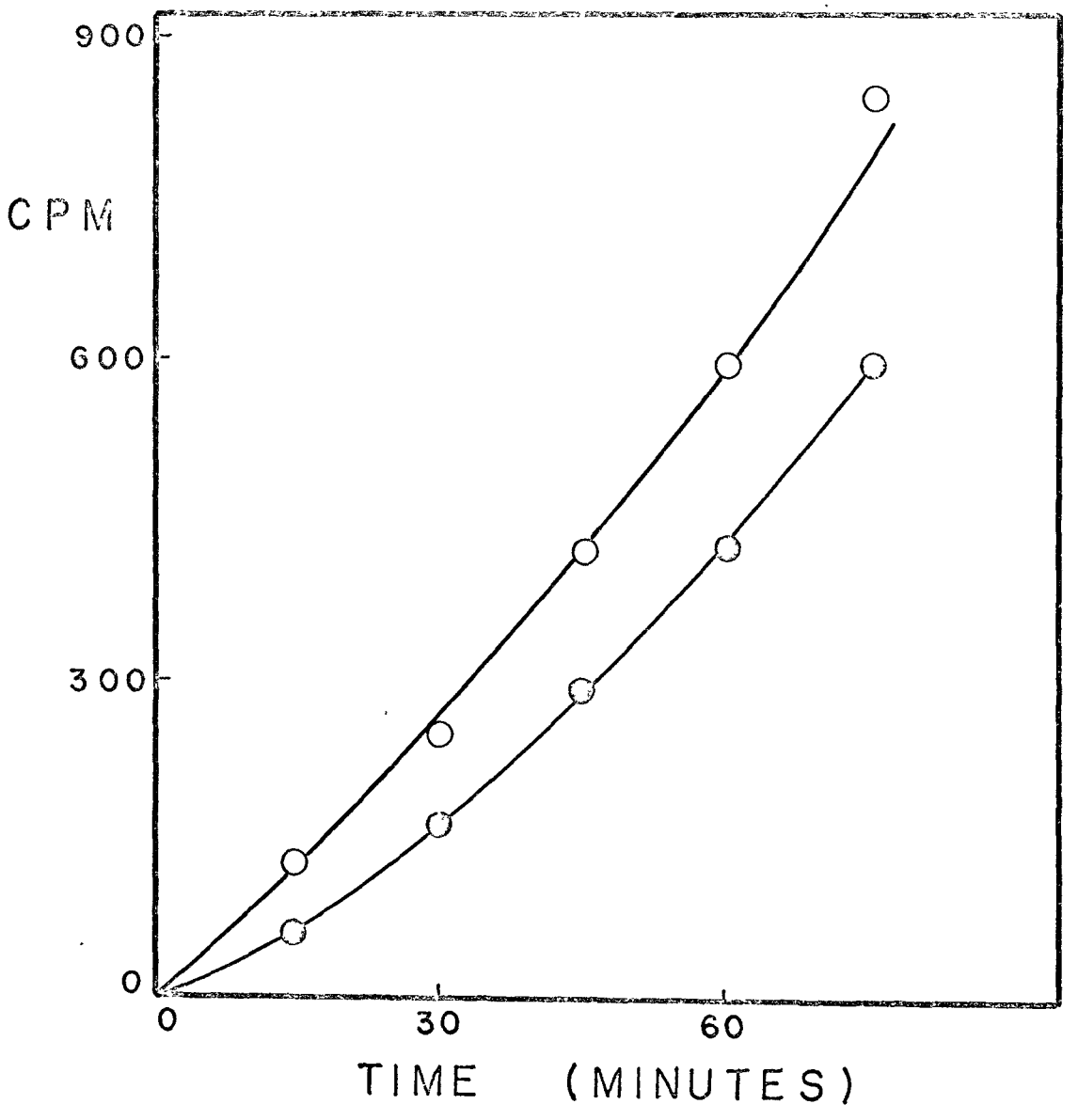


Figure 5c. Effect of 6 ug/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-stable portion of the TCA-insoluble material of E. coli B. Control, open circles; treated, closed circles.

Table VIII

Effect of Nitrosoguanidine on Macromolecule Synthesis in E. coli B

Concentration (µg/ml)	Time (min.)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
6	15	84	84	50
	30	87	81	67
	45	80	72	69
	60	77	72	71
	75	74	72	71
10	15	63	100	50
	30	74	100	38
	45	80	92	46
	60	80	80	60
	75	80	75	65
24	15	69	47	41
	30	73	49	33
	45	78	55	30
	60	83	60	33
	75	88	68	36
36	15	65	57	33
	30	54	43	17
	45	51	42	12
	60	48	44	11
	75	45	48	10

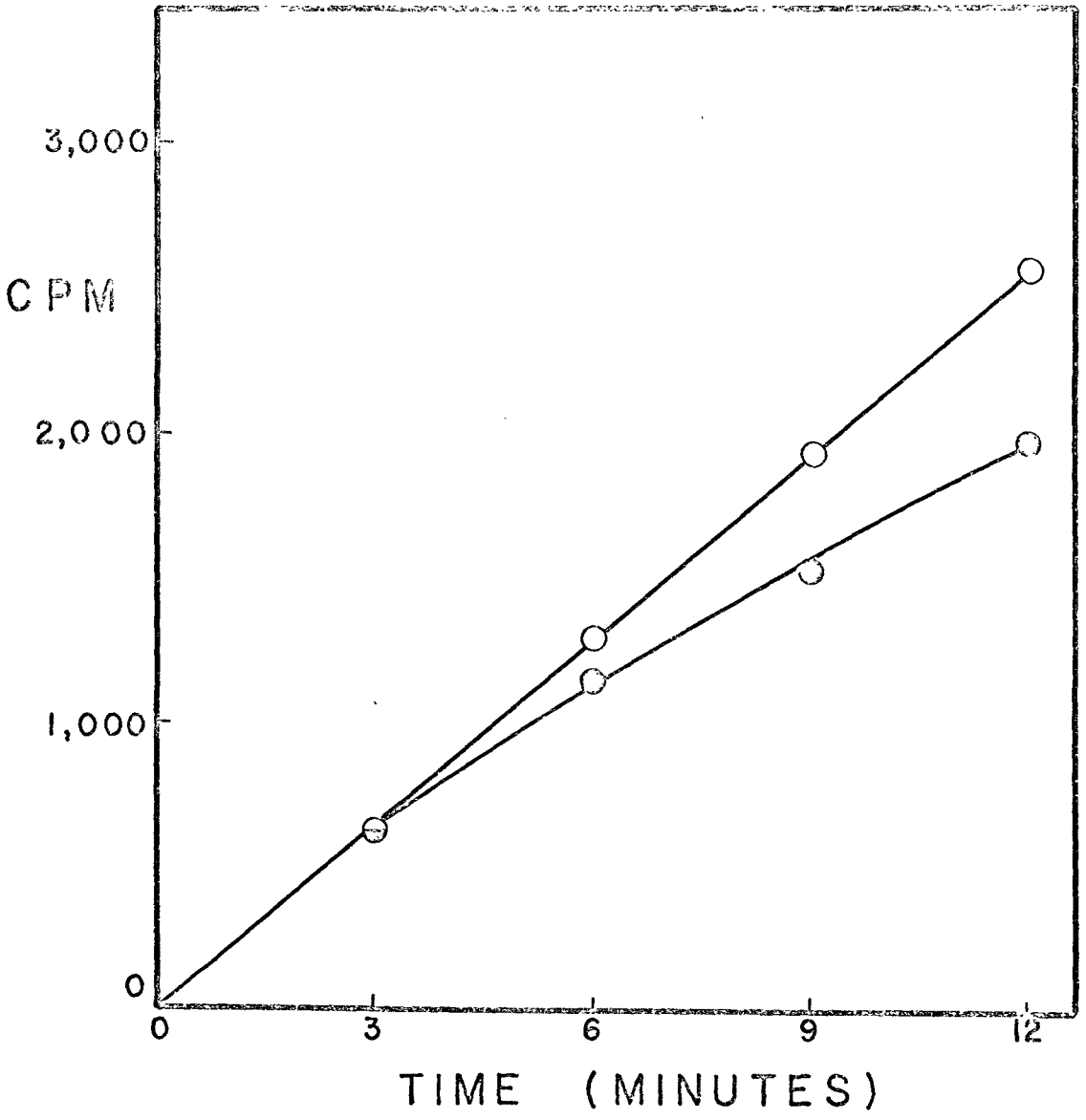


Figure 6a. Effect of 10 µg/ml nitrosoguanidine on the rate of incorporation of leucine-l-C^{14} into TCA-insoluble material of E. coli B at short times. Control, open circles; treated, closed circles.

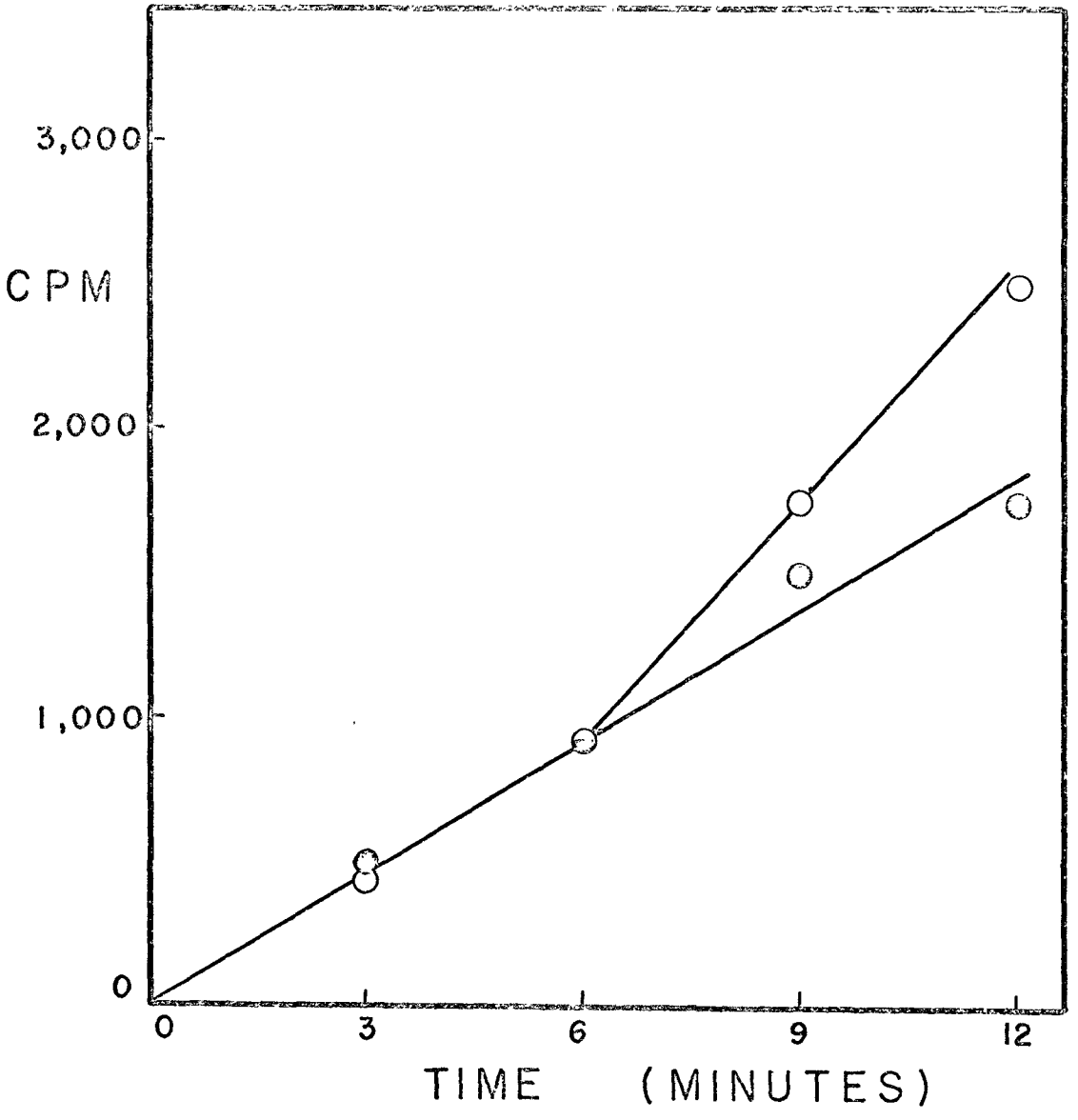


Figure 6b. Effect of 10 ug/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-labile portion of the TCA-insoluble material of E. coli B at short times. Control, open circles; treated, closed circles.

