ALTERATIONS IN NUCLEIC ACID METABOLISM DURING NUTRITIONAL DEFICIENCY IN *ESCHERICHIA COLI*

A Thesis submitted to the University of Oxford

for the Degree of Doctor of Philosophy

by

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ABSTRACT

ABSTRACT

This thesis concerns an investigation of the effects of thymine deficiency in thymineless mutants of Escherichia coli. Evidence was available which implicated damage to the DNA of the cell as the cause of the loss of viability (thymineless death) which accompanies thymine deficiency. DNA has been extracted from bacteria before and after thymineless death and examined for chemical and physical differences. There was evidence that the DNA extracted after thymineless death was labile to alkali since it could not be reprecipitated with acid after the accompanying RNA had been destroyed by alkali according to the method of Schmidt and Thannhauser. It had also been observed that incubation of the acid-insoluble residue from bacteria after thymineless death in N alkali for 16 hours at 37° resulted in an apparent decrease in material which reacted in the diphenylamine assay for DNA. It has been shown that the inability to reprecipitate the DNA is not a function of the DNA itself since if herring sperm DNA is added to the alkaline incubation mixture it too cannot be reprecipitated. The apparent decrease in diphenylamine-reacting material was shown to be due to the production during thymine deficiency of some material which when treated with alkali produced an inhibitor of the diphenylamine reaction. It has further been shown that incubation in alkali reduces the viscosity of DNA extracted after thymineless death by about the same amount as that of DNA extracted before thymineless death.

No evidence has been obtained of any physical differences between

DNA extracted before and after thymineless death from the use of sedimentation velocity, viscosity, melting-curve, band-widths in caesium chloride, autoradiography or cross-linking measurements. In spite of this, evidence has been obtained that suggests that a small amount of DNA synthesis occurs during thymineless death and death has been discussed in the light of the possibility that this DNA is damaged in some way due to the lack of thymine.

The observation (Riley,1962) that thymine deficiency is accompanied by the appearance of a deoxyribose compound in the culture medium has been extended to show that the compound does not absorb to charcoal-Celite columns or to Dowex-1 (formate) and is therefore probably free deoxyribose. It has also been shown that the appearance of deoxyribose in the medium during thymine deficiency is independent of thymineless death and that its maximum excretion requires protein and RNA synthesis. Excretion can also be inhibited by the restoration of thymine or bromouracil to the culture.

A method has been developed for mapping the intracellular acid-soluble nucleotides from bacteria. Using this method, no evidence of gross derangement of the nucleotide pools during thymineless death was obtained, although an overall increase in the total nucleotide content of the bacteria was observed. It has also been shown that preincubation of thymineless bacteria with deoxynucleosides has no effect on the kinetics of thymineless death when the cultures are subsequently deprived of thymine.

Maaløe and Hanawalt (1961) showed that if protein and RNA synthesis is inhibited in E.coli 15TAU, DNA synthesis stops after the

completion of one round of replication. These bacteria cannot now undergo thymineless death until they are allowed to initiate a new round of replication; a process which requires protein and RNA synthesis. This condition where cells cannot undergo thymineless death is called immunity, and it has been shown that 15TAU can be immunised just as effectively with bromouracil as with thymine. However, when immunised bacteria are resuspended in thymine-deficient medium in which they can now synthesise initiator for DNA replication, death is more rapid if the cells have been immunised with bromouracil rather than with thymine. Furthermore, if immunised cells are incubated in thymine or bromouracil after immunisation in bromouracil or thymine respectively, resuspension in medium in which initiator can now be synthesised results in death kinetics which are characteristic of the last medium in which the cells have been incubated. Since these effects have been effected at a time when it is expected that all DNA synthesis has ceased, it is argued that some cytoplasmic molecule which contains thymine is involved, and that when this is damaged thymineless death is induced.

ABBREVIATIONS

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
FUdR	5-fluorodeoxyuridine
Bu	5-bromouracil
BudR	5-bromodeoxyuridine
T	thymine
A	arginine
U	uracil
6MAP	6-methylaminopurine
UV	ultraviolet
Tris	tris(Hydroxymethyl)aminomethane
EDTA	ethylene diamine tetraacetate
dA	deoxyadenosine
dU	deoxyuridine
dG	deoxyguanosine
dC	deoxycytidine
dATP	deoxyadenosine triphosphate
dUMP	deoxyuridylic acid
THFA	tetra-hydro-folic acid
dTMP	deoxythymidylic acid

"After death DNA" is DNA extracted from bacteria after thymineless death has occurred.

"Before death DNA" is DNA extracted from bacteria before thymineless death has occurred.

Convention for describing medium supplements for growth of auxotrophs

In order to clarify the description of the arginine, uracil, thymine and bromouracil supplementation of growth media, the convention used by Maaløe and Hanawalt (1961) has been used:

When thymine is present and arginine and uracil are absent the medium is described as $T^+(AU)^-$ or $T^+A^-U^-$. Similarly, when 5-bromouracil, arginine and uracil are present, the medium is described as $Bu^+(AU)^+$.

In experiments where cells are transferred from one supplemented medium to another, the following method is used to indicate the successive transfers; the times of incubation in the respective media being indicated beneath the symbols:

$$T^+(AU)^+ \longrightarrow \underset{90\ \text{min.}}{Bu^+(AU)^-} \longrightarrow \underset{20\ \text{min.}}{T^+(AU)^-} \longrightarrow T^-(AU)^+$$

This means that E. coli 15TAU was grown in a medium supplemented with thymine, arginine and uracil, was then transferred to a medium supplemented with 5-bromouracil for 90 minutes, was then transferred to a medium supplemented with thymine for 20 minutes, and finally was transferred to a medium supplemented with arginine and uracil.

This convention has also been used for describing the growth media for E. coli $15T^-U^-$.

Bacterial strains are referred to throughout by their strain number i.e. B_3, $15T^-$ and 15TAU, and it is to be understood that whenever the specific name is not mentioned, the bacterium referred to is Escherichia coli.

INTRODUCTION

INTRODUCTION

With most auxotrophic bacterial mutants withdrawal of an essential growth factor (e.g. an amino-acid (Cohen, 1957), purine (Kaplan & Nowsden, 1964) or uracil (Barner & Cohen, 1954a)) stops growth and synthetic processes without causing a loss of viability. An exception is Escherichia coli $15T^-$ which requires thymine for growth, and which, in the absence of thymine, rapidly loses viability (Barner & Cohen, 1954a). The loss in viability is accompanied by an inhibition of DNA synthesis, but not of protein or RNA synthesis; restoration of thymine to such cultures restores DNA synthesis but not cell viability.

These processes were first discovered by Barner & Cohen (1954a), and the loss of viability is known as "thymineless death". It has been observed in other strains of E. coli (Okagaki, Tsubota & Sibatani, 1960; Gallant & Suskind, 1961a) and in thymineless mutants of Bacillus subtilis (Brabander & Romig, 1960) and Salmonella typhimurium (Okada, Homma & Sonohara, 1962). Thymineless death also occurs in B. subtilis inhibited with FUdR (Mennigmann & Szybalski, 1962a).

The Metabolic Lesion

The thymineless strains used in work on thymineless death have been obtained by a variety of methods: irradiation induction as in the case of $15T^-$ (Roepke, cited by Cohen, 1957) and Strains I and II (Zamenhof, De Giovanni & Rich, 1956); bromouracil (Bu) induction as in the case of B_3

(Brenner & Smith, 1959); and the aminopterin selection method of Okada et al. (1962).

Barner and Cohen, (1959) have shown that B_3 and 15T$^-$ lack thymidylate synthetase, an enzyme which catalyses the following reaction (Wahba & Friedkin, 1962; Pastore & Friedkin, 1962; Friedkin, Crawford, Donovan & Pastore, 1962):

$$\text{dUMP} \xrightleftharpoons[CH_3]{\text{THFA}} \text{dTMP}$$

There is no measurable activity of this enzyme in extracts of 15T$^-$ and 2.5% of the wild-type activity in B_3 extracts.

The thymineless lesion in bacteria may be simulated by inhibiting bacterial cells with FUdR, the nucleotide of which binds irreversibly to thymidylate synthetase (Cohen, Flaks, Barner, Loeb & Lichtenstein, 1958; Matthews & Cohen, 1963), or with sulphanilimide (Barner & Cohen, 1954b) which acts as a folic acid antagonist. Thymineless death occurs in both these cases. In addition, the omission of the alternative growth factors vitamin B_{12} or deoxyribonucleosides from the growth medium of Lactobacillus leichmannii also results in a loss of viability (Beck, Hook & Hurlock Barnett, 1962). This would suggest that the lethal event may be connected with another compound containing thymine rather than with an inhibition of DNA synthesis per se since vitamin B_{12} is known to be involved in the reduction of nucleosides to deoxynucleosides (Craven & Downing, 1963).

The lack of thymidylate synthetase will also result in the inability to synthesise thymidine diphosphate-L-rhamnose which is required for cell-wall synthesis (Okazaki, Strominger & Okazaki, 1963). This will presumably lead to a weakening of the cell-wall (Tomasz & Borek, 1962) during thymine deficiency.

The synthesis of thymine riboside in soluble RNA will not be affected by the absence of thymidylate synthetase since the uridylyl residue is apparently methylated *in situ* and the source of the methyl group is methionine rather than folic-acid (Fleissner & Borek, 1962).

Although the metabolic pathway leading to dTMP does not involve free thymine, a study (Crawford, 1958) of revertants of B_3 and 15T⁻ to wild-type suggests that a pathway for utilising thymine is induced in thymine-less bacteria and is retained in addition to the restored thymidylate synthetase in revertants. Permeability was not a factor since thymine could be shown to enter the pool of *E. coli* B even though it did not enter its DNA.

Morphological Changes During Thymine Deficiency

Morphological changes in thymineless *E. coli* were observed as early as 1947 (Roepke & Mercer, 1947), and it is characteristic of these cells growing in the absence of thymine that they become highly filamentous (Barner and Cohen, 1956a).

It is remarkable that although DNA synthesis is inhibited in the absence of thymine, protein and RNA synthesis are not. This results in a large increase in the protein/DNA and RNA/DNA ratios, and it was long thought that this "unbalanced growth" (Barner & Cohen, 1954a) was

responsible not only for the filamentous growth, but also for the characteristic loss of viability (Barner & Cohen, 1954a, 1956a; Deibel, Downing, Niven & Schweigert, 1956; Cohen, 1956). Filament production is not however peculiar to thymine deficiency since many conditions are known to give rise to it (Hughes, 1956), and it now seems improbable that unbalanced growth is the cause of thymineless death since unbalanced growth can occur under conditions where there is no effect on viability (Okagaki et al., 1960; Gallant & Suskind, 1960, 1961a; Maaløe & Hanawalt, 1961; Harold & Ziporin, 1958). Also, addition of Bu to the thymine deficient growth medium allows DNA synthesis to occur at a nearly normal rate, thus avoiding unbalanced growth, yet there is still a loss of viability and production of filamentous forms (Dunn & Smith, 1957; see also Stuy, 1958).

Maaløe and Hanawalt (1961), using fluorescence microscopy and acridine orange staining, have reported the formation of "empty spaces" in 15T⁻ during thymine deficiency. The DNA appeared to be distributed throughout the cell, though apart from this loss of compactness there was no evidence of any massive depolymerisation. This is consistent with the lack of release of ^{32}P (Maaløe & Hanawalt, 1961) and thymine (Barner & Cohen, 1954a) into the growth medium during thymine deficiency.

It is therefore clear that the morphological evidence so far available provides no clue to the mechanism of thymineless death.

Factors affecting the Kinetics of Thymineless Death

It is necessary to point out that the literature in this field is very inconsistent and contradictory. This is largely because work has not been confined to one thymineless strain; B_3 shows a much lower propensity towards thymineless death than does 15T⁻ and its derivatives on which most of the reported work has been done. In one instance, workers using Zamenhof's Strains I and II acknowledge that many of their results were unrepeatable (Okagaki et al., 1960), though in most respects Strains I and II seem to be similar to B_3. In most of the following discussion therefore, only work on B_3 and 15T⁻ and its derivatives has been considered.

The kinetics of thymineless death for 15T⁻ are shown in Fig. 1. After the removal of thymine from the synthetic growth medium there occurs a lag phase of about 30 minutes during which there is little or no increase in viable count. This is followed by an exponential death which proceeds over several decades. The loss of viability is dependent on the availability of an energy source and is prevented only by thymine, thymidine or some compound which is rapidly converted to these in vivo e.g. 5-hydroxymethyldeoxycytidine, 5-hydroxymethyldeoxyuridine (Cohen, Green & Barner, 1956). There is no change in the respiration rate of the bacteria on removal of thymine (Barner & Cohen, 1954b).