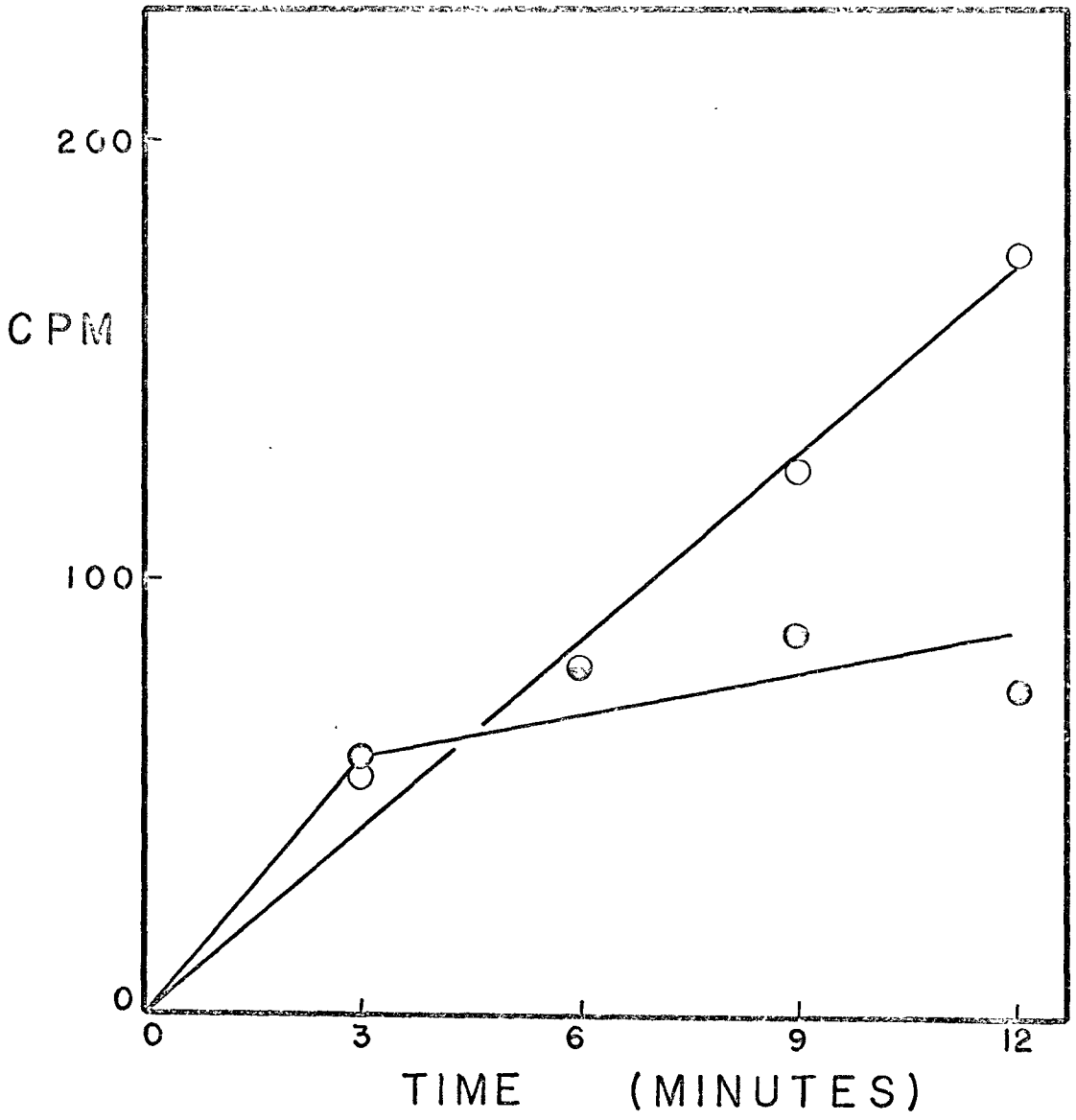


Figure 6c. Effect of 10 ug/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-stable portion of the TCA-insoluble material of E. coli B at short times. Control, open circles; treated, closed circles.

Table IX

Effect of Nitrosoguanidine on Macromolecule Synthesis in *E. coli* B at Short Times

Concentration (µg/ml)	Time (min.)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
10	3	100	100	100
	6	89	100	86
	9	82	85	61
	12	77	69	49
20	2	70	100	100
	5	75	100	90
	8	72	90	64
	11	69	70	40

The possibility that the observed inhibition of the synthesis of macromolecules might merely be a function of the surviving fraction of the original number of bacteria was examined. The results of a typical survival experiment are shown in Table X. Comparison of these data with incorporation data (Table VIII) show that no such simple relation exists. While a 45 minute exposure of E. coli B to 10 µg/ml nitrosoguanidine reduces survival by 8%, the synthesis of DNA is 50% of the control and protein synthesis 80% of control. Eighty per cent of the cells do not survive a 45 minute exposure to 20 µg/ml nitrosoguanidine while after the same period of time, 24 µg/ml reduces DNA synthesis to 30% of control and protein synthesis to 78% of control. Thus, the incorporation data are not merely the consequence of a fraction of cells in the population synthesizing macromolecules at the normal rate.

The conclusion that nitrosoguanidine is a general inhibitor of macromolecule synthesis in E. coli B has also been reached by Terawaki and Greenberg on the basis of using analytical data obtained over a 60 minute exposure interval (48). The present study has shown that a significant reduction in macromolecule synthesis occurs within ten minutes exposure of the bacteria to nitrosoguanidine. Terawaki and Greenberg have also noted a parallel between the inhibition of macromolecule synthesis and the inhibition of bacterial growth after a one hour treatment of E. coli B with 10 µg/ml nitrosoguanidine (48).

Although most other agents which induce prophage development in lysogenic bacteria inhibit DNA synthesis selectively in E. coli B, this does not appear to be a prerequisite for induction.

Table X

Effect of Nitrosoguanidine on Viability of *E. coli* B Cells

Concentration	Colonies formed per sample at:	
	0 time	45 minutes
0 (control)	264	588
10 µg/ml	302	276
20 µg/ml	339	70

D. Effects of Nitrosoguanidine and MNTS on Macromolecule Synthesis in Euglena

Low doses of nitrosoguanidine or MNTS bleach Euglena and inhibit growth temporarily while higher doses are lethal. As noted in the preceeding section, there appears to be a parallel between the inhibition of bacterial growth and the inhibition of bacterial macromolecule synthesis by nitrosoguanidine. It was, therefore, of interest to determine whether nitrosoguanidine and MNTS inhibit the growth of Euglena by inhibiting the synthesis of nucleic acids and protein. Techniques, similar to those just described for E. coli, were developed to detect early changes in the rates of macromolecule synthesis in Euglena treated with these agents.

Control experiments were carried out to establish that the incorporation of suitable labelled precursors represents a valid measure of protein, RNA and DNA synthesis.

Euglena cells were exposed to leucine-1-C^{14} for four hours and then extracted with cold methanol and with trichloroacetic acid solution. The residue was hydrolyzed with 6 N HCl. Chromatographic analysis (Figure 7) of the hydrolysate reveals only one radioactive component having an R_f value identical to that of leucine, indicating that exogenous leucine is incorporated into protein without detectable scrambling of the label. This result is similar to that obtained by Brawerman and Chargaff with stationary-phase Euglena (131).

With many organisms the DNA precursor of choice would be radioactive thymine or thymidine and the RNA precursor labelled uracil. However, pyrimidines are incorporated into the trichloroacetic

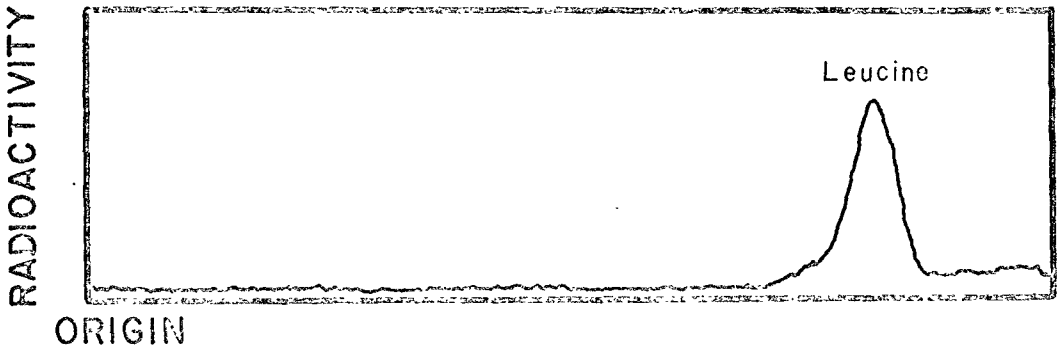


Figure 7. Radiochromatogram of Euglena hydrolysate following leucine-1-C^{14} incorporation. Euglena were exposed to labelled leucine for 4 hours and then collected, extracted with methanol and 5% TCA solution prior to hydrolysis in 6 N HCl. The hydrolysate was chromatographed in a butanol, acetic acid, water solvent.

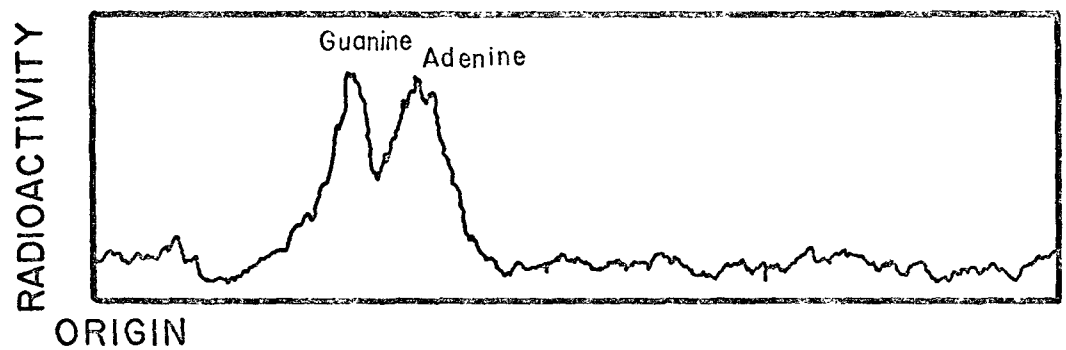


Figure 8. Radiochromatogram of nucleic acid bases from alkaline hydrolysates of Euglena grown in the presence of adenine-8-C^{14}. The nucleotides liberated by alkaline hydrolysis of the TCA-insoluble material were converted to purine and pyrimidine bases by heating with 88% formic acid in a sealed tube at 175° for 30 min. The bases were chromatographed in an isopropanol-HCl solvent (120).

acid-insoluble material of Euglena at a very slow rate. Furthermore, most of the trichloroacetic acid-insoluble counts from tritiated thymidine treatment of Euglena are not found in the DNA at all but are associated with RNA and protein (133). Since purines are known to be incorporated into Euglena nucleic acids much more efficiently than are pyrimidines, adenine was selected with the hope that, after extraction of the TCA-insoluble fraction with alkali to remove RNA, the remaining radioactivity would be due to DNA. The difficulty encountered by Ray (102) in achieving useful levels of incorporation of radioactive adenine was overcome by the use of Euglena culture medium of pH 6.8 instead of pH 3.5 used by Ray.