Factors affecting the Lag Phase

Factors which lengthen the lag phase comprise conditions under which: (1) death is inhibited due to the provision of a thymine-like compound

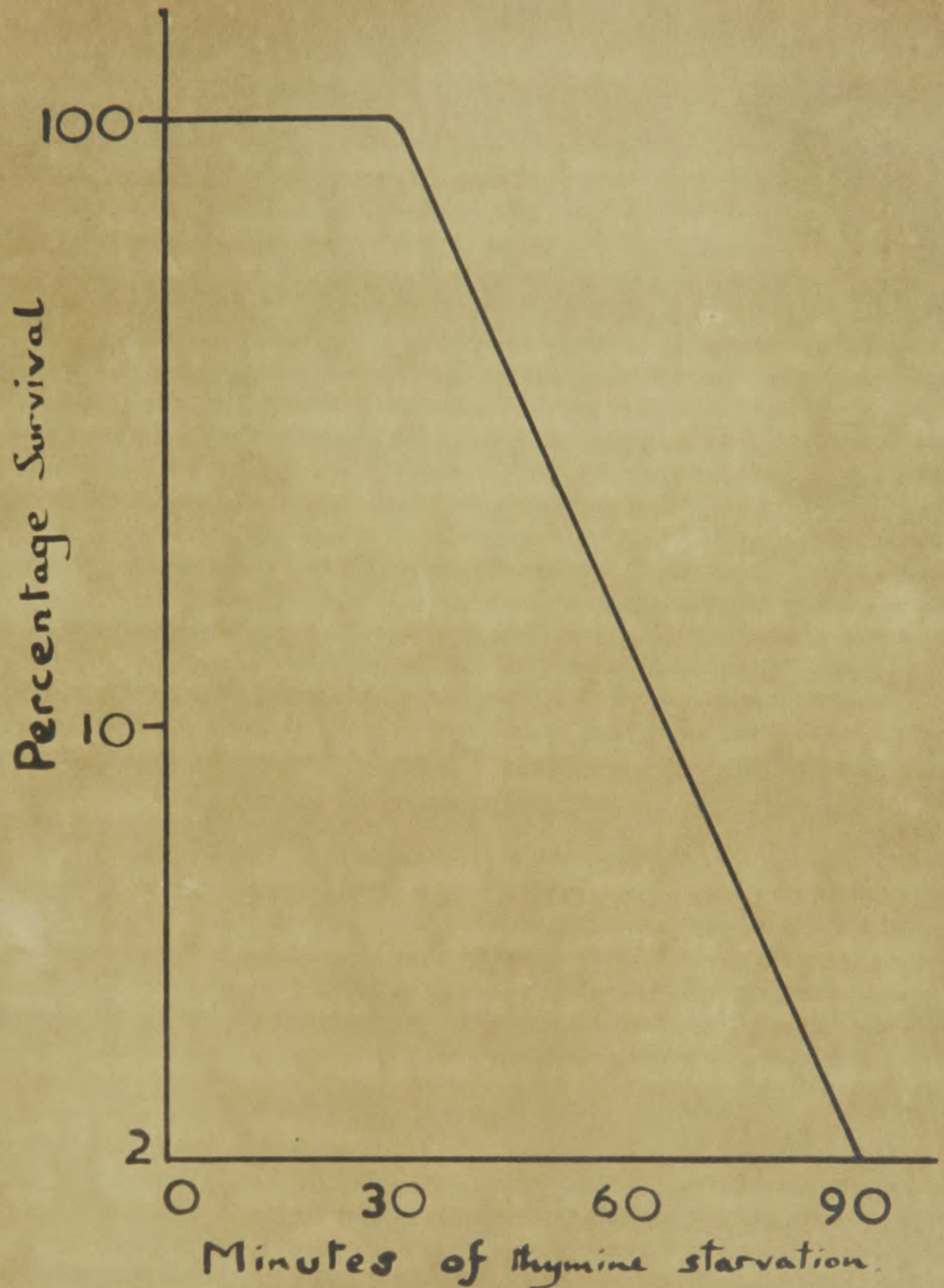


Fig.1. The kinetics of thymineless death in E.coli 15T⁻.
(From Maaløe and Hanawalt, 1961)

by cross-feeding.

(2) the cell is in a physiologically immune state,

(3) the lethal process is specifically inhibited.

(1)

(a) When cells of 15T⁻ are transferred to broth from which the thymine has been exhausted by supporting the growth of 15T⁻ under thymine deficient conditions, these transferred cells remain immune to thymineless death for at least an hour (Barner & Cohen, 1954a). Growth of 15TAU in T⁻(AU)⁻, whilst producing thymineless death, does not give rise to this factor in the growth medium (Maaløe & Hanawalt, 1961). It therefore seems that the excretion of this "immunising factor" requires protein and/or RNA synthesis.

(b) When B_3 cells are grown in minimal medium at a high cell density (5×10^8 cells/ml) they remain viable for an unlimited period (Gallant & Suskind, 1961a). B_3 cells at low cell densities (5×10^5 cells/ml) undergo death with kinetics very similar to those shown by 15T⁻ (Gallant & Suskind, 1961a).

(c) When B_3 cells are grown at low cell density in minimal medium at 25° instead of at the normal 37°, no loss of viability is incurred (Gallant & Suskind, 1961a). Also even after extensive death at 37°, further death may be blocked by transfer to 25° (Gallant & Suskind, 1960).

(d) A culture of 15TAU which is grown at 25° and transferred to T⁻(AU)⁺ at 25° exhibits a lag of 45 minutes instead of 30 minutes before

undergoing exponential death at a rate characteristic of growth at 25° (Maaløe & Hanawalt, 1961).

In the case of (a), it is argued (Berner & Cohen, 1954a) that some thymine-like compound has been released into the medium during death. In the other cases, cross-feeding (Gallant & Suskind, 1961a) together with leakiness may well be sufficient to maintain the viability of B_3. See also Crawford (1959). However, more recently Gallant & Suskind (1962) have shown that growth of B_3 at 37° in the presence of FUdR followed by resuspension at 25° still protects cells against thymineless death. Since the FUdR would be expected to inhibit any residual thymidylate synthetase, it was concluded that leakiness is not an important factor in lengthening the lag phase in this case.

(2)

(a) When cells of strain 15TAU are incubated in a medium containing thymine but no arginine or uracil i.e. $T^+(AU)^-$, for 90 minutes, they become immune to thymineless death when transferred to $T^-(AU)^-$ (Maaløe & Hanawalt, 1961).

(b) When cells of 15TAU are grown in $T^+(AU)^+$ until stationary phase is reached, they become similarly immune on transfer to $T^-(AU)^-$ (Davison, unpublished results cited by Maaløe, 1963).

These are both cases where the cells are not carrying out DNA replication on being placed in $T^-(AU)^-$ and will be discussed further in 'Involvement with the DNA Replication Cycle'.

(3) Many observations have been made concerning the effect of protein and RNA synthesis on thymineless death (Barner & Cohen, 1956a, 1959; Nakada, 1960; Billen, 1959, 1960a; Gallant & Suskind, 1961a, 1962; Maaløe & Hanawalt, 1961; Brock & Brock, 1960; Hanawalt, 1963).

(a) Many of the results regarding a requirement for protein synthesis for thymineless death are contradictory. Gallant and Suskind (1961b, 1962) have shown that the result depends greatly on the concentration of chloramphenicol used to inhibit protein synthesis. However, in an experiment where 2 µg/ml of chloramphenicol, which is slightly stimulatory to RNA synthesis, was used (Gallant & Suskind, 1962) together with 5-methyl tryptophan to inhibit protein synthesis, no effect on the lag phase of thymineless death was observed, although an increase in the rate of exponential death was obtained. This is consistent with the fact that death occurs in 15TAU whether nett protein and RNA synthesis occurs or not (Hanawalt, 1963).

It would seem therefore that protein synthesis is not a requirement for thymineless death.

(b) Experiments performed with thymine deficient cells growing in a limiting phosphate medium have shown that thymineless death in B_3 depends on the availability of phosphate whereas it does not in 15TAU (Hanawalt, 1963). This difference has been attributed to the generally lower susceptibility of B_3 to thymineless death, but without any real evidence.

(c) Transfer of cells of the mutant 15T⁻U⁻, isolated from UV-irradiated

15T⁻ by Barner and Cohen (1958), from T^+U^+ to T^-U^- increased the lag phase to about 1 hour (Barner & Cohen, 1958). The transfer of 15TAU to $T^-A^+U^-$ however showed no such effect (Kanazir, Barner, Flaks & Cohen, 1959). This difference could be explained on the grounds of the leaky uracil requirement shown by 15TAU (Barner & Cohen, 1958). Indeed, in a strain designated 15TAU-bar, which has a far more stringent uracil requirement, removal of thymine and uracil from the growth medium results in only 30% death after 3 hours (Hanawalt, 1963). On removing thymine, uracil and any or all four of the required amino-acids of this strain (arginine, proline, methionine, tryptophan) there was an increase in the killing rate, all the susceptible fraction being killed in 3 hours.

The above results are all consistent with thymineless death requiring RNA synthesis but not protein synthesis for its expression. Since a great deal of RNA synthesis can be inhibited without affecting death, as in 15TAU, it would seem that only a small amount is necessary. It has been suggested (Hanawalt, 1963) that this would be consistent with messenger RNA turnover being a requirement for death, and in particular for the kinetics of the lag phase.

Factors which shorten the lag phase:

(a) Incorporating ^{32}P into the DNA of 15T⁻, followed by storage at -196° for various times up to several weeks, leads to a progressive decrease in the length of the lag phase of thymineless death when the cells are thawed and incubated in thymine deficient medium (Fuerst & Stent, 1957).

(b) Exposure of B_3 to increasing doses of UV-irradiation followed by resuspension in thymine deficient medium results in a progressive decrease in the length of the lag phase (Gallant & Suskind, 1960, 1961a).

Both the above treatments are known to damage the bacterial DNA and these observations are discussed further in 'The Effect of Thymineless Death on the DNA of the Cell'.

(c) Incubation of B_3 at low cell densities in the absence of thymine at 25°, at which temperature thymineless death does not occur, followed by resuspension in thymine deficient medium at 37° results in a decrease in the lag phase (Gallant, 1961).

(d) Incubation of 15TAU in a complete medium containing only 0.05 µg/ml of thymine for 1-2 hours, followed by resuspension in $T^-(AU)^+$ results in a decrease in the lag phase (Maaløe, 1963).

(e) $15T^-$ cells which have not actually died are, however, not unaffected. If after 40 minutes (50% survival) thymine is restored and diluted out again after 20 minutes the lag before the onset of death again is not more than 15 minutes and it takes 60 minutes of T^+ recovery to re-establish the normal 30 minutes lag (Maaløe, 1963).

The observations described in (c)-(e) have all been discussed (Gallant & Suskind, 1960; Pauling & Gallant, 1963; Maaløe, 1963) in terms of "sub-lethal damage", though this may not necessarily be directly in the DNA itself.

Thus, all factors affecting the lag phase can be regarded in terms of either damage to the DNA or some closely related compound in the cell, or an inhibition of RNA turnover, and it was one of the aims of this study

to attempt to elucidate this problem.

Factors Affecting the Rate of Exponential Death

(1) Maaløe and Hanawalt (1961) have shown with 15TAU that the kinetics of thymineless death are independent of the composition of the growth medium, and therefore independent of the rate of cytoplasmic synthesis. Growth at different temperatures however gives rates of death which are characteristic of the temperature at which death occurs and independent of the temperature of cell growth prior to transfer to the thymine deficient medium. The death rate decreases with the temperature (Maaløe & Hanawalt, 1961).

(2) The rate of death is also affected by the presence of analogues of thymine, but these will receive more detailed treatment in a later section. The presence of a high concentration of uracil in the growth medium does not affect the rate (Barner & Cohen, 1956b).

(3) As mentioned earlier, the removal of thymine, uracil and amino-acids from 15TAU-bar increases the killing rate. This has been interpreted as being due to a sparing effect of the uracil from messenger RNA turnover because of the lack of nett RNAsynthesis (Hanawalt, 1963).

Factors Affecting the Extent of Thymineless Death

It was first recognised by Barner and Cohen (1957) that growth of their amino-acid-less, thymineless mutants in T^-aa^- medium resulted in a much higher survival compared with that of $15T^-$. These observations were extended by Maaløe and Hanawalt using 15TAU. They found that transfer of 15TAU to a medium containing $T^-(AU)^-$ resulted in a small percentage of the

cells remaining immune to thymineless death. Furthermore, when 15TAU were put through these conditions in media in which the mean generation times of the bacteria were different, e.g. glucose, succinate, then a different percentage of the population was immune. Thus, the longer the mean generation time the higher the percentage which is immune (Maaløe, 1961).

Involvement with the DNA Replication Cycle

It was first suggested by Maaløe and Hanawalt (1961) that immunity of cells to thymineless death is a function of the physiological state of the cells. Using autoradiographic techniques (Hanawalt, Maaløe, Cummings & Schaechter, 1961) it was possible to show that the DNA replication cycle takes a constant time in a given type of cell whatever the mean generation time. 15TAU cells which were immune as judged by the above criteria were not undergoing DNA synthesis at the moment of resuspension in thymine deficient medium. Furthermore, if cells were allowed to finish their DNA replication cycle in the absence of protein and RNA synthesis e.g. 15TAU incubated for 90 minutes in $T^+(AU)^-$, then the entire cell population gradually became immune. During renewed growth in $T^+(AU)^+$ the acquired immunity was gradually lost and, in parallel, DNA synthesis was resumed. It was argued that the steps leading to DNAsynthesis required protein and/or RNA synthesis and occurred as efficiently in the presence as in the absence of thymine. These results are consistent with the work of Billen (1960b) and of Maruyama and Lark (1961) with regard to the requirement for protein and/or RNA synthesis for the initiation of DNA replication.

Further evidence comes from the observation that restoration of thymine to a culture of 15T⁻ after ½ hour thymine starvation results in a complete rescue from thymineless death and synchrony of DNA synthesis and division over four generations (Barner & Cohen, 1955).

Thus the available observations provide excellent evidence for an intimate connection between thymineless death and the DNA replication cycle.

Effects of Thymine Analogues on Thymineless Death

Analogues which are not incorporated into DNA seem to have no effect on the viability of thymineless cells even though they inhibit DNA synthesis e.g. azathymine, 5-nitrouracil and spongothymidine (Barner & Cohen, 1956b) and 5-aminouracil (Dunn & Smith, 1958). Bu, however, is incorporated into the DNA of thymineless bacteria (Barner & Cohen, 1954b; Maaløe & Hanawalt, 1961; Weygand, Wacker & Dellweg, 1952; Strelzoff, 1962a) with little inhibition of RNA and protein synthesis (Barner & Cohen, 1954b; Strelzoff, 1962a). This only occurs in log phase bacteria, however, since, contrary to the reports of Weygand et al. (1952) and of Zamenhof et al. (1956a,b), Michaelson and Mandel (1962) find no evidence of any exchange between Bu and thymine in the DNA of stationary phase E.coli. In spite of the fact that Bu can support DNA synthesis it only slightly protects 15T⁻ against loss of viability (Barner & Cohen, 1954b; Strelzoff, 1962a), even though 15TAU remains viable in $Bu^{+}(AU)^{-}$ indefinitely (Hanawalt et al., 1961). It has also been shown that DNA in which 70% of its thymine has been substituted by Bu is fully functional with respect to its specific transforming activity (Szybalski, Opara-Kubinska, Lorkiewicz,

Ephrati-Elizur & Zamenhof, 1960), and that no gross distortion of the deoxyribonucleotide sequences occurs during its incorporation (Burton, 1962; Trautner, Swartz & Kornberg, 1962). There is also evidence to suggest that the incorporation of Bu into the DNA of 15TAU does not affect the number of growing points on the chromosome (Bonhoeffer & Gierer , 1963).

The role of Bu in this system is very puzzling since though DNA synthesis occurs apparently normally it does not prevent death. Addition of Bu after 1/2 hour thymine starvation with 15T⁻ does not result in any division and there is a loss of viability after a 60 minute lag. Synchronous DNA synthesis still occurs (Barner & Cohen, 1956c; Strelzoff, 1962a). In these cases where Bu does not effectively replace thymine, a ratio of 200 µg/ml of thymine to 10 µg/ml Bu is sufficient to reverse the deleterious effects (Barner & Cohen, 1956b), although it is not clear to what extent this may represent the absence of Bu from the newly synthesised DNA.

In general, thymidine analogues are more effective than thymine analogues in reversing the effects of thymine deficiency in these systems (Barner & Cohen, 1956b) even though the growth rate of 15T⁻ is identical in thymine and thymidine (Cohen, 1957). Thus, Bu or BudR, whilst allowing DNA synthesis to occur, does not prevent loss of viability although it does affect the kinetics of death markedly.

Effects of Thymineless Death on the DNA of the Cell

Estimates of the amount of DNA synthesised after the removal of thymine from a culture of thymineless bacteria vary greatly (Barner & Cohen, 1954a, 1958; Gallant & Suskind, 1960; Fuerst & Stent, 1957; Korn &

Weissbach, 1962), though it is fairly certain that there is no turnover of DNA during death (Barner & Cohen, 1954a, 1958; Maaløe & Hanawalt, 1961). B_3 appears to make a little more than 15T⁻ which makes little or none at all. Whilst this is what would be expected from the greater leakiness of B_3, it is difficult to decide how efficiently thymine is removed from the culture media during the washing procedures which accompany harvesting.

The fact that enzymes could be induced equally well before or after death suggested that the DNA was probably functionally largely intact (Barner & Cohen, 1954b; Wainwright & Nevill, 1956; McFall & Magasanik, 1960; Nakada, 1962a,b). There was evidence however that there were chemical and physical differences in the DNA after death e.g. much difficulty has been encountered in extracting DNA from bacteria after death (Hennigmann & Szybalski, 1962a; Dunn & Smith, 1955a,b, 1958; McFall & Magasanik, 1962b; Smith, 1962). This suggested that the DNA might have been damaged in some way, and this appeared to be consistent with the apparent alkali lability of the DNA after death. Cohen and Barner (1954a) found difficulty in reprecipitating this "after death DNA" after the cells had been extracted with alkali, and Riley (1962), using the diphenylamine reaction for estimating DNA (Burton, 1956) found that the DNA after thymineless death appeared to be partially destroyed by alkali.

Meanwhile, it had been reported (Coughlin & Adelberg, 1956) that thymine deficiency in 15T⁻ resulted in as much as a 1000-fold increase in

mutation rate in certain auxotrophic markers of bacteria surviving after 3 hours. This was confirmed (Kanazir in Barner & Cohen, 1958; Kanazir, Barner, Flaks & Cohen, 1959; Weinberg & Latham, 1956) for a number of other auxotrophic markers and Kanazir also showed that the most mutagenic stage of thymine deficiency was during lag phase. The absence of uracil in 15T⁻U⁻ markedly inhibited this effect. This was suggestive evidence of a change in the DNA during thymineless death, but that this was only an early event and a uracil-requiring step was perhaps necessary for its expression.

Dunn and Smith (1955a,b, 1958, 1960) showed that the proportion of the unusual base 6-methylaminopurine (6MAP) increased in the DNA during conditions of limiting thymine from 0.4% to 3.1% of total bases. A similar increase also occurred when 5-aminouracil, 2-thiothymine or 5-chlorouracil were added to the growth medium, but they report that they could show no increase in 6MAP content under conditions of complete thymine deficiency (Dunn & Smith, 1958). This could be because insufficient DNA synthesis occurs under these conditions for detectable incorporation, however Theil & Zamenhof (1962) have shown that neither B_3 nor B. subtilis inhibited with 5-aminouracil exhibits an increase in 6MAP content and Korn & Weissbach (1962) have shown that no increase occurs in thymineless $K_{12}(\lambda)$ and it may be that 6MAP is irrelevant to thymineless death. Addition of 6MAP to the growth medium of 15T⁻ inhibits growth, an effect that is reversed by adenine and not thymine, yet it does not result in any increase in 6MAP content in the DNA

(Dunn & Smith, 1958). An increase in 6MAP content could be mutagenic since the methyl group would tend to interfere with the hydrogen bonding of adenine with thymine.

In order to investigate the physical nature of the DNA before and after death, Luzzatti and Revel (1962) studied the melting curves of DNA prepared by the method of Marmur (1961) and by centrifugation of a whole lysate followed by ribonuclease treatment and dialysis. The curves were identical in respect of melting point, hyperchromicity and the range over which the DNAs melted. In addition they showed that there was no difference in the behaviour of before and after death DNAs in a caesium chloride gradient in the analytical ultracentrifuge, either with or without heat denaturation. This suggested that there was no massive fragmentation of the after death DNA in E.coli as was later suggested by Mennigmann and Szybalski (1962b) in 15T⁻ and FUdR-inhibited B.subtilis.

In 1962 (Mennigmann & Szybalski, 1962a) it was briefly reported that extraction of the DNA from FUdR-inhibited B. subtilis after 4 hours death ($\leqslant$ 1% survival) gave DNA which had a 3 to 5-fold lower specific transforming activity than DNA extracted from normal bacteria. Also that addition of thymidine after 2 hours of FUdR inhibition results in a restoration of DNA synthesis without restoration of viability, and that this DNA has a specific transforming activity 20-fold less than that of normal DNA. They reported that the DNAs as isolated after death were of

low molecular weight and did not form fibres in alcohol, and that if extensive deproteinisation was not carried out then the specific transforming activity was only moderately (sic) low. They suggested that the DNA must be fragile after death though they did not mention the possibility that extraction and deproteinisation of DNA from bacteria which had undergone unbalanced growth might be a more violent process and therefore result in the lower molecular weight etc.

In 1962 Mennigmann and Szybalski (1962b) further reported that the DNA as isolated by phenol deproteinisation of FUdR-inhibited B.subtilis had only 5% of the viscosity of the before death DNA and the DNA was more susceptible to shear when forced through a syringe needle. They claimed to have shown the same effect with the DNA of 15T⁻ isolated after death. We had already shown that DNAs prepared by Marmur's chloroform deproteinisation method from 15T⁻ had essentially the same intrinsic viscosity before and after death, and we extended this to show that phenol-deproteinised DNA (as used by Mennigmann and Szybalski) was no different; a fact which has since also been shown by Davison and Freifelder (unpublished experiments, cited in Maaløe, 1963).

Pauling and Gallant (1963) have briefly reported that 4 hours thymine deficiency of B_3 at 25° followed by incubation at 37° with thymine resulted in the 4 to 6-fold synthesis of DNA before division was resumed. (See also the Discussion to Maaløe's paper, 1963). Induction of 2-aminopurine mutants at this stage is less efficient/unit of DNA and they suggested that

some of this DNA may be non-functional.

Recently Lark and Pritchard have briefly reported (1963) the effect of thymine starvation on sequential chromosome synthesis in E. coli. The work of Nagata (1963), Yoshikawa and Sueoka (1963) Cairns (1963) and Bonhoeffer and Gierer (1963) shows that DNA replication starts at one point, the origin, on the bacterial chromosome and travels round the circular chromosome until it arrives at the origin again. Using pulses of tritiated thymidine and Bu density labelling (Lark, Repko & Hofmann, 1963), Lark and Pritchard (1963) have evidence that thymine starvation causes the DNA synthesis that occurs on subsequent addition of thymine to take place at three sites: at the site at which DNA synthesis was arrested, and at the two sites formed by the replicated origin. This could provide an explanation for the induction of the DNA synthesis synchrony observed by Barner and Cohen (1955), since all cells, irrespective of the position of the growing point relative to the origin, would begin a new round of DNA replication at the same time.

As mentioned earlier, ^{32}P-suicide and UV-inactivation act additively with thymine deficiency in causing cell death. Not only does ^{32}P-suicide shorten the lag phase of thymineless death, but pre-starvation of 15T⁻ for 30 minutes in thymine deficient medium before cold storage and and subsequent ^{32}P-decay results in an increased initial rate of inactivation (Fuerst & Stent, 1957)

UV-irradiated cells of B_3 show a shorter lag before thymineless death

(Gallant & Suskind, 1961) as mentioned earlier. On target theory, UV-inactivation of normal cells follows single-hit inactivation with five sensitive units. When B_3 cells are grown at 25° or 37° in the absence of thymine there is a progressive increase in their sensitivity to UV-light. However, at 37° but not at 25°, a rapid disappearance of UV-sensitive sites also occurs, and the inactivation kinetics rapidly approximate to those expected for single-hit inactivation by the time death has become exponential. There is also a decrease in mean lethal dose under these latter conditions.

Dissection of these two effects by studying the effects of the absence of protein synthesis caused by 2 µg/ml of chloramphenicol on the mean lethal dose and the number of sensitive sites showed that the decrease in mean lethal dose was due to unbalanced growth, and the decrease in sensitive sites was due to thymineless death (Gallant & Suskind, 1961). Although the change from five sensitive sites to one sensitive site gives a rough approximation to the kinetics of thymineless death (Gallant & Suskind, 1961), the break away in 15T⁻ is in fact too sharp to be explained simply on this basis (Maaløe, 1963).