Brawerman and Chargaff have reported that in non-growing Euglena the label from adenine-8-C^{14} is recovered from the alkaline hydrolysate of the TCA-insoluble material as adenylic and guanylic acids (131). Figure 8 shows the results of a similar experiment with log-phase Euglena administered adenine-8-C^{14} for four hours. The nucleotides liberated by alkaline hydrolysis of the TCA-insoluble material were converted to purine and pyrimidine bases prior to chromatography. Only adenine and guanine were labelled. (Labelled guanine is to be expected since adenine is readily converted into guanine by Euglena (132).)

In order to show that adenine-8-C^{14} was incorporated into DNA the following experiment was performed. Nucleic acids were extracted from a large batch of Euglena cells which included a small proportion of radioactive cells labelled by exposure to adenine-8-C^{14} for 4 hours, along with the main portion of unlabelled cells which were

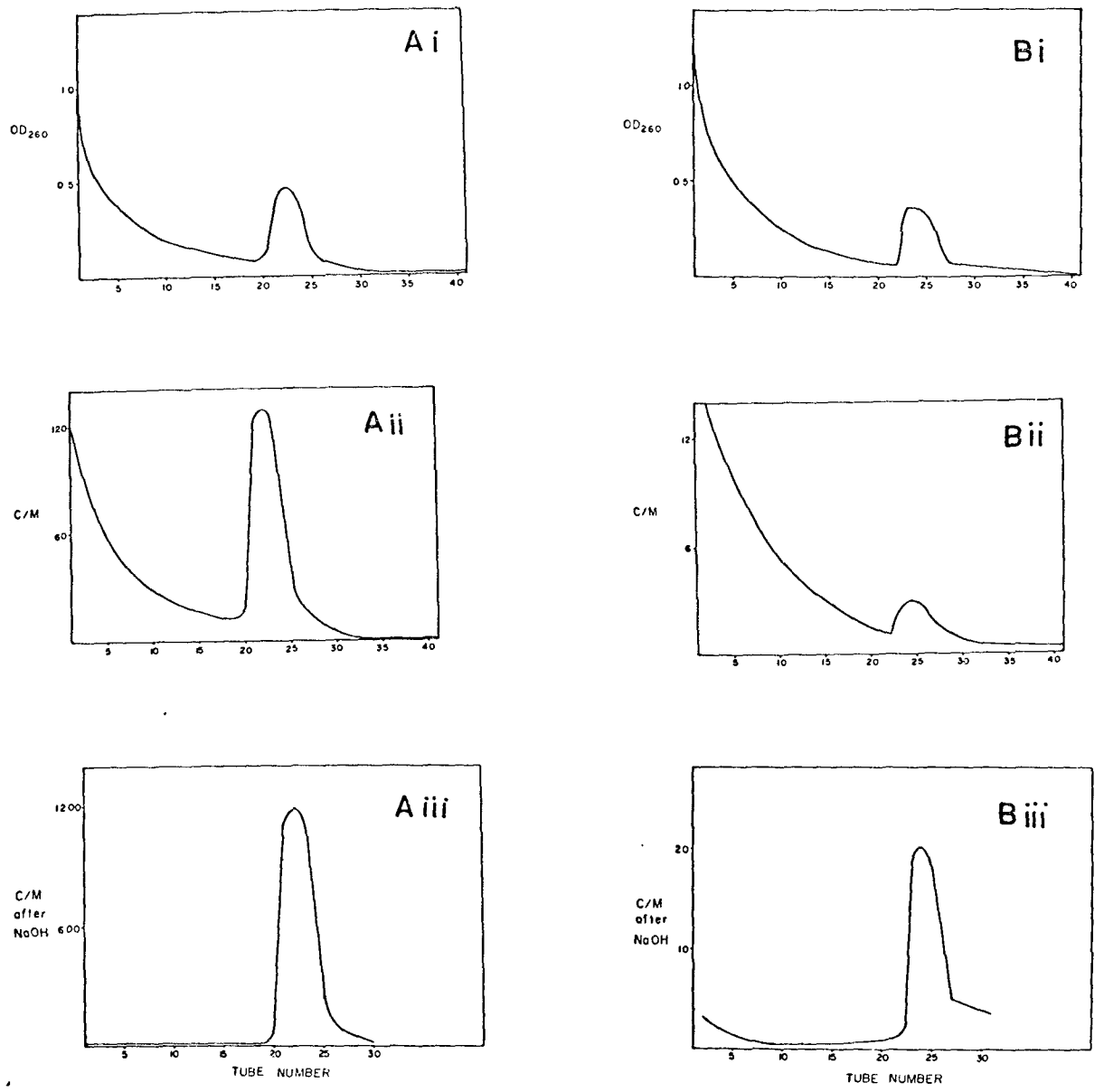


Figure 9. Density-gradient centrifugation of nucleic acids from Euglena grown with adenine-8-C^{14} for 4 hours. After labelling cells were diluted with unlabelled cells to provide "carrier" DNA. The cells were then lysed and, after deproteinization, the extracts were examined by density-gradient centrifugation. A, control; B, 20 µg/ml nitrosoguanidine. (i) OD_{260} (due mainly to carrier DNA); (ii) radioactivity of total nucleic acids; (iii) alkali-stable radioactivity.

used to provide "carrier". The nucleic acids were subjected to cesium chloride density gradient ultracentrifugation. The contents of each centrifuge tube were fractionated and analyzed. Figure 9i shows the variation of absorbance at 260 mμ along the length of the centrifuge tube. In all 3 parts of Figure 9 the right-hand plot (B) refers to a culture, the radioactive portion of which was exposed to nitrosoguanidine. Figure 9ii shows the distribution of radioactivity along the tube. The marked reduction in radioactivity in the nucleic acids from the cells treated with nitrosoguanidine is striking. Figure 9iii which shows the distribution of radioactivity along the tube after treatment of the samples with dilute alkali shows that the radioactivity at the bottom of the tube is due to RNA. The alkali-stable radioactivity is, therefore, attributable to DNA. While this work was in progress other workers presented evidence that purines are suitable precursors of Euglena DNA (134,135).

Thus, incorporation of leucine-1-C^{14} into acid-insoluble material represents protein synthesis. Incorporation of the label from adenine-8-C^{14} into the alkali-labile portion of the acid-insoluble material represents RNA synthesis and into the alkali-stable portion of the acid-insoluble material represents DNA synthesis.

The data of Table XI and of Figure 10 show that nitrosoguanidine inhibits the incorporation of labelled precursors into protein, RNA and DNA. The synthesis of all three types of macromolecule is inhibited to approximately the same extent, a result which might have been expected on the basis of results of similar experiments with E. coli B. An interesting feature of these data is the low inhibition of leucine-

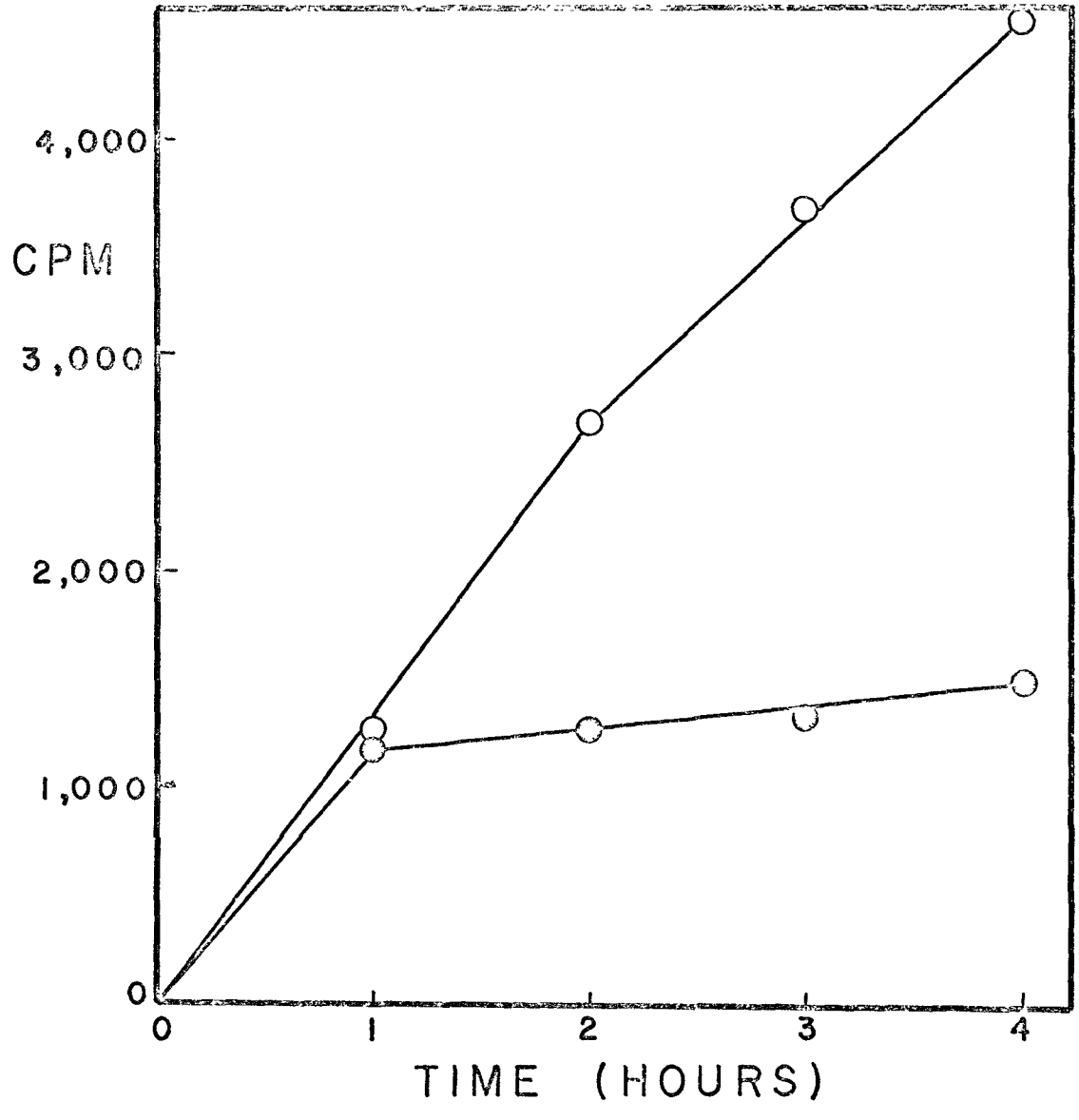


Figure 10a. Effect of 10 µg/ml nitrosoguanidine on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