Effects of Thymineless Death on the Acid-Soluble Components of the Cell

One of the effects of thymineless death on 15T⁻ is to cause the excretion of UV-absorbing materials which support twice as much growth of a uracil-requiring organism than do the excretions of E.coli B (Barner & Cohen, 1954a). This has also been shown with thymineless

mutants of B.subtilis (Brabander & Romig, 1960). Charcoal-celite column absorption shows that the materials contain uracil, hypoxanthine and orotic acid, and that since these compounds will incorporate ^{14}C during thymineless death, they are elaborated during death and are not merely autolytic products (Barner & Cohen, 1954b).

Some evidence for the control of DNA biosynthesis by regulation of the deoxynucleotide or nucleotide precursor pools is available (Prussoff, 1959; Morris & Fischer, 1960; Lark, 1960; Langer & Klenow, 1960). Little is known about regulation at the level of reduction from nucleotide to deoxynucleotide, however in vitro studies have indicated that some kind of feedback mechanism may exist (Reichard, 1960; Reichard, Canellakis & Canellakis, 1961), and in view of the effect of vitamin B_{12} deficiency in Lactobacillus leichmannii, this may be important.

Bacteria do not in general exhibit a gross build up to deoxynucleotides prior to synchronous DNA synthesis (Lark, 1963). In addition Lark (1961) found that inhibition of DNA synthesis does not lead to a measurable increase in accumulated deoxyribonucleotides. However, when E.coli B is irradiated with UV-light such that DNA synthesis is inhibited without affecting RNA and protein synthesis, an accumulation of acid-soluble pentose and deoxypentose compounds is obtained (Kanazir, 1954; Kanazir & Errera, 1954). Munch-Petersen (1960) found that the inhibition of DNA synthesis by deoxyadenosine in Ascites tumour cells (Langer & Klenow, 1960) led to an accumulation of dATP: only previously

observed in rat carcinoma (LePage, 1957). More recently Munch-Petersen and Neuhard (1964) have evidence for an accumulation of dATP in 15TAU during thymineless death. It is not yet clear what this observation means (Maaløe, 1963), though since it occurs in other conditions where DNA synthesis has gone awry it may be a result of thymineless death rather than a cause.

In addition to the UV-absorbing materials which are excreted during death, Riley (1962) showed that there was a massive excretion of diphenylamine-reacting material. The quantities excreted corresponded to the amounts which would have been incorporated into DNA had synthesis occurred at the rate characteristic of wild-type bacteria under the same conditions.

Thus it is clear that thymineless death, as well as affecting the DNA of the cell also causes considerable derangement of the acid-soluble precursors. The elucidation of some of these effects was another of the subjects of this study.

Site of the Lethal Event of Thymineless Death

Much evidence suggesting an alteration in the DNA after thymineless death has been reported: the co-operative effects of UV-inactivation and ^{32}P-suicide with thymineless death; the apparent alkali lability of the DNA as measured by its acid precipitability and by the decrease in diphenylamine colour; the fact that cells have to be actually engaged in DNA synthesis at the time of thymine deprivation for death to occur; the increase in mutation rate; the fact that death induces lysogenic

phages (Korn & Weissbach, 1962; Melechen & Skaar, 1962) and colicins (Sicard, 1961; Sicard & Devoret, 1962) just as they are induced by agents which damage DNA such as UV-irradiation which produces thymine dimers (Wacker, Dellweg & Jacherts, 1962), Mitomycin C which produces cross-links (Iyer & Szybalski, 1963) and Streptonigrin which also damages the DNA of the cell (Cohen, Shaw & Craig, 1963; Levine & Borthwick, 1963). Further it seems likely that RNA synthesis is required to express the lesion, for example by synthesising faulty messenger from the damaged DNA: although all the RNA experiments so far performed could equally well be explained by assuming that the synthesis of a small amount of DNA was necessary for death to occur, and that processes which deplete the cytosine and uracil pools for RNA synthesis also deplete the pools for DNA synthesis.

Evidence relating to changes in other components of the cell during thymineless death is very limited. There is evidence to suggest that there is no change in the ribosome distribution in the cells (Melechen, Stevens & Skaar, quoted by Gallant & Suskind, 1962), that the RNA formed during death is just as stable as normal RNA and enhances the cell's capacity for RNA and enzyme synthesis quite normally (McFall & Magasanik, 1962a). It has been observed that the rate of messenger RNA production falls off during thymine deficiency (McFall & Magasanik, 1962b), but this has been convincingly interpreted (Nakada, 1962) in terms of catabolite repression (Pardee, 1961) rather than its being due to damage due to

thymine deficiency. The only report of a change in other cell components due to thymine deficiency is of the appearance of a new ribosomal component (Roberts, 1960) in 15TAU, sedimenting at 42S. There has been no further report on this observation and it is difficult to assess its importance.

The brief reports (Sicard, 1961; Sicard & Devoret, 1962) that the induction of a colicin is the cause of the rapid loss of viability in 15T⁻ is of great interest since this would be consistent with some damage to the DNA being the primary event in thymineless death thus inducing colicin production, a step requiring RNA synthesis for its expression. A number of factors however are difficult to reconcile with this observation. Induction of a colicin would be expected to show similar kinetics to the induction of a lysogenic phage (Adams & Wasserman, 1956) with the observed lag phase representing the period of induction and the exponential death being caused by the activity of the colicin. Maaløe (1963) found however that cells which were in a culture in the exponential phase of death, yet were still viable, could be completely rescued by adding thymine for 60 minutes. Also the breakaway from lag phase into exponential death is too sharp to be solely due to the colicin killing although it was admitted that considerable loss of viability occurred in a strain in which there was no evidence of colicins at all, and the superimposition of the two sets of kinetics may be sufficient to explain the sharpness of the breakaway. Insufficient evidence was published however to decide on this possibility.

It is difficult also to explain the fact that protein synthesis does not appear to be necessary for death if a proteinaceous colicin is involved. Colicin killing is known to inhibit cell metabolism immediately and to render phage infection abortive (Fredericq, 1957), however oxygen uptake is apparently not affected during thymineless death (Barner & Cohen, 1954a) and phage T2 can successfully infect 15T⁻ before or after death (Barner & Cohen, 1954a).

It might be argued as Maaløe has done recently (1963) that death occurs in two stages: a build up of potential and the expression of this potential requiring a complete medium i.e. the agar plate. In this case expression of the induction of a colicin may require thymine as does the expression of the induction of λ by thymineless death (Korn & Weissbach, 1962). It appears that it is indeed the case (Devoret, personal communication) that colicin production requires the presence of thymine. Thus, although colicin-killing may be involved in producing the particular kinetics of death observed, it is probably irrelevant to the actual cause of thymineless death itself.

The present work

The first aim of this study has been to elucidate the effects of thymineless death on the DNA of the cell; particularly to investigate the apparent alkali lability of the DNA and its physical structure by means of viscosity, sedimentation and melting curve determinations,

the measurement of band-widths in caesium chloride gradients; the search for cross-links; and autoradiography. The second aim has been to elucidate the factors governing the excretion of deoxyribose compounds during thymine deficiency and also to attempt to dissect the observations arising from the use of thymine analogues.

MATERIALS AND METHODS

Materials and Methods

Bacterial Strains

Escherichia coli B_3, obtained from Dr. S. Brenner, and E. coli $15T^-$, obtained from Dr. S. Cohen are thymineless strains which grow optimally on 2 µg/ml of thymine. They were maintained on hard agar slopes containing 10 µg/ml of thymine.

E. coli 15TAU, obtained from Dr. O. Maaløe, was derived from $15T^-$ by UV-irradiation (Barner & Cohen, 1958) and had additional requirements for arginine and uracil. This strain was maintained on hard agar slopes supplemented with 10 µg/ml thymine, 25 µg/ml arginine-HCl and 10 µg/ml uracil.

E. coli B96, obtained from Dr. J. Cots, is a purine requiring mutant which grows optimally on agar supplemented with 10 µg/ml histidine, 10 µg/ml thiamine and 10 µg/ml adenine. The purine requirement is due to its inability to convert 4-amino 5-imidazole carboxamide ribotide to inosinic acid (Cots, 1953).

Method of culture

A minimal medium (Cohen & Arbogast, 1950) buffered with phosphate at pH 7.4 and containing 1 g./litre mannitol as carbon source was used for all media unless otherwise indicated. Supplements were added as necessary. 16 µg/ml of thymine was routinely used to ensure that sufficient thymine was always available. The mean generation time of E. coli in this medium was about 55 min.

In experiments where a medium with a low phosphate concentration was required, the mannitol medium was modified by buffering with 0.1M Tris at

pH 7.4. The phosphate concentration in these cases was 0.3 μmoles/ml and the sodium and potassium requirements were satisfied by the addition of 1g. each of NaCl and KCl. The mean generation time of the bacteria in this medium was also about 55 min.

Bacterial cultures were prepared by inoculating a small volume of medium from the stock agar slopes which were maintained at 4°, and incubating this at 37° with aeration overnight. The following morning this inoculum was diluted 10-fold in prewarmed fresh medium, and this culture was incubated at 37° with aeration until the culture had an extinction of 0.3 when read in a cell of 1 cm. light path at 650 mμ in a Beckman DU spectrophotometer. This corresponds to about 5×10^8 viable cells per ml. of culture.

Where necessary, harvesting was carried out by chilling the culture in an ice-bath and centrifuging at 5,420 g for 10 minutes in a Mistral 6L refrigerated centrifuge or at 3,600g for 15 minutes in an MSE International centrifuge. The supernatant culture medium was then discarded, and the bacterial pellet was resuspended twice in ice-cold saline (0.1M NaCl, 0.01M $MgCl_2$) and centrifuged before final resuspension in an equal volume of prewarmed medium for an experiment.

Herring DNA was prepared by the method of Kay, Simmons and Dounce (1952) and was a gift from Dr. K. Burton.

Micrococcus lysodeikticus DNA was prepared by the method of Marmur and was a gift from Dr. G. Petersen.

Preparations of DNA from E. coli

Since so many reports have appeared in the literature claiming that DNA is more difficult to extract after thymineless death, particular attention has been focussed on obtaining good yields of DNA. This should help eliminate the possibility that only intact DNA has been extracted for characterisation.

Preparation 1

The DNA prepared for the determination of the effect of alkali on its viscosity was prepared from 15T⁻ by the chloroform deproteinisation method of Marmur (1961). "Before death" DNA was prepared from 15T⁻ grown with 16 µg/ml thymine; "after death" DNA was prepared from bacteria which had undergone a further 2½ hours growth in the absence of thymine. "Wild-type" DNA was prepared from E. coli B.

These preparations had the characteristics shown in Table 1.

The DNAs were dissolved in M/100 NaCl. The pH of the solutions was 7. A drop of chloroform was added to each solution to prevent bacterial growth, and the solutions were stored at 4°. Preparations stored in this way showed no change in intrinsic viscosity over a period of three months suggesting the absence of any deoxyribonuclease activity.

Table 1 shows that Marmur's method gives similar DNA yields in both after death and wild-type preparations. It is also clear that such DNA preparations are relatively free of protein and RNA.

TABLE 1

DNA	yield of bacteria in g. wet wt.	yield of DNA in mg.	RNA content as % total sugar	protein as % w/w	DOR/P
Wild type	8.1	27	1	0.1	0.47
Before death	8.0	13	0.5	0.2	0.45
After death	8.0	25	1	0.2	0.45

Analysis of 15T⁻ DNA. Preparation I

Preparation II

A second preparation of DNA from before and after death $15T^-$ was made for viscosity work using the method of Marmur. The after death bacteria had been starved of thymine for 3 hours (survival < 0.3%). The after death DNA could not be freed of protein and RNA after two treatments with ribonuclease and subsequent chloroform deproteinisation. The DNA was sufficiently free however for the viscosity measurements required. The DNAs were dissolved in M/100 NaCl and the pH was 7. Table 2 shows the characteristics of the preparations used. These DNAs were subsequently deproteinised further using redistilled phenol according to the method of Davison and Freifelder (1962). This method is the most gentle method available for deproteinisation and minimises the shear degradation caused by violent shaking. 1 ml. of each of a DNA preparation (1 mg/ml in M/100 NaCl) was added to an equal volume of ice-cold, redistilled phenol and rocked gently at 4° for ½ hour. The phenol layer was then removed and the process was repeated with another quantity of phenol. The phenol layer was again removed and the remainder of the phenol was removed by 5 washes with ether which had been redistilled and stored over $FeSO_4$ to trap peroxides.

The resulting DNAs had no detectable protein associated with them.

Preparation III

These DNAs were prepared by an application of phenol deproteinisation to whole bacterial lysates. Cells were grown and harvested before and after three hours of thymine deficiency (< 0.5% survival). The bacterial

TABLE 2

DNA	bacterial yield in g. wet wt.	DNA yield in mg.	RNA content as % total sugar	protein as % w/w	DOR/P
Before death 15T⁻	17	47	0	0.1	0.50
After death 15T⁻	23	54	2	2	0.46

Analysis of 15T⁻ DNA. Preparation II.

pellets were washed in saline-EDTA (Marmur, 1961) and resuspended in 10 ml of Tris, pH 8.0. The cell-walls were digested with 200 µg/ml lysozyme at 30° for 10 minutes, and lysis was effected with 1% sodium dodecyl sulphate at 37°. As soon as lysis had occurred (3-4 minutes) the lysate was chilled in an ice-bath and an equal volume of cold, water-saturated, redistilled phenol was added and the whole mixture was rocked for 1½ hours at 4°. The phenol and aqueous phases were separated by centrifuging at 2,500 g for 5 minutes and the phenol layer removed. Five washes with equal volumes of ether were used to remove the last traces of phenol, and the ether was removed by blowing air over the surface of the solution at 30°. RNA was hydrolysed by treatment with 50 µg/ml ribonuclease at 37° for ½ hour, and papain was then added to digest the ribonuclease and itself. The solution was then centrifuged in an MSE Superspeed-40 at 3° for 24 hours at 160,000 g. The pellet was washed and resuspended in 0.1 M NaCl and the centrifugation repeated. The pellets contained all the DNA and were dissolved in 0.1 M NaCl. The preparations still contained about 20% w/w RNA which was ribonuclease resistant, and this was removed by centrifuging in CsCl at 160,000 g for 24 hours. 1.25 x the weight of DNA solution was the weight of CsCl used (Sinsheimer, Starman, Nagler & Guthrie, 1962) to give a mean density of 1.71 g cm^{-3}. The DNA banded free of RNA and protein, and CsCl was removed by dialysing this fraction against 0.005M phosphate, pH 7.2, 0.1M NaCl.

Sedimentation analysis of this preparation in the analytical

ultracentrifuge showed that the DNAs had sedimentation coefficients of 29.1 S in 0.005M phosphate pH 7.2, 0.05M NaCl, both for the before and after death DNA preparations. Using the equation of Rubinstein, Thomas and Hershey (1961):

$$S_{w,20} = 0.00244 \times M_w^{0.543}$$

where $S_{w,20}$ is the sedimentation coefficient at 20° in water expressed in Svedberg units ($1S = 1 \times 10^{-13}$ sec).

The equation is based on a scale standardised against autoradiographic estimates of the molecular weight (M_w) and gives values rather higher than those obtained previously (Doty, McGill & Rice, 1958).

The above equation gives a molecular weight of about 40×10^6 for the DNAs of Preparation III.

Preparation IV

This preparation was prepared for cross-linking studies. Before and after death DNA was prepared from 15T⁻ by phenol deproteinisation as in Preparation III. Instead of centrifuging down the DNA in the Speed 40 however, the ribonucleotides produced by the ribonuclease action and the amino-acids produced by the papain action were removed by dialysis against three changes of 1½ litres of 0.01M phosphate buffer, pH 7.2, 0.5M NaCl followed by three changes of 1½ litres of 0.01M phosphate buffer, pH 7.2, 0.01M NaCl at 4° for 72 hours.

DNA was prepared from 15T⁻ before death, after 1½ hours' death, after three hours' death and after 1½ hours thymine deficiency followed by 1 hour with 16 µg/ml of thymine.

The DNAs thus prepared had large quantities of RNA still associated and this was removed by shaking the DNA dissolved in 0.14M NaCl with activated charcoal (Zamenhof & Chargaff, 1951). This gave preparations which contained the contaminants shown in Table 3. Since the CsCl gradient separates these contaminants from the DNA, it was possible to use these preparations without further purification.

Sedimentation analysis of these four DNAs showed that the sedimentation constants were identical in dilute saline-citrate (see Table 4). This value compares favourably with the value of 30S obtained by Eigner, Schildkraut and Doty (1962) for the DNA of E.coli K 12 dissolved in dilute saline-citrate, pH 7.0 and corresponds to a molecular weight of about 40×10^6 (Rubinstein et al., 1961).

Caesium Chloride was supplied by British Drug Houses Ltd., Poole, Dorset, and was a grade designated "For Ultracentrifuge Work".

Deoxyribonucleosides were supplied by California Foundation for Biochemical Research, Los Angeles, California, U.S.A.

Papain was used as supplied in the crystalline form by British Drug Houses and diluted 1:2 with M/100 cysteine.

Ribonuclease was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Before use the enzyme was heated at 80° for 5 min. in Tris or phosphate buffer at pH 7.0 to inactivate any deoxyribonuclease activity present.

5-bromodeoxyuridine was supplied by Schwartz Bioresearch Inc., Orangeburg, New York, U.S.A.

Table 3

Analysis of 15T⁻ DNA - Preparation IV

Type of DNA	protein	RNA
Before death	5%	3%
After 1½ hr. death	2%	4%
After 1½ hr. death followed by 1 hr. with thymine	4%	4%
After 3 hr. death	4%	5%

Table 4

Sedimentation coefficients of the DNAs of Preparation IV

Type of DNA	Sedimentation coefficient
Before death	30.5
After 1½ hr. death	30.6
After 1½ hr. death followed by 1 hr. with thymine	30.6
After 3 hr. death	30.4

Chloramphenicol was supplied by Parke, Davis & Co., Hounslow, Middlesex.

Phenylethyl alcohol was supplied by British Drug Houses, and was redistilled before use.

5-Bromouracil was supplied by Sigma Chemical Company. A concentration of 200µg/ml was used in all cases unless otherwise stated.

Mitomycin C was supplied by Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

E. coli phosphomonoesterase was a chromatographically purified material suspended in 65%$(NH_4)_2SO_4$ (Worthington Biochemical Corporation, Freehold, N.J., U.S.A.). The suspension was diluted with 2 vol. of 0.01M $MgCl_2$ and dialysed against 0.01M Tris buffer pH 8.0 containing 0.01M $MgCl_2$. The preparation was then heated at 85-90° for 10 mins. and quickly chilled.

Radio-isotopes: Tritiated thymidine, labelled in the 6-position and of high specific activity (14.2 c/mmole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

^{32}P-labelled phosphate was carrier-free and of high specific activity. This was also obtained from the Radiochemical Centre.

Formaldehyde: was redistilled into water immediately before use and assayed according to Vogel (1958).

Dilute saline-citrate is a buffered saline containing 0.015M NaCl plus 0.0015M trisodium citrate (Marmur, 1961).

Saline-EDTA: contains 0.15M NaCl plus 0.1M EDTA, pH 8.0. Both EDTA and high pH inhibit deoxyribonuclease activity (Marmur, 1961).

Viscometry

All viscosimetric measurements were made at 25° using a viscometer described by Zimm & Crothers (1963) and constructed by Mr. M.G. Smith. The advantage of this apparatus over all other viscometers is that shear rates are obtainable that are sufficiently low for the measured viscosities to be entirely independent of shear.

Relative viscosities, η_{rel}, were obtained by measuring the rotor rotations in the DNA solution and in the solvent alone:

$$\eta_{rel} = \frac{\eta_{solution}}{\eta_{solvent}}$$

The specific viscosity η_{sp} is given by the relationship

$$\eta_{sp} = \eta_{rel} - 1$$

The intrinsic viscosity, $[\eta]$, is then given by the intercept on the ordinate when η_{sp}/c is plotted against c, where c is the concentration of the solute. $[\eta]$ is a direct measure of the molecular weight of the solute (Yang, 1961).

The measurement of the absolute viscosity of a 10% w/w solution of glycerol using this instrument gave a value of 1.151 centipoises. The published value is given as 1.153 centipoises at 25° (Lange, 1956).

Determination of Melting Curves of DNA

This was carried out in a Unicam SP500 spectrophotometer fitted with an adjustable constant temperature device. The temperature of the circulating water was raised by 1° intervals and the temperature measured

in the blank cell by an immersed thermometer. When the cell temperature was constant for three minutes it was assumed that the temperature of the DNA solution was the same, and its extinction at 260 mμ was measured. Evaporation of the DNA solution was prevented by using the special 1 cm. stoppered cuvettes supplied for this purpose by Unicam Ltd.

Sedimentation Analyses

These were performed at 39,460 r.p.m. in a standard 12 mm cell in the Spinco Model E. Analytical Ultracentrifuge fitted with UV optics. The temperature was held at 20° by an automatic thermostat device supplied with the instrument. Photographs of the sedimenting material were taken every four minutes with 15 sec. exposures. The optical density profile of the film was measured in an Analytrol integrating densitometer (Beckman Instruments Inc., California, U.S.A.) and the sedimentation constant was calculated graphically by standard methods. No correction was made to zero concentration of DNA (Shooter & Butler, 1956), but all measurements were done at the same DNA concentration with each sample. Corrections for concentration effects would not be more than 2S at these concentrations (Eigner et al., 1962), and therefore do not affect the conclusions reported.

Determination of the Band-width of DNA in Caesium Chloride Gradients

The methods used throughout were those of Thomas and Berns (1961a,b) and Thomas and Pinkerton (1962). 2μg/ml of DNA from Micrococcus lysodeikticus was used as a density marker, and densities were adjusted by means of a differential refractometer to give a mean density of 1.710g. cm^{-3} (Vinograd & Hearst, 1962). DNA of Preparation III, at

5 µg/ml final concentration was dissolved in 50 mM phosphate, pH 7.2, 12.4mM NaCl (final concentration) with CsCl. Photographs of the rotating cells which were spinning at 44,770 r.p.m. for 30 hrs. were taken using a 30 sec. exposure. An aperture mask was used to allow the independent photography of the two samples which were centrifuged simultaneously.

The densities of the DNA bands were calculated from their distance from the M. lysodeikticus marker by the method of Sueoka (1961).

Determination of Cross-linking in DNA

The method of Iyer and Szybalski (1963) was used. The DNA (Preparation IV), heated in 1% formaldehyde for 6 minutes at 100° and rapidly cooled in ice-water, was dissolved at 5 µg/ml final concentration in a CsCl solution whose final composition was 0.6mM NaCl, 0.06mM trisodium citrate, pH 7.0. The density was adjusted to 1.710 $g.cm^{-3}$ as before, and the solutions were centrifuged at 44,770 r.p.m. for 30 hours. Photographs of the spinning cells were taken as before and densitometer tracings also obtained. Calculation of the densities of the bands was by the method of Sueoka (1961) and the areas under the bands were taken as being proportional to the amount of that class of DNA present in the solution.

Measurement of DNA synthesis during thymine deficiency

250 ml of 15T⁻ were grown to 5 x 10^8 cells/ml in a mannitol medium containing low phosphate. After harvesting, the bacteria were resuspended in 250 ml of the same medium but deficient in thymine. 1 mC of ^{32}P was added on resuspension and 20 ml samples were taken at various

times for 1½ hours. The samples were immediately pipetted into 16 ml of ice- cold 0.5N perchloric acid and spun at 2500 g at 4° for 10 minutes. The pellets were washed once with cold 0.2N perchloric acid and then washed with ethanol:$CHCl_3$::3:1 and ether to extract the phospholipids. After drying the pellets 2 ml N NaOH were added and the mixture was incubated overnight at 37° to destroy the RNA. The solutions were then neutralised with 0.4 ml 5N HCl and 2 volumes of ethanol were added to reprecipitate the DNA (McFall & Magasanik, 1962b). The solutions were then spun for 1 hour at 2,500g to pack the precipitated DNA into compact pellets. These pellets were washed with 0.2N perchloric acid and spun for another hour at 2,500 g. The pellets were finally dissolved in 2 ml of 66% formic acid and these solutions were divided into two parts. To one was added 0.02g diphenylamine (Burton & Petersen, 1960) and all tubes were incubated for 16 hours at 30°.

The inorganic phosphate released from the inter-purine phosphate links was extracted from the DNA digests with ammonium molybdate in HCl (Martin & Doty, 1949) saturated with isobutanol and an equal volume of water-saturated benzene and of water-saturated isobutanol (Nielsen & Lehninger, 1955). The isobutanol layer was made up to 10 ml with isobutanol and the radioactivity in the solution was counted in an Ecko Autoscaler liquid counter (Ecko Electronics Ltd.).

Photography

UV Films. All films taken with UV Optics in the analytical ultracentrifuge were developed in Kodak 19b developer for 7 mins. at 19°. The plates

were then washed in tap water and fixed for ½ hour in Kodak Acid Fixer with Hardener. Finally the films were washed for two hours in running water and ½ hour in distilled water before drying in the air at room temperature.