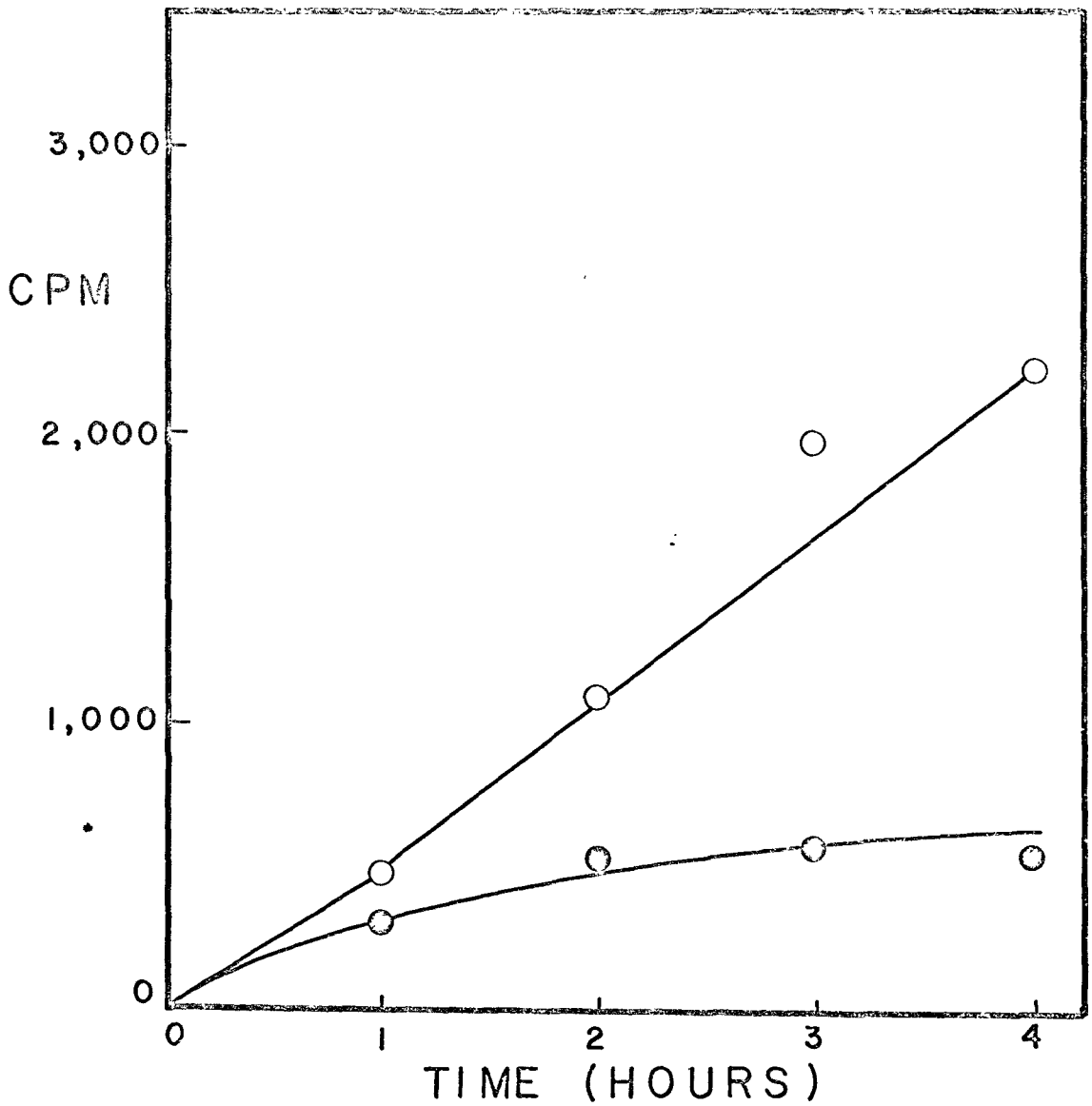


Figure 10b. Effect of 10 µg/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-labile portion of the TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

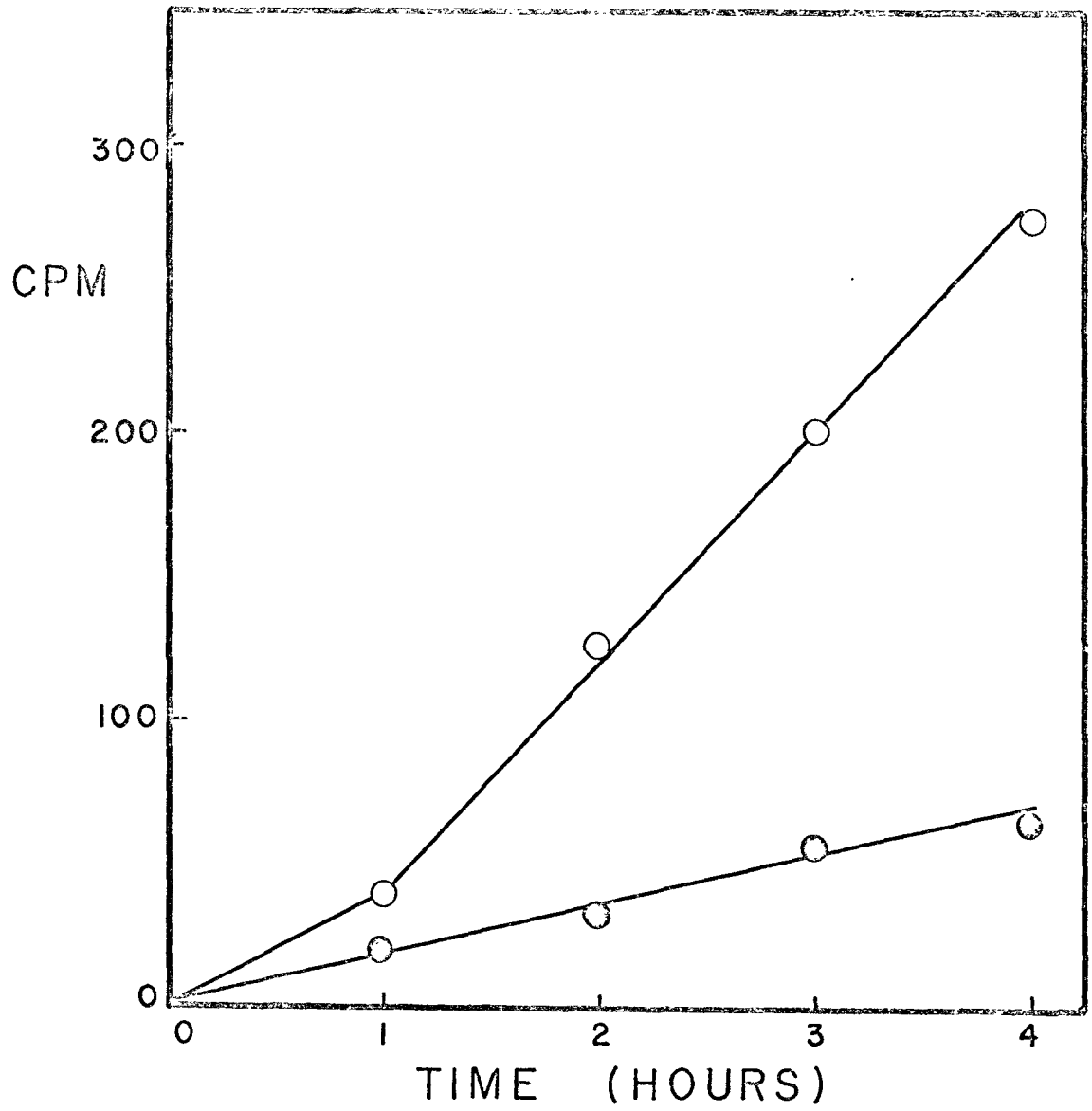


Figure 10c. Effect of 10 ug/ml nitrosoguanidine on the rate of incorporation of adenine-8-C^{14} into the alkali-stable portion of the TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

Table XI

Effect of Nitrosoguanidine on

Macromolecule Synthesis in Euglena

Concentration (μg/ml)	Time (hours)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
10	1	94	64	52
	2	47	49	26
	3	36	29	28
	4	33	24	24
25	1	54	25	23
	2	40	13	16
	3	27	13	15
	4	30	15	10

1-C^{14} incorporation at the one hour point both with 10 µg/ml and 25 µg/ml nitrosoguanidine. This observation prompted examination of leucine-1-C^{14} incorporation at times shorter than one hour. Figure 11 shows that while nucleic acid synthesis is significantly inhibited by 15 minutes exposure to nitrosoguanidine, the synthesis of proteins is inhibited only after 45 minutes exposure to the agent.

The data of Table XII show that from one hour exposure onwards the inhibition of macromolecule synthesis in Euglena by MNTS is non-selective, as was the case with nitrosoguanidine inhibition. In addition, Figure 12 shows that with MNTS the inhibition of adenine-8-C^{14} incorporation preceeds inhibition of leucine-1-C^{14} incorporation. Again this is reminiscient of the effect of nitrosoguanidine.

The growth of Euglena is temporarily inhibited by low concentrations of nitrosoguanidine (44) and of MNTS (McCalla, unpublished results). It is apparent that the inhibition of macromolecule syntheses by similar concentrations of these agents could account for the inhibition of growth. McCalla has recently described a mutant strain of Euglena which is resistant to the effects of nitrosoguanidine (136). Nucleic acid synthesis in this strain is much less sensitive to nitrosoguanidine than is the corresponding process in the sensitive parental strain, a result which is consistent with the notion that inhibition of nucleic acid synthesis might contribute to the inhibition of growth. Evidence is presented in the final section of this thesis which indicates that MNTS reacts with Euglena nucleic acids in vivo, suggesting that nucleic acid synthesis could be

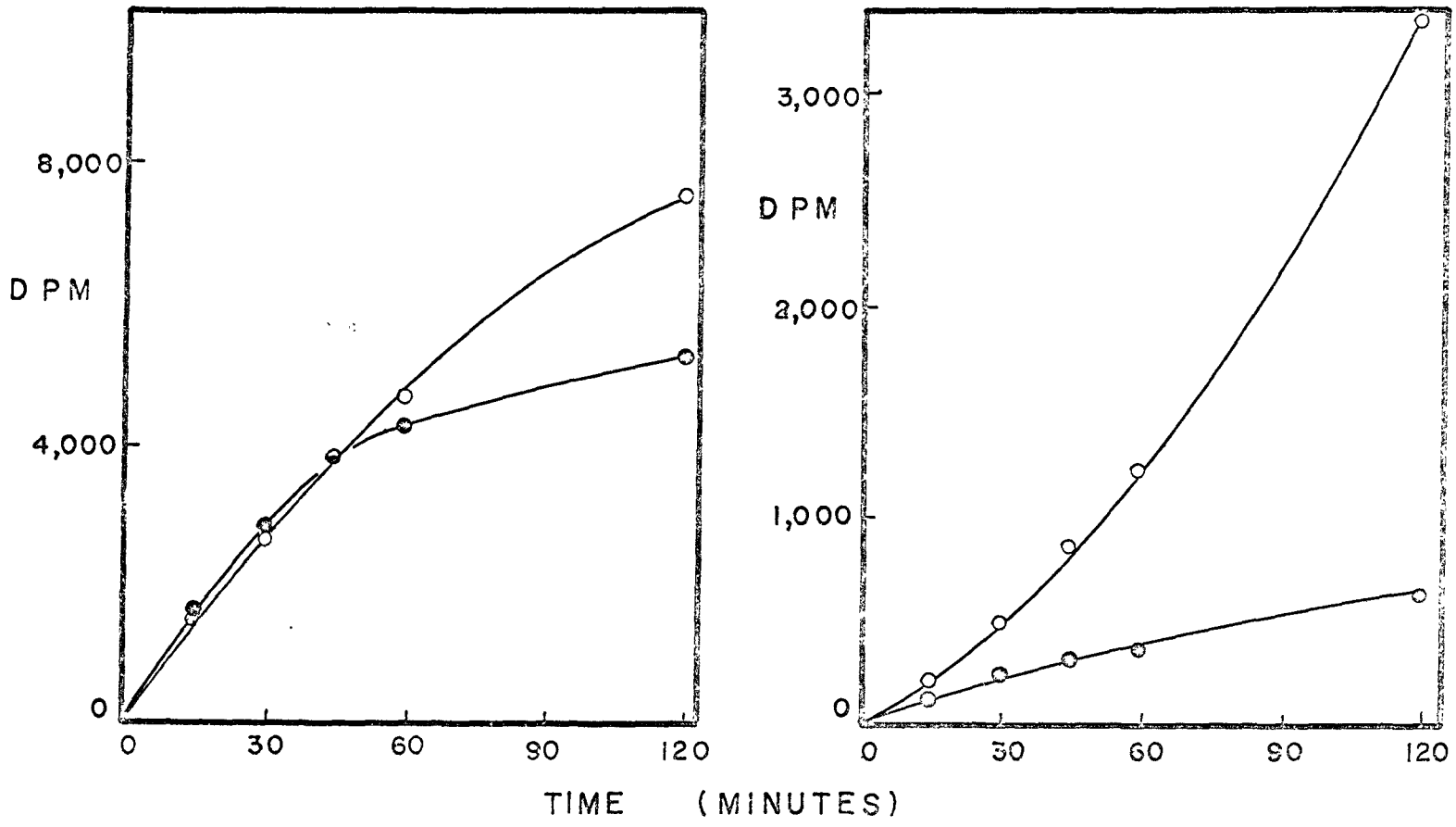


Figure 11. Effect of nitrosoguanidine on macromolecule synthesis in Euglena at short times. The left-hand plot shows the effect on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material. The right-hand plot shows the effect on the rate of incorporation of adenine-8-C^{14} into TCA-insoluble material. Open circles, controls: closed circles, 10 ug/ml nitrosoguanidine.