Photographs of bacteria and autoradiographs were taken by phase contrast in a Zeiss light microscope with a camera attachment. Exposures of 6 sec. were used, and the films were developed and fixed in the same way as with the UV films.

Electron Microscopy

Samples of bacteria were prepared for electron microscopy as follows: 15TAU was grown in $T^+(AU)^+$ to an extinction of 0.3 at 650 mμ, harvested, washed and 1/3 resuspended in each of $T^-(AU)^-$, $T^+(AU)^-$ and $Bu^+(AU)^-$. Those in $T^-(AU)^-$ were aerated at 37° for 4 hours. Those in $T^+(AU)^-$ were aerated for 90 minutes before being divided into two and being resuspended and aerated in $T^-(AU)^-$ and $T^-(AU)^+$ respectively for 3 hours.

Those in $Bu^+(AU)^-$ were aerated for 90 minutes before being resuspended in $T^-(AU)^-$ and aerated for 3 hrs. at 37°. At the end of these treatments the cells were centrifuged at 5,000g for 5 min. and a concentrated suspension (about 10^{11} bacteria/ml) used as the starting material.

These samples were fixed in osmium tetroxide and dehydrated in ethanol by standard procedures. The samples were then embedded in methacrylate which had been partially prepolymerised to reduce embedding artifacts, and was hardened into a solid block by heating at 50° for two days. Ultrathin sections (about 80 mμ thick), were cut with a microtome and

loaded onto Formvar coated grids and dried. Samples were then examined in a Siemens Elmiskop I, and pictures were taken of typical fields.

Autoradiography of Bacterial Chromosomes

The procedures used were exactly as those described by Cairns (1962, 1963). In these cases, 15T⁻ was labelled for about 1½ generations with high specific activity tritiated thymidine (14.2 curies/mmole) in the presence of 250 µg/ml deoxyadenosine which effectively increases the thymidine concentration by acting as an alternative substrate for phosphorylase, hydrolase and N-glycosyltransferase activities (Boyce & Setlow, 1962).

0.08 ml of culture at an extinction of 0.3 at 650 mµ were taken in thymine deficient medium and to this was added 0.24 ml $\frac{4}{3}$ x concentrated mannitol medium and 0.08 ml tritiated thymidine (160 µC, 2 µg/ml final concentration) labelled in the 6 position. This suspension was made 250 µg/ml with respect to deoxyadenosine and incubated with aeration at 37°. The bacteria were harvested, washed and resuspended in 0.4 ml of thymidine-deficient mannitol medium and incubated with aeration.

2 µl samples were taken at zero time (Before death) and after 1½ hr. After 1½ hr. the culture was divided into two parts and to one part was added more deoxyadenosine and tritiated thymidine and incubation continued for a further ½ hour before taking a 2 µl sample. The other half of the culture was incubated for a further 1½ hr. in the absence of thymidine and a 2 µl sample taken at the end of this incubation period (a total of 3 hr. thymidine deficiency).

The 2 µl samples were pipetted immediately into ice-cold "lysis medium" containing 1.5M sucrose, 0.01M KCN, 0.05M NaCl, 0.01M EDTA, pH 8.0 and diluted 400-fold in the same solution. 0.5 ml of these bacterial suspensions were then pipetted into glass dialysis cylinders made especially for this purpose: See Fig. 2.

5.6µg herring sperm DNA was added as carrier to each cylinder (8 autoradiographs were taken for each sample), and a VM Millipore filter, 50 mµ pore size, was used as the dialysis membrane. The cylinder was assembled with two small elastic bands keeping the two glass sections together, and the join was coated with silicone grease to render it water-tight. The cylinders were suspended from threads in the following dialysis media:

1.5M sucrose, 0.05M NaCl, 0.01M EDTA, 1% sodium dodecyl sulphate, pH 8.0 for 2 hr. at 37°.

0.4M NaCl, 0.1M EDTA, pH 8.0 for 1 hr. at 37°.

0.01M EDTA, 0.04M NaCl, pH 8.0 for 36 hr. (3 changes) at 4°.

The Millipore filters were then punctured and the fluid in the dialysis chambers allowed to run out. The filters were dried at room temperature, and cemented with Millipore Cement or Cow Gum onto microscope slides subbed in 5% gelatin plus 0.5% chrome alum. When dry, the filters were overlaid with A.R.10 Kodak stripping film using a wet mounting procedure, and the slides were dried in air at room temperature overnight. The slides were sealed in a light-tight box with silica gel and placed in a

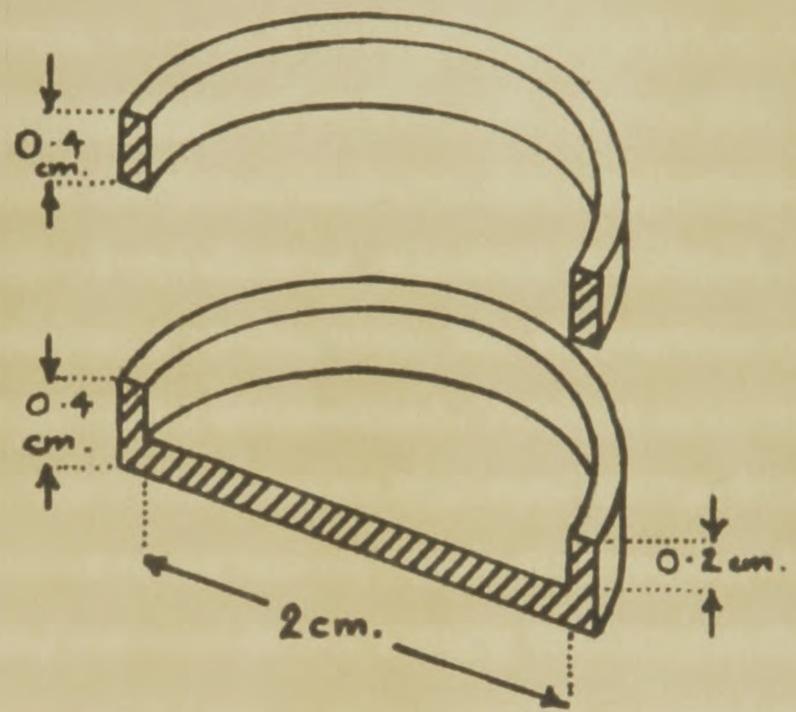


Fig. 2. Sagittal section of a glass cylinder used for dialysing small volumes of liquid: a Millipore filter is placed between the two glass rings and the whole apparatus is held in position with rubber bands.

desiccator with silica gel and a CO_2- atmosphere in a refrigerator. After 6 weeks the films were developed in Kodak 19b for 7 min., fixed in acid fixer for ½ hour and washed for several hours in tap water. The films were floated off the slides and spread out to dry on clean microscope slides. They were then examined under phase contrast and photographs taken of suitable chromosomes.

Charcoal-Celite chromotography

The method of Cohn (1963) was used with the modification that the alkaline washing stage of the charcoal contained 1% toluene. This acted by plugging the irreversible binding sites for UV-absorbing materials on the charcoal (Cohn, personal communication). Before nucleotide solutions were applied to the (1cm x 4 cm) column they were made approximately pH 5 with HCl. Darco G-60 charcoal was used instead of Barneby-Cheney 1654. Dowex-1, 200-400 mesh, was used in the formate form as described by Burton & Petersen (1960).

Dowex-50, 200-400 mesh, was used in the H^+ form as described by Moore and Stein (1951).

Mapping procedure for Acid-Soluble Nucleotides from 15T⁻

Acid-soluble nucleotides from 1 litre of bacteria at 5 x 10^8 cells/ml give sufficient material for one map.

1 litre of bacteria were harvested by adding 300 g. of ice to the culture and centrifuging at 5,420g at 4°. The bacterial pellets were immediately washed with ice-cold 0.2N perchloric acid and the washings were combined in a total volume of 50 ml. These were then spun at 5420g

at 4° for 10 min. to remove the acid-insoluble material, and one drop of phenol red was added as indicator and the extract neutralised with ice-cold 5N KOH (Hurlbert, Schmitz, Brumm & Potter, 1954; O'Donnell, Mackal & Evans, 1958). After standing for 45 min. in ice the solution was spun at 5,420 g for 10 min. to remove the precipitated $KClO_4$. The pH was then adjusted with HCl to about 5 and the extract was applied to a charcoal-Celite column. Salts were washed through with 10 bed-vols. of water. The UV-absorbing materials were then eluted with 10 ml of ethanolic-ammonia and the eluate freeze-dried. Dissolved in a small quantity of water the eluted nucleotides were spotted onto Whatman No. 1 chromatography paper (47cm. x 57cm.) and the paper dipped, except round the spot where it was sprayed from an atomiser, in ammonium formate buffer (Petersen, 1963) to which M/1000 EDTA had been added. The pH of the buffer was 2.7. A Perspex electrophoresis apparatus, essentially the same as that described by Rushizky and Knight (1960) was used to separate the nucleotides according to their charge. This was done at 500 volts at room temperature for about 10 hours. The time taken for the separation varied a great deal according to the efficiency with which the salt was removed from the extract, but the course of the separation could be followed by means of the phenol red which acted as a marker. After drying the electrophoretogram in air, the nucleotides were separated in a second dimension by descending chromatography (twice if necessary) in isopropanol/water::70/30. The resulting chromatogram was photographed using a UV-lamp by making a contact print on Ilford 50M document paper, the shortest practicable exposure time (Markham & Smith, 1949) being used. As can be seen from

Plate 1, this procedure satisfactorily separates the mono-, di-, and triphosphates from each other and confirms the absence of large quantities of cytosine nucleotides in E. coli (O'Donnell et al., 1958). Spots were then cut off the paper and concentrated in the tips of the cut-out spots by dipping one end into water and allowing capillary action to transport the nucleotide to the farther end. The nucleotide was eluted from the tip with 2 ml of water and identified from its acid, neutral and alkaline spectrum measured in the Beckman DK-2 recording spectrophotometer, and from its sugar and phosphate content. Of 16 spots this method allowed unequivocal identification of 11. 3 of the remaining 5 contained adenine: ribose:phosphate in the ratio 1:1:2, and may be degradation products of NAD. The other two spots remained unidentified.

It was essential to extract the nucleotide rapidly from the bacteria. Glucose was used as carbon source in preference to mannitol since metabolism continues anaerobically in this case and there is a greater chance that the nucleotide pattern will reflect the normal state of affairs. If the acid-soluble nucleotides are not isolated rapidly the ratio of the tri- to di- to monophosphates decreases rapidly. There is also a rapid accumulation of hypoxanthine and xanthine unless rapid extraction is achieved.

Determination of Bacterial Respiration Rates

15TAU were grown and harvested under standard conditions; half the culture was resuspended in $T^{+}(AU)^{-}$ for 90 minutes and the other half was divided into four aliquots and resuspended in 1/4 of the volume of $T^{+}(AU)^{+}$, $T^{-}(AU)^{-}$, $T^{+}(AU)^{-}$ and $T^{-}(AU)^{+}$ respectively. After the first half of the culture had completed its 90 minute incubation it was again

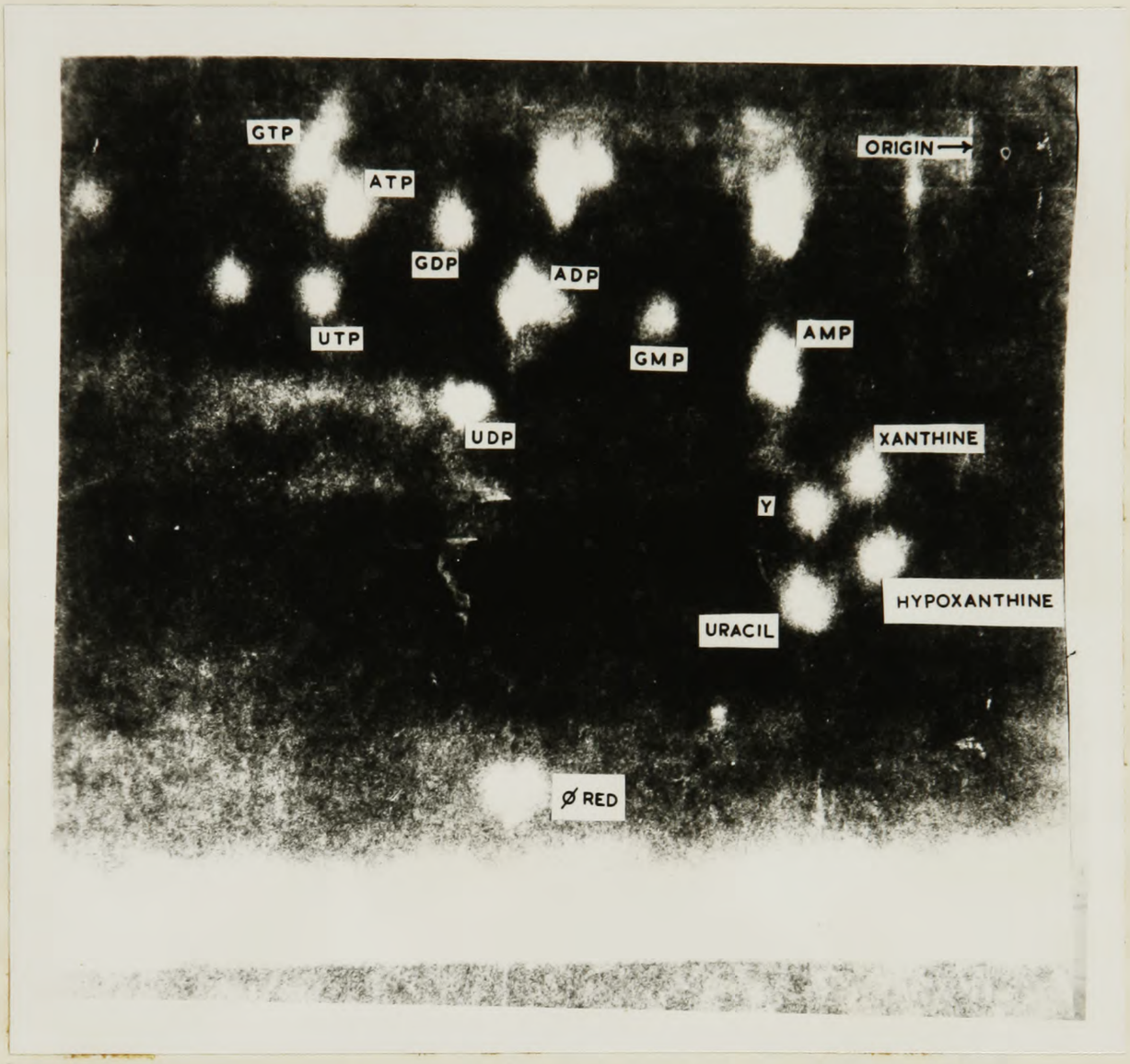


Plate 1. Separation of acid-soluble nucleotides from 15T⁻ by paper electrophoresis and chromatography. Phenol red was present from the neutralisation stage of the acid-extraction and acted as a marker. Paper electrophoresis was carried out in the first dimension (from right to left across the photograph). Chromatography was carried out in the second dimension (from top to bottom of the photograph).

harvested and resuspended in 1/4 of the volume of $T^-(AU)^-$ and $T^-(AU)^+$ respectively.

3 ml of each of the 6 concentrated bacterial suspensions were pipetted into Warburg flasks containing 0.2 ml 5N KOH in the centre well to absorb CO_2. The oxygen uptake was measured at 37° using the usual manometric techniques.

Viable counts:

These were determined by the double layer method (Adams, 1950). Samples of bacteria were taken from the culture at suitable times, appropriately diluted in sterile saline (0.1M NaCl, 0.01M $MgCl_2$) and plated with suitably supplemented soft agar onto hard agar in a sterile Petri dish. After 18 hr. incubation at 37°, the number of colonies were counted and the viable count was calculated.

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

RNA was assayed by the method of Ogur & Rosen (1950).

DNA and deoxyribose was assayed by the method of Burton (1956).

Inorganic Phosphate was estimated by the method of Chen, Toribara & Warner (1956).

Total Phosphorus was estimated by the modified Chen procedure used by Burton & Petersen (1960).

EXPERIMENTAL AND RESULTS

Experimental

Investigations on the DNA of E. coli 15T⁻

In order to investigate the apparent alkali-lability of the DNA from a thymineless mutant after thymine starvation two aspects were considered. The non-precipitability of the DNA after neutralisation of the alkaline incubation mixture with acid (Garner & Cohen, 1954a; McFall & Magasanik, 1962b), and the decrease in diphenylamine reacting material (Riley, 1962).

Non-precipitability of 15T⁻ DNA after alkali-treatment

The DNA from E. coli 15T⁻ after thymineless death was extracted by the method of Marmur and the reprecipitability tested at each stage. Bacteria were harvested, washed, resuspended in saline-EDTA and lysed with 1% sodium dodecyl sulphate. The lysate was then made N with respect to sodium perchlorate and deproteinised by shaking with chloroform-isoamyl alcohol. The aqueous layer was treated with two volumes of ethanol and the DNA collected as spooled fibres. Samples were taken at each stage and made N with respect to NaOH and incubated for 1 hour at 70°. The incubated mixtures were then neutralised with 5N HCl and spun at 2,500g for 10 minutes. The supernatants were decanted from the precipitates and both fractions were assayed for DNA. Controls were performed by treating identical DNA preparations with N alkali followed by immediate neutralisation. The results are shown in Table 5; expressed as percentages of the total DNA present in the samples.

This clearly suggested that the DNA was not itself alkali-labile at all, but that alkaline incubation of some other factor in the bacteria

Table 5

Stage of purification	Unheated control		Heated in alkali	
	supernatant	pellet	supernatant	pellet
acid washed pellet	9	91	96	4
crude lysate	11	89	56	44
aqueous layer after first deproteinisation	17	83	33	67
protein interface from first deproteinisation	0	100	0	100
ethanol pptd. DNA	0	100	4	96

Precipitability of 15T⁻ DNA after thymineless death at various stages of the Marmur extraction

was resulting in the formation of a complex with the DNA which would not precipitate on neutralisation of the solution. To test this some herring sperm DNA was added to the acid-washed precipitate of the after and before death 15T" pellets at the same time that the alkali was added. After incubation at 70° for 1 hour, neutralisation failed to reprecipitate either the E. coli or the herring sperm DNAs although both DNAs could be precipitated quite normally in the unheated controls. Thus it was clear that from this stand-point at least, the alkali-lability of the DNA was not a function of the DNA, nor did it have anything to do with thymineless death as has been suggested (McFall & Magasanik, 1962b) since the apparent alkali-lability is also observable before death has occurred. Consistent with this is the observation that after 3 hours incubation of B_3 cells at high cell density in the absence of thymine (<10% death), the DNA does not show this inability to reprecipitate after alkali-treatment.

Decrease in Diphenylamine-Reacting Material after Alkali Treatment.

It was noticed during the assay of the DNA in the various fractions of the Marmur-extraction that alkali-treatment of some of the fractions resulted in an apparent decrease in diphenylamine-reacting material rather more than in others, but that the final alcohol-precipitated DNA was quite stable to alkali. See Table 6.

Riley had suggested that in E. coli B_3 the DNA becomes more alkali-labile during thymine starvation since he had also observed this decrease

Table 6

Fraction	%age decrease of diphenylamine-reacting material due to alkali treatment
aqueous layer from $CHCl_3$ deproteinisation	15
protein interface layer from $CHCl_3$ deproteinisation	50
ethanol pptd. DNA	0

Decrease in diphenylamine-reacting material after alkali-treatment at various stages of the Marmur extraction

in diphenylamine colour and observed that the effect increased during thymine starvation. It seemed possible that the observed alkali-lability was in fact due to the formation of an inhibitor of the diphenylamine reaction during alkaline incubation with some component of the cell which increased during the period of unbalanced growth.

An investigation of B_3 DNA extracted by Marmur's procedure from the cells after 2½ hr. thymine deficiency showed that the purified DNA was perfectly stable in alkali at 37° overnight or at 70° for 1 hr.

Examination of the alkali-lability of the DNA at the various stages of the purification however showed that whilst the DNA in the aqueous layer from the first $CHCl_3$-deproteinisation showed no alkali-lability, the alkali-treated DNA which was trapped in the denatured-protein interface and which had been solubilised by overnight incubation with crude papain gave a <u>yellow</u> colour in the diphenylamine reaction with no trace of the normal blue colour. Dilution of the low-speed (1000g for 10 min.) supernatant from the papain treatment with an equal volume of water completely precipitated the DNA. This DNA showed no alkali-lability. Recombining some of the DNA and the alkali-treated papain supernatant resulted in a complete inhibition of the diphenylamine reaction. Tests showed that alkali-treated casamino-acids or bovine serum albumin (1mg) will also inhibit the diphenylamine reaction to give a yellow colour. 0.35mg of cystine but not of cysteine, histidine, tryptophan or tyrosine will produce a 20% inhibition if heated in N alkali for 1 hr. at 70°. It is therefore clear that from this standpoint also, the DNA is not alkali-labile after thymine starvation.

An experiment where unbalanced protein synthesis was inhibited by the presence of 2 µg/ml of chloramphenicol did not show any correlation between the protein/DNA ratio and the production of inhibitor under the above conditions.

Effect of N alkali on the viscosity of DNA after thymineless death

To confirm the alkali stability of the DNA, more DNA was extracted by Marmur's procedure from 15T⁻ before and after 2½ hours thymine starvation (0.3% survival), and also from the wild-type E. coli B (Preparation I). The DNA was treated with N NaOH overnight at 37° and the solutions were then neutralised with HCl to pH 7.0. The viscosity of the DNA solutions was then measured. High pH's are known to denature DNA and the viscosities of the resulting solutions were very low. However the viscometer used could measure intrinsic viscosities of this magnitude to an accuracy of about ± 15%. Thus any great alkali-lability would at once be evident since even one break per molecule would reduce the viscosity by 25%. All viscosimetric measurements were carried out in a final concentration of 0.41M NaCl.

Controls which had not been incubated at 37° overnight were prepared by neutralising immediately after the addition of the alkali. The results are shown in Table 7 and Fig. 3.

The $[\eta]$ before alkali treatment of the DNAs was about 130dl/gm. Thus the percentage drop in viscosity on incubation in N alkali at 37° is:

Table 7

Effects of alkali on the viscosity of DNA before and after thymineless death

DNA solution and its concentration in μg/ml	η_{rel} for various dilutions					η_{sp}/c in dl/gm					[η]
	1.0x	0.8x	0.6x	0.4x	0.2x	1.0x	0.8x	0.6x	0.4x	0.2x	dl/gm
Wild type DNA unincubated control 191	1.113	1.103	1.073	1.050	1.027	5.91	6.73	6.38	6.54	7.06	6.5
Wild type DNA alkali treated 157	1.053	1.044	1.039	1.025	1.014	3.38	3.50	4.14	3.98	4.46	4.0
Before death 15T⁻ unincubated control 183	1.114	1.054	1.077	1.050	1.026	6.24	7.04	7.02	6.84	7.10	6.8
Before death 15T⁻ alkali treated 203	1.054	1.044	1.034	1.020	1.012	2.70	2.71	2.78	2.50	2.99	2.8
After death 15T⁻ unincubated control 184	1	1.044	1.090	1.070	1.032		7.88	8.15	8.51	8.69	8.2
After death 15T⁻ alkali treated 206	1.055	1.116	1.045	1.014	1.010	2.74	2.86	3.73	1.75	2.49	2.8

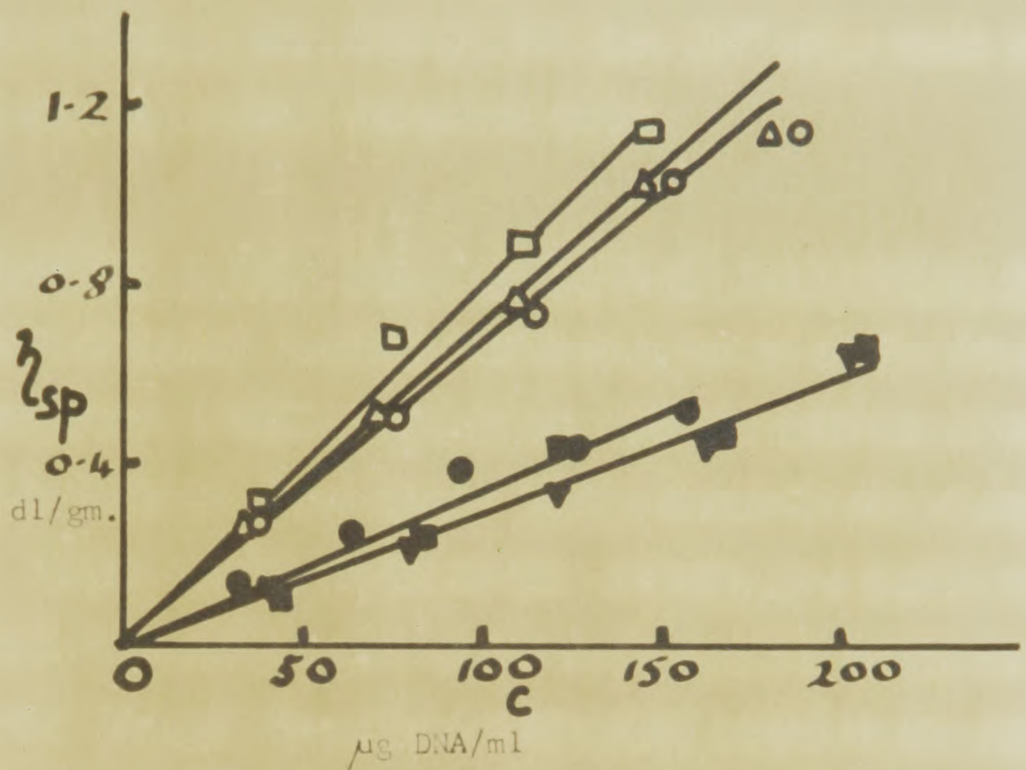


Fig. 3. The effect of incubation in alkali on the viscosity of DNAs before and after thymineless death. DNA extracted from E.coli B and 15T⁻ before and after 2½ hours thymineless death was incubated in N NaOH for 16 hours at 37°. The specific viscosity was measured at different DNA concentrations at 25° and compared with controls which were neutralised immediately after the addition of alkali. The solvent was 0.41M NaCl. Wild-type treated with alkali = (●); before death treated with alkali = (▼); after death treated with alkali = (■); wild-type control = (○); before death control = (△); after death control = (□).

wild-type	40%
before death	57%
after death	65%

The results therefore show that the DNA from 15T⁻ after death is no less stable towards alkali than DNA before death.