Table XII

Effect of MNTS on Macromolecule Synthesis in Euglena

Concentration (µg/ml)	Time (hours)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
2.5	1	56	55	42
	2	54	48	36
	3	50	46	29
	4	52	45	29
10	1	-	21	20
	2	-	14	9
	3	-	13	12
	4	-	12	8

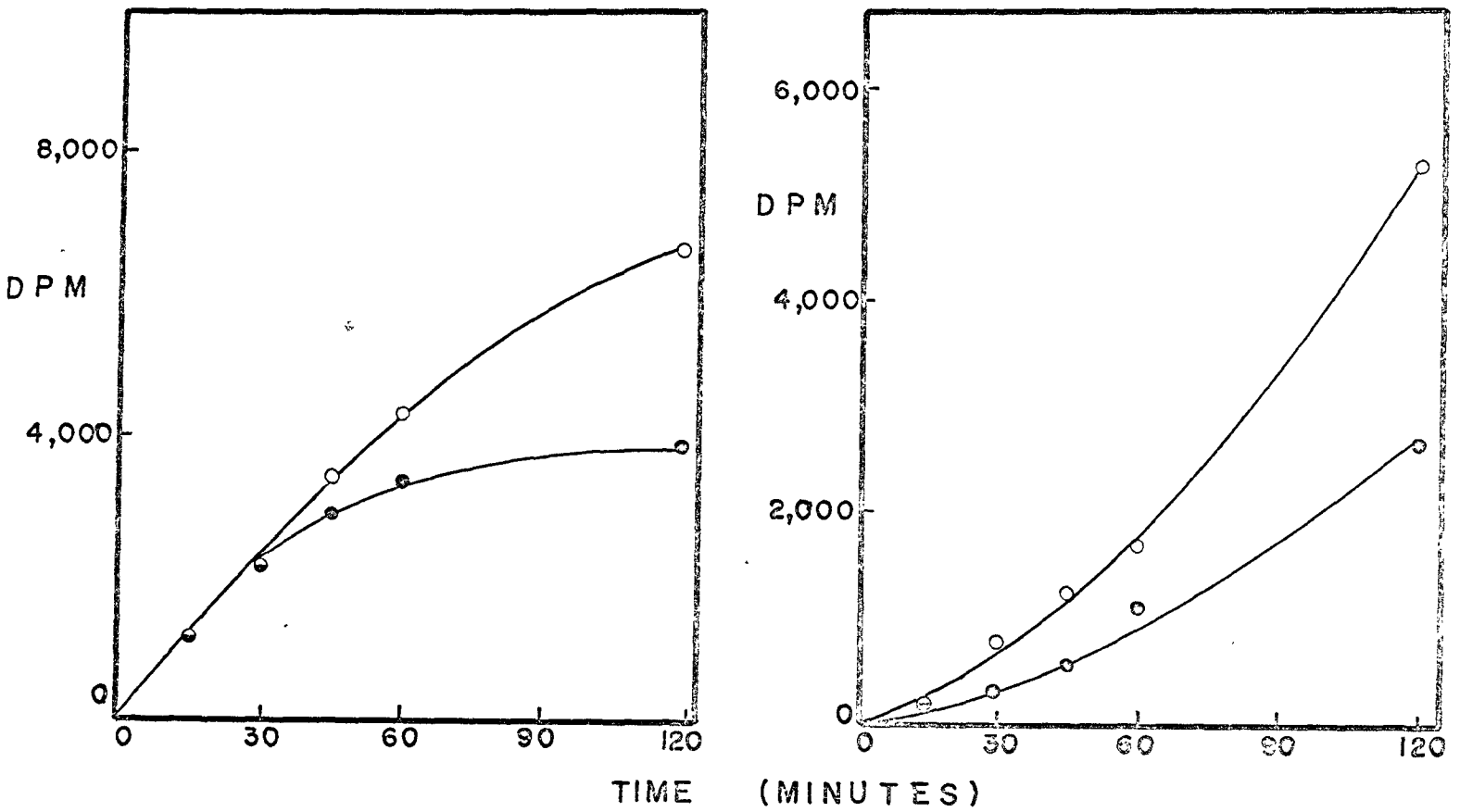


Figure 12. Effect of MNTS on macromolecule synthesis in Euglena at short times. The left-hand plot shows the effect on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material. The right-hand plot shows the effect on the rate of incorporation of adenine-8-C^{14} into TCA-insoluble material. Open circles, controls; closed circles, 2.5 ug/ml MNTS.

inhibited by damage to the nucleic acid primers.

The initial lag in inhibition of protein synthesis when Euglena is treated with nitrosoguanidine or MNTS raises the question of whether protein synthesis might be inhibited as a consequence of the reduced amount of m-RNA being synthesized or whether some other effect is involved. The former possibility might be tested by using Actinomycin D which selectively inhibits DNA-dependent RNA synthesis in many organisms (137). Unfortunately, however, Actinomycin D does not appear to permeate Euglena grown in pH 6.8 medium. Using pH 3.5 medium, Pogo and Pogo (97) found that concentrations of Actinomycin D which inhibit RNA synthesis about 50% have no effect on protein synthesis for a period of about 9 hours, suggesting that Euglena m-RNA is stable during that period of time. While this result does not necessarily apply to Euglena grown in pH 6.8 medium, it seems highly improbable that a change in the pH of the growth medium would reduce the stability of m-RNA, by a factor of about ten; that is, to the point where the amount of m-RNA would be appreciably reduced one hour after exposure to nitrosoguanidine. It is concluded, therefore, that the observed inhibition of protein synthesis is not a consequence of inhibition of m-RNA production.

Another possibility, which seems more likely, is that some component of the protein-synthesizing system is slowly inactivated by nitrosoguanidine and by MNTS, while DNA and RNA syntheses are immediately inhibited, perhaps as a result of damage to DNA.

E. Effects of NFT and Nitrofurantoin on Macromolecule Synthesis in Euglena

Frequently, the known biochemical effect of an agent on a particular organism is used as the basis for interpretation of observations made with other organisms. Based upon results obtained with E. coli it might have been expected that NFT would be a specific inhibitor of DNA synthesis in Euglena while nitrofurantoin, nitrosoguanidine and MNTS would inhibit macromolecule synthesis non-selectively. Results presented in the preceeding section indicate that this is the case with nitrosoguanidine and MNTS except at short times of exposure. The same techniques as described in the preceeding section have been used to measure the effects of NFT and nitrofurantoin on macromolecule synthesis in Euglena.

It is apparent from the data of Table XIII and of Figure 13 that NFT is not a specific inhibitor of DNA synthesis in Euglena. Low concentrations of NFT have a strongly inhibitory effect on protein and RNA synthesis as well as on DNA synthesis. Experiments at short times (Figure 14) show that the syntheses of both proteins and nucleic acids are inhibited from fifteen minutes onwards. This is in striking contrast to the delayed effect of nitrosoguanidine and of MNTS on protein synthesis noted in the preceeding section.

The finding that NFT inhibits the synthesis of protein, RNA and DNA in Euglena whereas only the synthesis of DNA is inhibited in E. coli B serves as a reminder that one must exercise due caution when extrapolating data obtained with one organism to others.

A comparison of these incorporation data with data on the

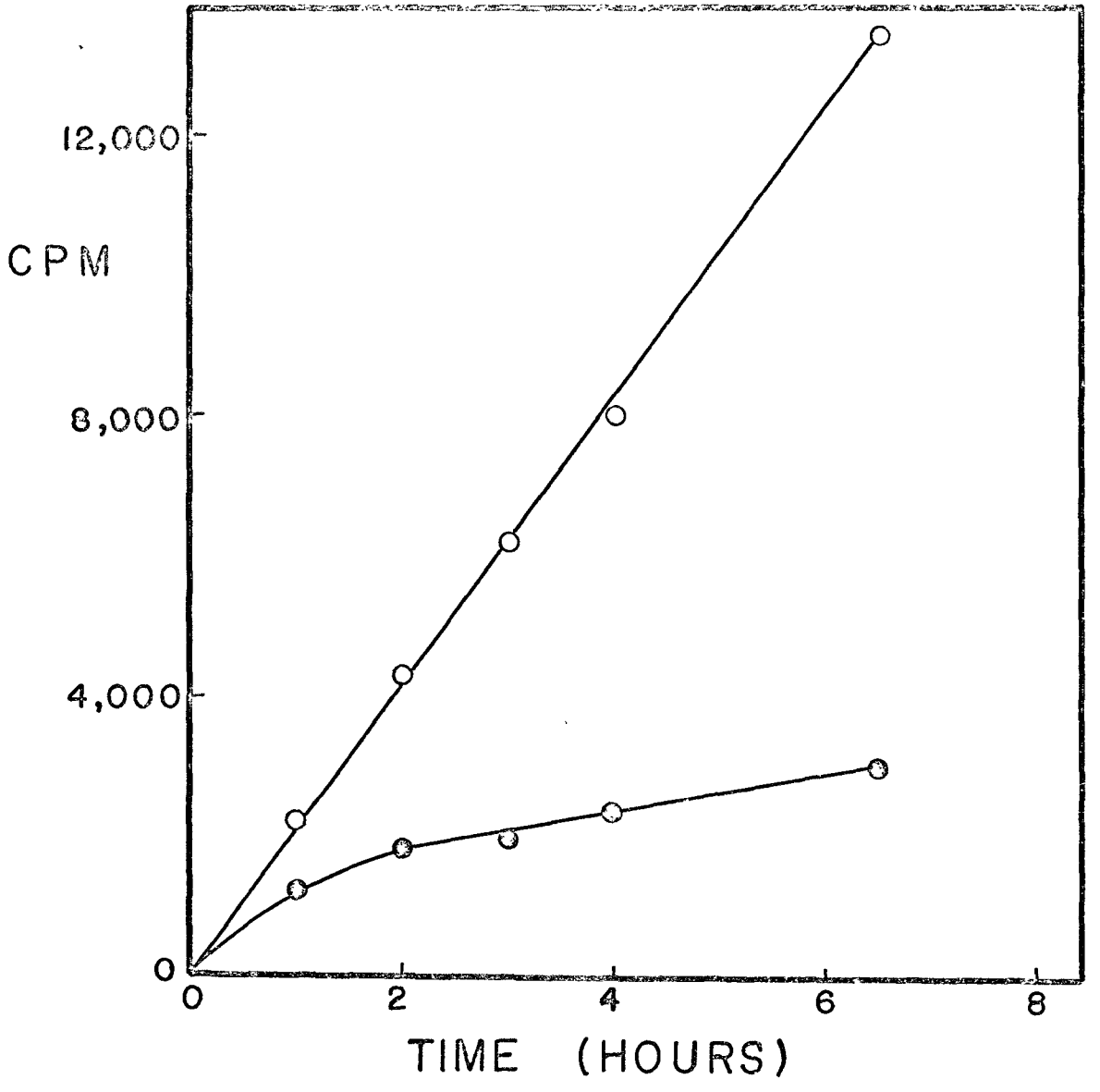


Figure 13a. Effect of 1.5 µg/ml NFT on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

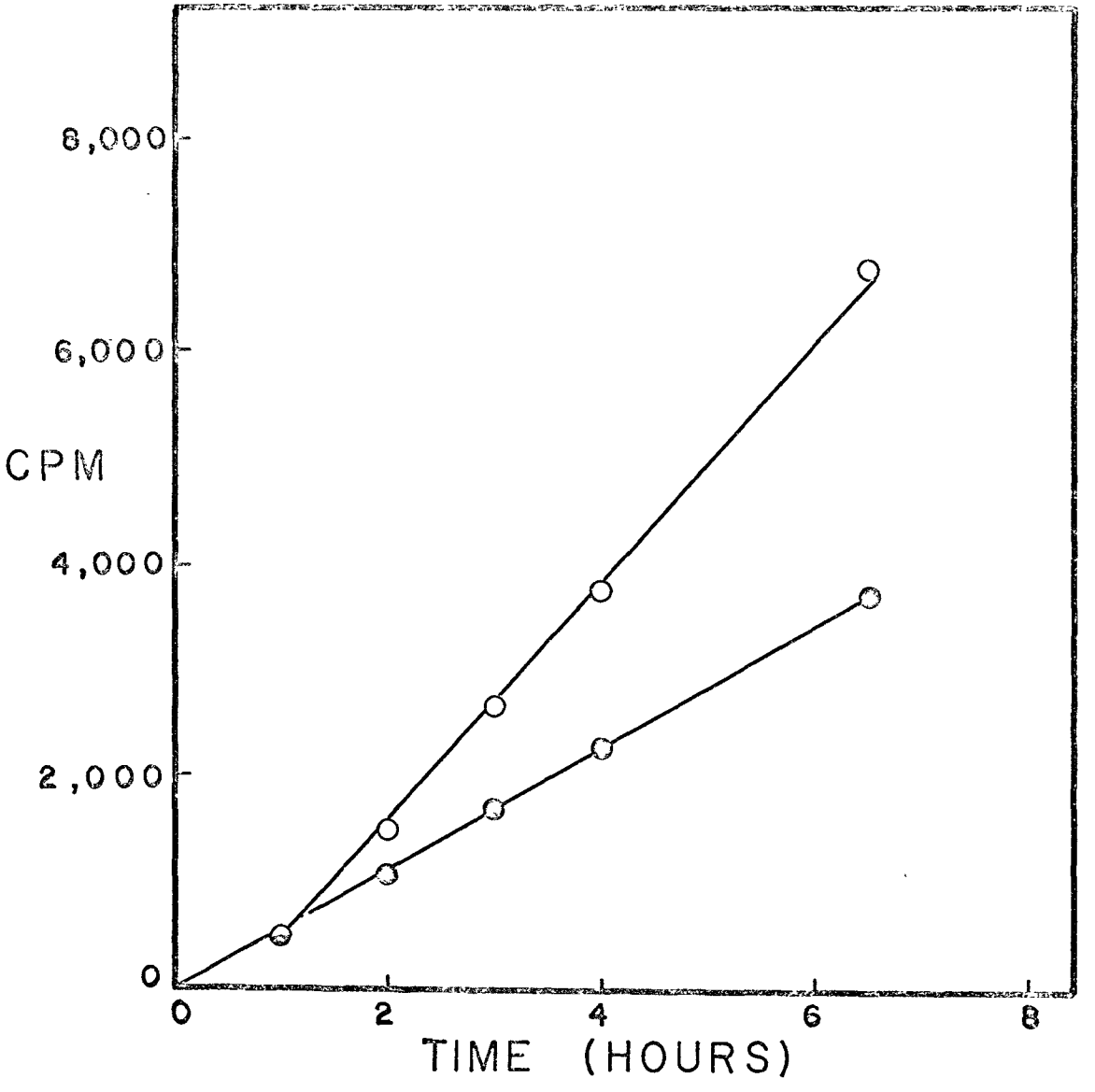


Figure 13b. Effect of 1.5 µg/ml NFT on the rate of incorporation of adenine-8-C^{14} into the alkali-labile portion of the TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

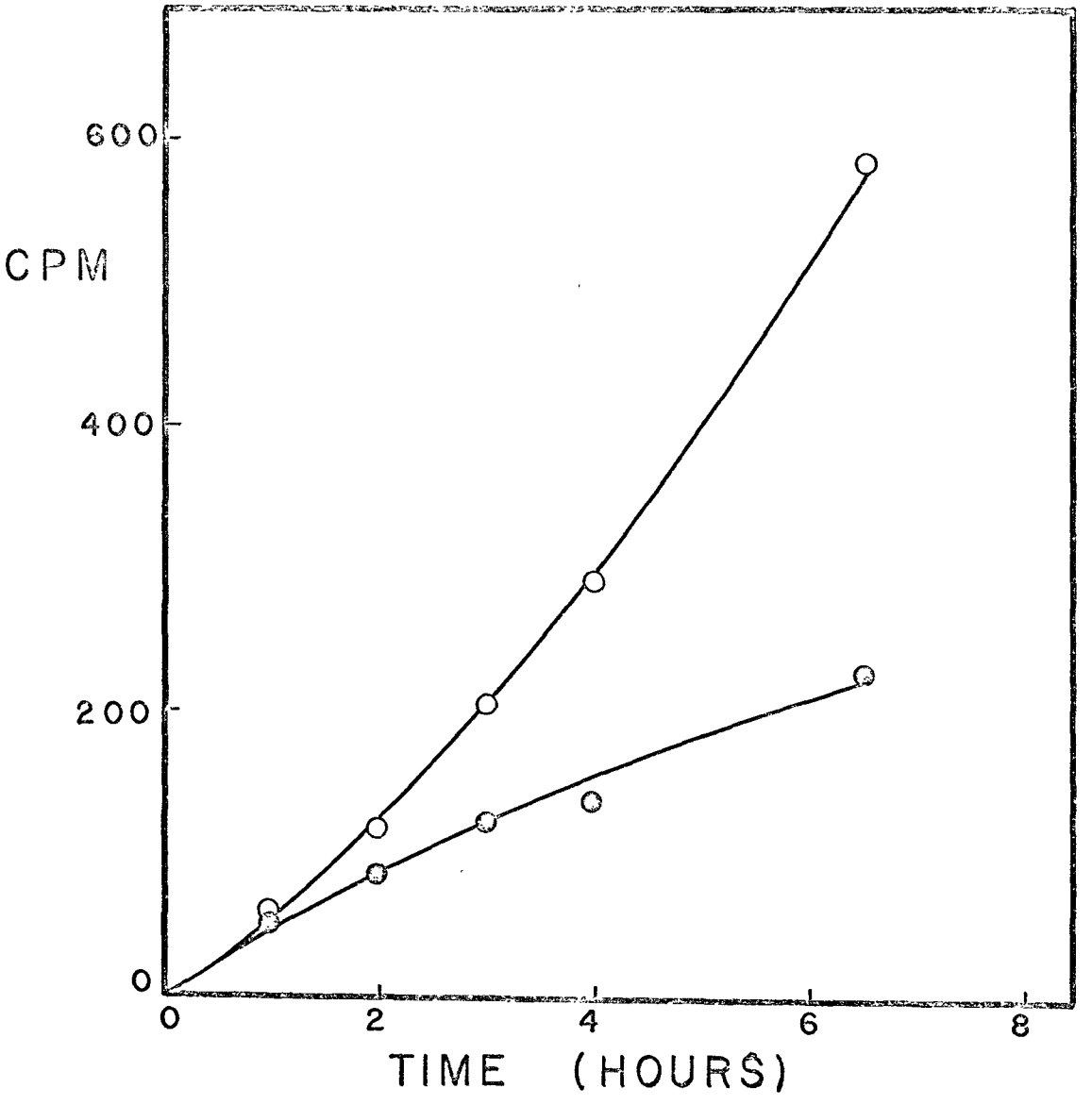


Figure 13c. Effect of 1.5 ug/ml NFT on the rate of incorporation of adenine-8-C^{14} into the alkali-labile portion of TCA-insoluble material of Euglena. Control, open circles; treated, closed circles.

Table XIII

Effect of NFT on Macromolecule Synthesis

in Euglena

Concentration (µg/ml)	Time (hours)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
1.0	1	83	97	100
	2	65	75	82
	3	64	80	71
	4	60	76	55
2.5	1	46	52	51
	2	25	33	35
	3	22	28	28
	4	22	24	18

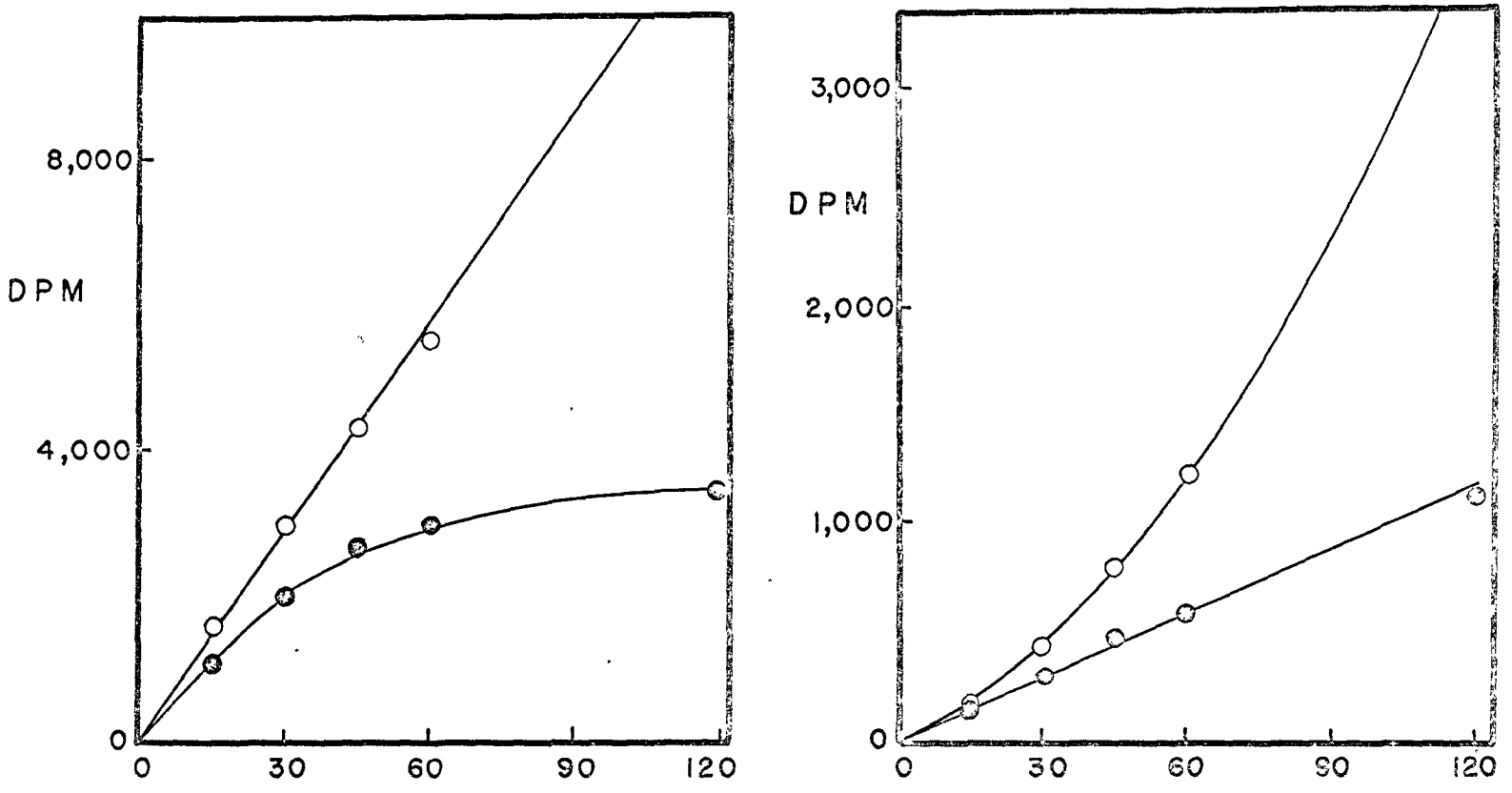


Figure 14. Effect of NFT on macromolecule synthesis in Euglena at short times. The left-hand plot shows the effect on the rate of incorporation of leucine-1-C^{14} into TCA-insoluble material. The right-hand plot shows the effect on the rate of incorporation of adenine-8-C^{14} into TCA-insoluble material. Open circles, controls; closed circles, 1.5 µg/ml NFT.

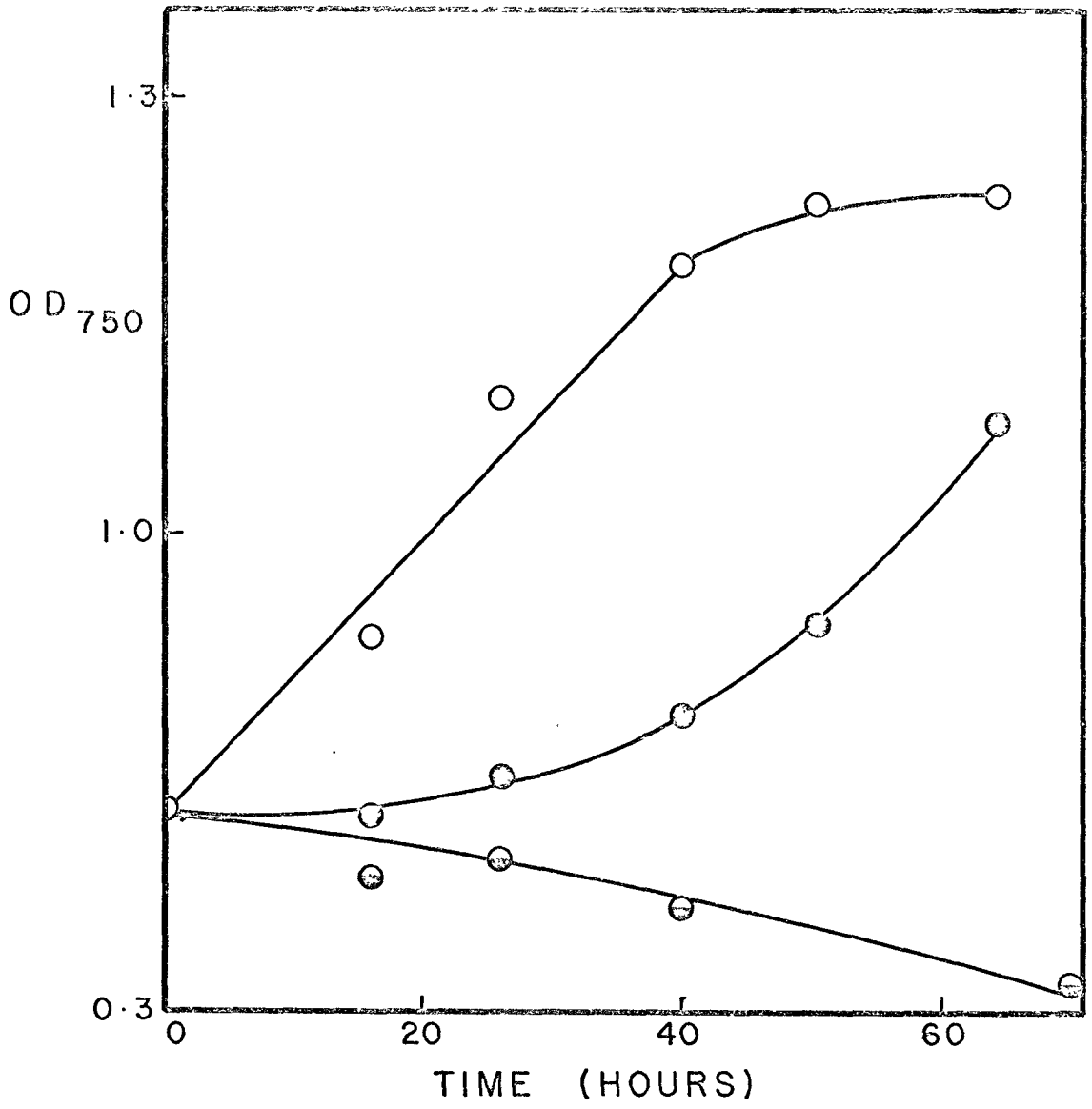


Figure 15. Growth of Euglena in the presence of NFT. Open circles, control; closed circles, 1.5 or 2.5 ug/ml NFT; half-closed circles, 5 ug/ml NFT.

effect of NFT on the growth of Euglena (Figure 15) reveals that concentrations of NFT which inhibit growth also have a strongly inhibitory effect on the synthesis of macromolecules. Similar parallels have been noted previously for nitrosoguanidine and MNTS.

Table XIV shows that nitrofurantoin, at a concentration of 64 µg/ml, has little effect upon macromolecule synthesis in Euglena over a period of four hours. The testing of higher concentrations was precluded by the limited solubility of nitrofurantoin in the growth medium.

Table XIV

Effect of Nitrofurantoin on Macromolecule Synthesis in Euglena

Concentration (µg/ml)	Time (hours)	Relative Incorporation into TCA-insoluble material (Per Cent of Control)		
		Leucine-1-C^{14}	Alkali-labile adenine-8-C^{14}	Alkali-stable adenine-8-C^{14}
64	1	85	98	98
	2	94	103	117
	3	83	98	97
	4	90	97	85

The fact that bleaching agents such as nitrosoguanidine, MNTS and NFT inhibit the synthesis of nucleic acids as well as the synthesis of protein may be pertinent to a consideration of their mode(s) of action. Chloramphenicol is a specific inhibitor of protein synthesis in many organisms and Pogo and Pogo have shown this to be the case in non-dividing Euglena (138). They have found that protein synthesis is reduced by chloramphenicol more strongly in the chloroplast fraction than in either the microsomal or supernatant fractions of Euglena. Nevertheless, chloramphenicol does not bleach Euglena (138). Although none of the bleaching agents tested, nitrosoguanidine, MNTS or NFT, has a selective effect on the synthesis of a particular class of macromolecules in Euglena, each agent has an inhibitory effect on nucleic acid synthesis. Taken together with Pogo's results, these data are consistent with the notion that the effect of these bleaching agents on cellular nucleic acid synthesis may be an important event in the bleaching process.

F. Studies with N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide

It was pointed out in the Historical Introduction that it has been suggested that agents which bleach Euglena might conceivably do so by selectively damaging chloroplast DNA (64). Chloroplasts damaged by exposure to a bleaching agent would not be expected to retain the capacity to replicate and hence, if cell division continued, apoplastidic cells would arise. It is necessary to postulate "selectivity" since the nuclear genetic determinants are apparently not inactivated. It was suggested that one possible basis for such selectivity might be the differences in base compositions of the chloroplast and nuclear deoxyribonucleic acids. (The adenine plus thymine content of the chloroplast DNA is approximately 75% while that of the nuclear DNA is approximately 50% (85,86,87).) Since virtually nothing is known about the mechanism of action of most bleaching agents it is, at the present time, impossible to evaluate this hypothesis critically. Qualitatively, the available data are consistent with this notion: ultraviolet light, known to form thymine dimers in bacteria, bleaches Euglena; alkylating agents, known to alkylate guanine principally, do not bleach Euglena; nitrous acid, known to deaminate guanine and cytosine more rapidly than adenine, does not bleach Euglena; and finally, hydroxylamine, known to react in vitro almost exclusively with cytosine (although in vivo with all bases) (139), does not bleach Euglena.

In principle, the question of selectivity should be susceptible to experimental investigation through the use of labelled bleaching agents. Such an investigation was carried out using N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide.

Some N-methyl-N-nitroso compounds are unstable in aqueous solution (McCalla, unpublished results). Preliminary experiments were, therefore, carried out to test the possibility that the labelled methyl group of MNTS would find its way into the "one-carbon pool" of Euglena. Since carbon atoms 8 and 2 of the purine ring are derived from one-carbon units, incorporation of radioactivity into adenine and guanine was taken to indicate labelling of the one-carbon pool. Figure 16 shows that after four hours exposure of Euglena to labelled MNTS there is significant, and about equal, incorporation into adenine and guanine (as estimated by comparing the areas under the peaks on the radiochromatogram). Figure 17 shows the results of a similar experiment using Euglena which were exposed to labelled MNTS for only one hour. It can be seen that little of the label corresponds to the adenine and guanine positions on the chromatogram and that other components are labelled. Hence it was concluded that measurement of the relative specific activities of chloroplast and nuclear deoxyribonucleic acids would be meaningful if the Euglena cells were exposed to labelled bleaching agent for one hour.

Preliminary information regarding the possible identities of some of the other labelled compounds can be obtained from Figure 17. The peak of radioactivity near the origin of the chromatogram corresponds well with the position expected for 7-methylguanine in this solvent, although 1-methyladenine cannot yet be excluded. The major portion of the radioactivity is found at the solvent front and the identity of the compound or compounds responsible was not investigated.

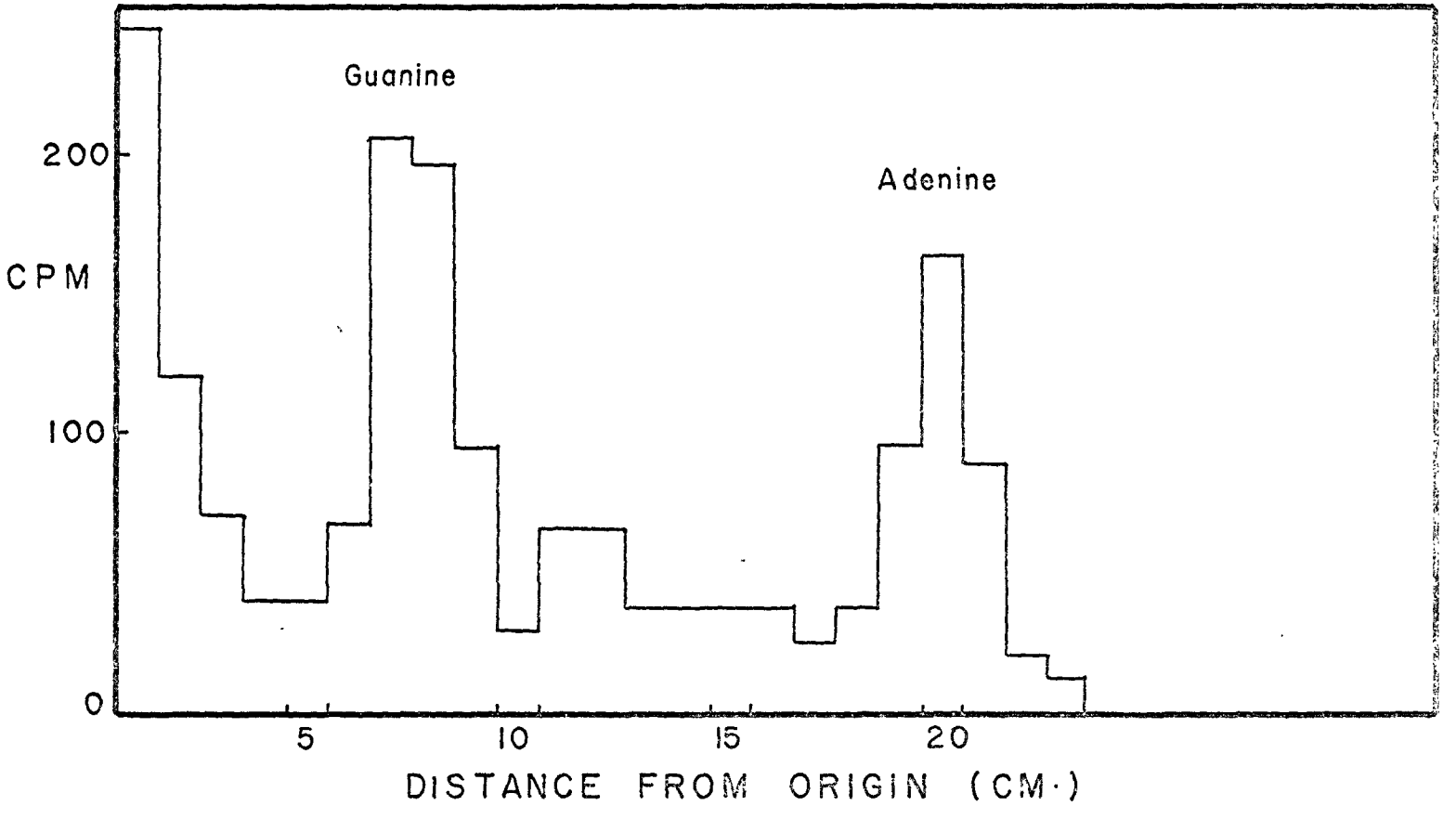


Figure 16. Radiochromatogram of nucleic acid bases from Euglena exposed to radioactive MNTS for four hours. After collection and extraction with methanol labelled cells were hydrolysed in 0.5 N NaOH. The liberated nucleotides were converted to free bases by hydrolysis in 88% formic acid in a sealed tube at 175° and the bases chromatographed in a butanol, ammonia, water solvent.

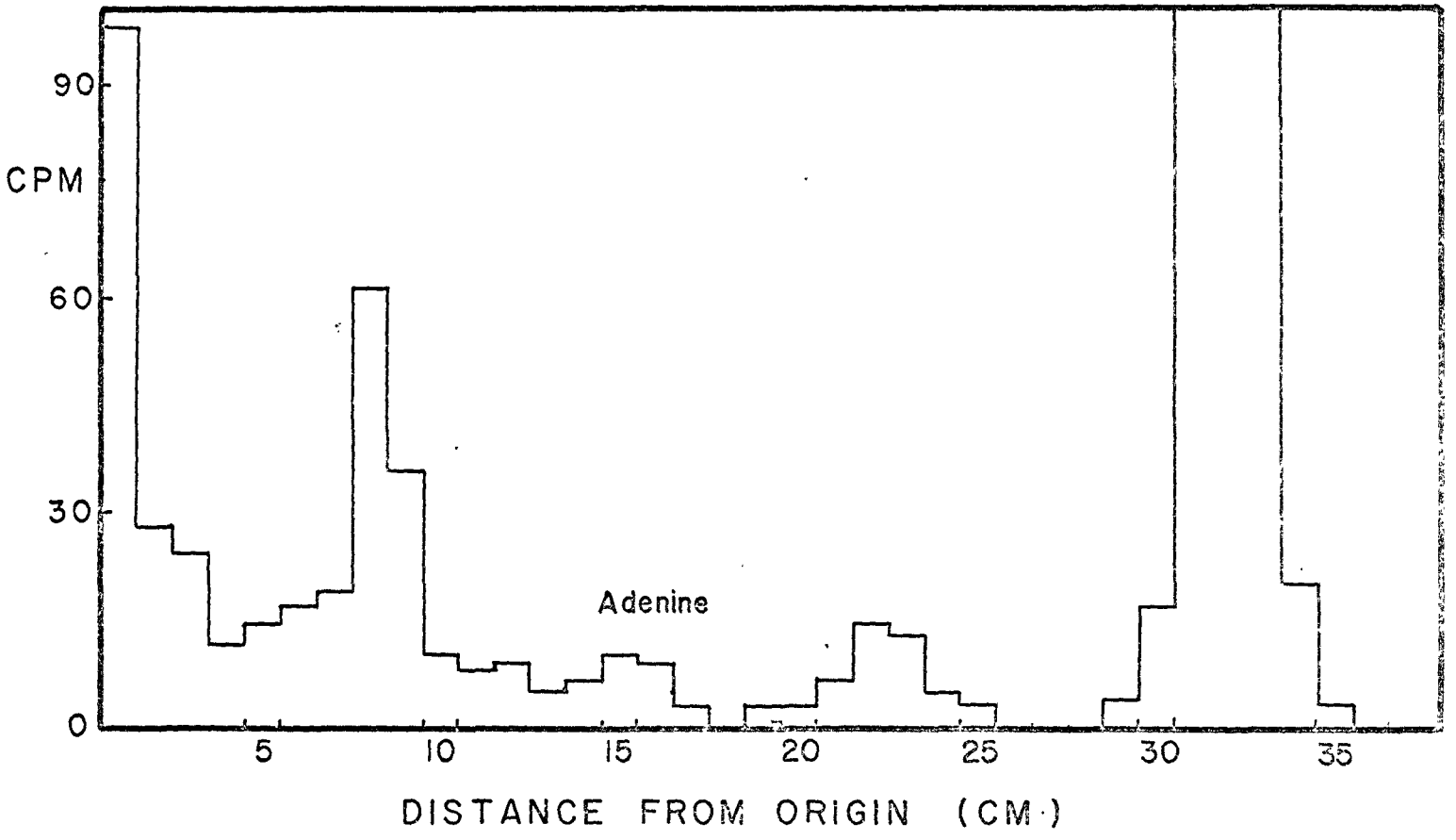


Figure 17. Radiochromatogram of nucleic acid bases from Euglena exposed to radioactive MNTS for one hour. After collection and extraction with methanol labelled cells were hydrolysed in 0.5 N NaOH. The liberated nucleotides were converted to free bases by hydrolysis in 88% formic acid in a sealed tube at 175° and the bases chromatographed in a butanol, ammonia, water solvent.

Relative Specific Activities of Chloroplast and Nuclear Deoxyribonucleic acids

Radioactive MNTS was administered to a culture of Euglena for one hour after which nucleic acids were extracted both from whole cells and from a purified chloroplast preparation. The relative specific activity of each nucleic acid preparation is shown in Table XV.

Table XV

Relative Specific Activities of Nucleic Acids from Euglena Exposed to Radioactive MNTS for One Hour

Origin of Nucleic Acids	net d/m	OD_{260}	net d/m/OD_{260}
whole cells	335	6.2	54
purified chloroplast preparation	6.6	0.49	13.5

The relative specific activity of the nucleic acids from whole cells is 4 times that from purified chloroplasts.

After treatment of each solution of nucleic acids with ribonuclease followed by deproteinization and dialysis, the relative specific activities were again measured. The results are recorded in Table XVI.

Table XVI

Relative Specific Activities of DNA from Euglena

Exposed to Radioactive MNTS for One Hour

Origin of DNA	net d/m	OD_{260}	net $d/m/OD_{260}$
whole cells	55.5	0.38	146
purified chloroplast preparation	19.5	0.39	50

The relative specific activity of the DNA from whole cells is 3 times that of the DNA from the purified chloroplast preparation. A preferential labelling of DNA is indicated by the fact that both for whole cells and for purified chloroplasts the relative specific activity of the DNA is approximately 3 times that of the total nucleic acids.

To determine the purity of the chloroplast preparation used in this investigation, a similar preparation of nucleic acids extracted from purified nonradioactive chloroplasts was subjected to cesium chloride density gradient centrifugation in an analytical ultracentrifuge. Figure 18 indicates that the preparation contained approximately 70% chloroplast DNA and 30% nuclear DNA.

On this basis the relative specific activities of chloroplast and nuclear deoxyribonucleic acids obtained in experiments with radioactive MNTS would suggest that the chloroplast DNA was not labelled. The thirty per cent of the DNA preparation attributable to nuclear DNA has a relative specific activity of 146 d.p.m./O.D. unit and if diluted with seventy per cent unlabelled chloroplast DNA would result in a

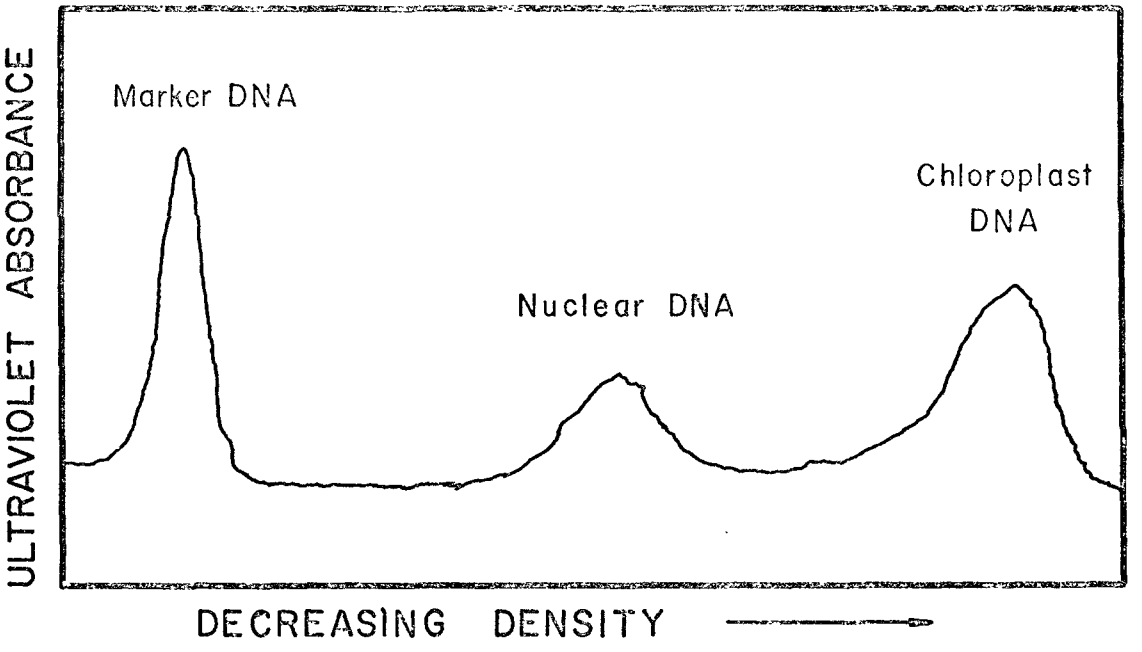


Figure 18. Densitometer tracing of the ultraviolet absorption photograph taken after equilibrium density gradient centrifugation of DNA extracted from a purified chloroplast preparation.

relative specific activity of 44 d.p.m./O.D. unit for the DNA from the chloroplast preparation, a value very close to that found. The same considerations apply to the total nucleic acid preparations which contain mainly RNA and thus it is apparent that chloroplast RNA is not labelled selectively.

These conclusions appear to be in marked conflict with the suggestion that bleaching agents act selectively on chloroplast DNA. The interpretation of the data, however, is limited by the fact that the Euglena cells did not remain in the presence of MNTS long enough to become bleached. The one-hour treatment of Euglena with labelled MNTS was, of necessity, short in comparison to the required several-hour bleaching treatment. It would appear that this particular bleaching agent has an effect other than an early penetration of the chloroplast and reaction with chloroplast DNA. It is quite possible, of course, that eventual penetration of the chloroplast takes place with subsequent damage to the chloroplast DNA but experiments at longer times using labelled MNTS are precluded by the scrambling of the radioactivity. There seems to be little merit, at this time, in indulging in further speculation.

SUMMARY

The main conclusions of this thesis are summarized below.

An evaluation of the relative importance of biochemical effects of 5-nitrofurans on Escherichia coli has revealed that bacteriostasis by these compounds is more likely a result of inhibition of DNA synthesis rather than of inhibition of respiration.

N-Methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) has been shown to induce the development of prophage in a lysogenic strain of E. coli. Measurement of the incorporation of appropriate labelled precursors of macromolecules into acid-precipitable material has shown that after only 5 to 10 minutes exposure to nitrosoguanidine the synthesis of proteins, RNA and DNA in E. coli is reduced appreciably. Although most other agents which induce prophage development in lysogenic strains of bacteria inhibit DNA synthesis selectively in E. coli B, this does not appear to be a requisite for induction.

The effects of nitrosoguanidine, N-methyl-N-nitroso-p-toluenesulfonamide (MNTS) and 3-amino-6-[2-(5-nitrofuryl)-vinyl]-1,2,4-triazine (NFT) on macromolecule synthesis in Euglena gracilis have been measured at concentrations which cause "bleaching" and temporary inhibition of growth. Nitrosoguanidine and MNTS inhibit nucleic acid synthesis immediately while protein synthesis becomes inhibited after 45 to 60 minutes. From one hour onwards these compounds are general inhibitors

of macromolecule synthesis in Euglena as is the case in E. coli B. On the other hand, NFT inhibits the synthesis of nucleic acids and proteins in Euglena from, at most, 15 minutes onwards, in sharp contrast to the situation in E. coli B where NFT is a specific inhibitor of DNA synthesis.

The hypothesis that bleaching agents may exert their effect by selectively damaging chloroplast DNA has been tested using N-methyl-C^{14}-N-nitroso-p-toluenesulfonamide. It was found that nuclear DNA extracted from cells treated with radioactive bleaching agent was labelled, while DNA extracted from a purified chloroplast preparation was unlabelled. Although a definitive statement is precluded by limitations inherent in this experiment, there is an indication that MNTS may bleach Euglena by a process other than selective damage to chloroplast DNA.

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