Search for single strand breaks in 15T⁻ DNA after thymineless death

In view of so much evidence implicating damage to the DNA as the cause of thymineless death, a search was made for evidence of breaks in the DNA. These could be of two types: either breaks in one strand or in both strands. In the case of breaks in both strands, on a random basis one could expect some breaks in one strand to be opposite breaks in the other strand of the double helix and would therefore result in the DNA having a lower molecular weight than normal DNA. In either case the rigidity of the double helix would be reduced and it would be expected that the DNA would be more susceptible to breakage by hydrodynamic shear. This would be manifested as a lower molecular weight of the extracted DNA after thymineless death. Since viscosity is a function of the length of rigid molecules, and therefore of the molecular weight, measurement of the intrinsic viscosity of 15T⁻ DNA before and after death would give an unequivocal answer to this possibility.

Using the same DNA preparations (Preparation I, prepared by $CHCl_3$ deproteinisation) but dissolved in M/100 NaCl the calculated $[\eta]$ for both "after death" and "before death" DNAs was 125dl/gm. (see Fig. 4).

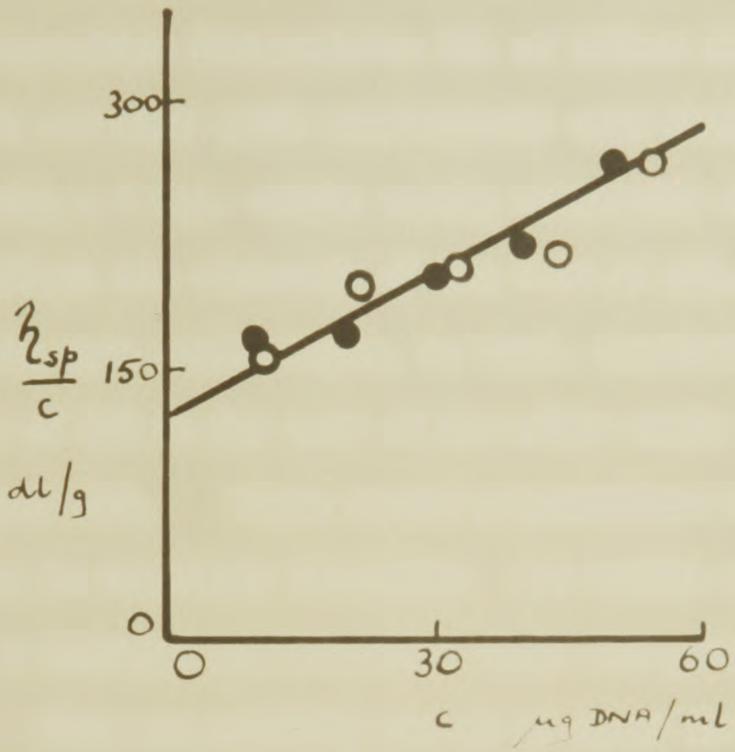


Fig. 4. The viscosity of DNAs from 15T⁻ before and after death (Preparation I). The specific viscosity of the DNAs was measured at different DNA concentrations and the intrinsic viscosity obtained from the intercept on the ordinate. The solvent was 0.01M NaCl. 15T⁻ DNA before death = (○); 15T⁻ DNA after death = (●).

This showed quite clearly that there was no difference in the molecular weight of the DNAs before and after death.

To investigate the DNA's susceptibility to shear, solutions of a second preparation (Preparation II) of before and after death DNA at 46.0 µg/ml and 49.6 µg/ml respectively were forced through a no. 19 needle using a 2 kgm. weight acting vertically downwards. This treatment did not affect the viscosity of the DNAs at all, but forcing the solutions through the same needle twice, by hand, resulted in a drop in specific viscosity of 20% in the case of the after death DNA and of 25% for the before death DNA. These second preparations however showed a difference in intrinsic viscosity: the before death 15T⁻ DNA gave $[\eta]$ = 180dl/gm and the after death DNA gave $[\eta]$ = 140dl/gm (see Fig. 5). This was probably due to an error in the estimations of the DNA concentrations since the slopes of the straight lines given by η_{sp}/c v. c were different and correcting the relative DNA concentrations to give identical slopes gives intrinsic viscosities which are almost the same. This is a reasonable correction to make since there is no other evidence from this work to suggest that the Huggins' constants, k', in the equation $\eta_{sp}/c = k'c + k''$ (Eigner, 1960) of the DNAs before and after death are different.

At this stage of the experimental work, Mennigmann and Szybalski (1962b) published a report in which they claimed that DNA isolated from 15T⁻ after 2 hours thymine deficiency (0.3% survival) had a viscosity of only 1% of that from normal 15T⁻. These workers however purified their

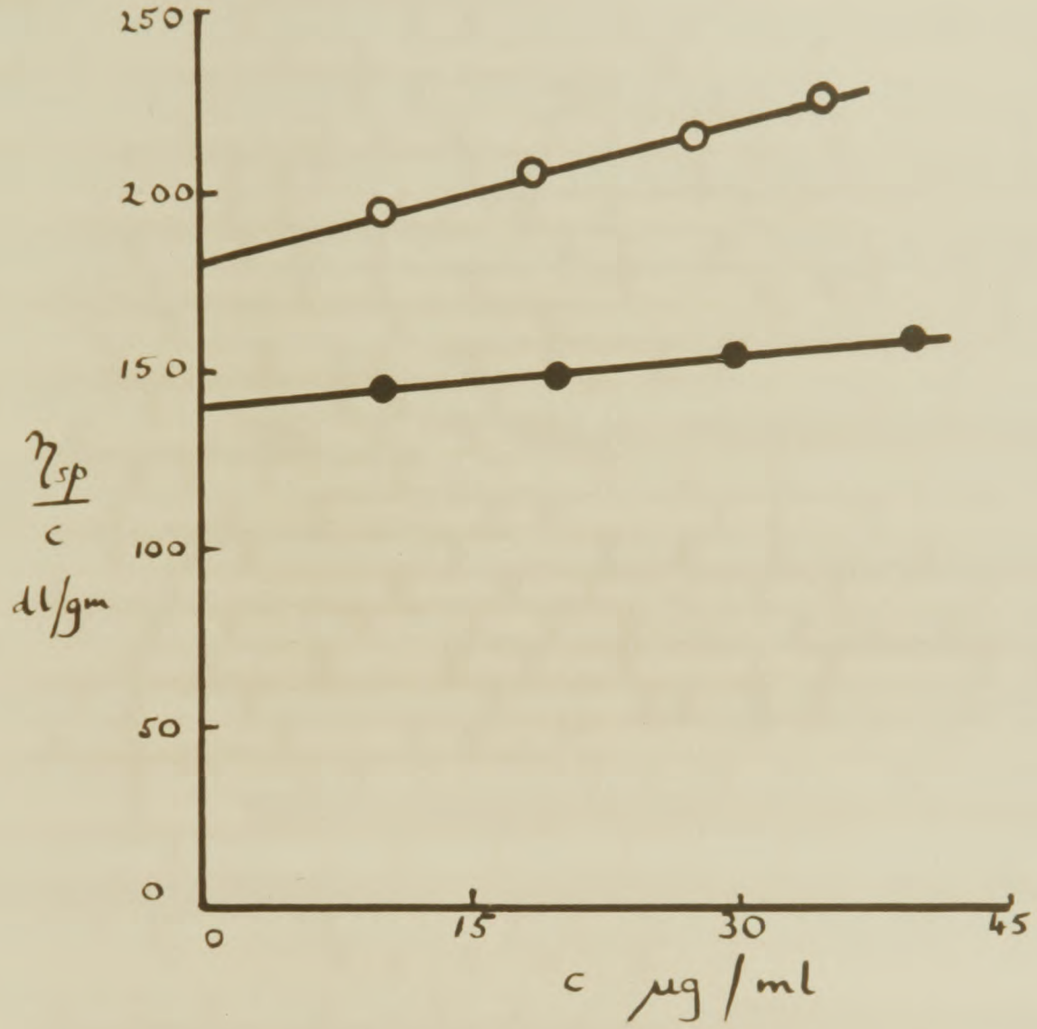


Fig. 5.

The viscosity of DNAs from $15T^-$ before and after death (Preparation II). The specific viscosities of the DNAs were measured at different DNA concentrations at 25° and the intrinsic viscosity obtained from the intercept on the ordinate. The solvent was 0.01M NaCl. $15T^-$ DNA before death = (O); $15T^-$ DNA after death = (●).

DNAs by a phenol deproteinisation method and differences between phenol- and $CHCl_3$-deproteinised DNAs have previously been reported (Ginoza & Guild, 1961). They have been attributed to the fact that $CHCl_3$ always leaves a residual quantity of protein in the DNA preparation which can subsequently be removed by phenol. Since this residual protein may have been functioning as a support to the more fragile DNA from bacteria after thymineless death it was important to test whether this could have explained the discrepancy between Szybalski's results and ours.

Further treatment of some $CHCl_3$-deproteinised DNA (Preparation II) with phenol by the method of Davison and Freifelder (1962) yielded DNAs which had no detectable protein and very similar intrinsic viscosities; 130dl/gm for the before death DNA and 110dl/gm for the after death DNA. Shearing of these preparations through a no. 21 syringe needle with the 2kgm weight resulted in no decrease in the intrinsic viscosity. Squirting the solutions through the same needle as hard as possible by hand gave a reduction of specific viscosity of 12% for the before death DNA (50µg/ml) and 11% for the after death DNA (35µg/ml).

It therefore was quite clear that the massive differences in intrinsic viscosity found by Mennigmann and Szybalski in 15T⁻ were an artifact, and more recently it has been reported by Maaløe (1963) that Freifelder, in unpublished experiments, has been unable to find any evidence for single-strand breaks in the DNA of 15TAU after thymineless death. It is not ruled out, of course, that the multiple fragmentation reported by Mennigmann and Szybalski may be the cause of death in B. subtilis inhibited with FUdR.

Determination of the Melting Curves of Before and After Death DNA after $CHCl_3$ and Phenol Deproteinisation.

Another method of showing differences in the physical structure of a DNA is to measure the hyperchromicity changes on heating. Measurement of the melting curves of the DNAs of Preparation II either after $CHCl_3$ deproteinisation or after $CHCl_3$- and phenol-deproteinisation showed that the DNAs had exactly the same melting points (T_m = 75.0 ± 0.5°), hyperchromicity changes (31 ± 1%), and range of temperatures over which melting occurred (66-80°). See Fig. 6.

Once again this provides no evidence of many single-strand breaks in the DNA after thymineless death and confirms and extends the observations of Luzzatti and Revel (1962) who also showed the absence of differences between the melting curves of $CHCl_3$-deproteinised DNA before and after thymineless death.

Sedimentation Analysis of the DNA from 15T⁻ before and after thymineless death.

If the DNA contains single-strand breaks then heating in the presence of formaldehyde should separate the strands, keep them apart during rapid cooling and result in populations with different sedimentation characteristics in the ultracentrifuge according to the distribution of the breaks. See Fig. 7.

Sedimentation velocity determinations on before and after death DNA

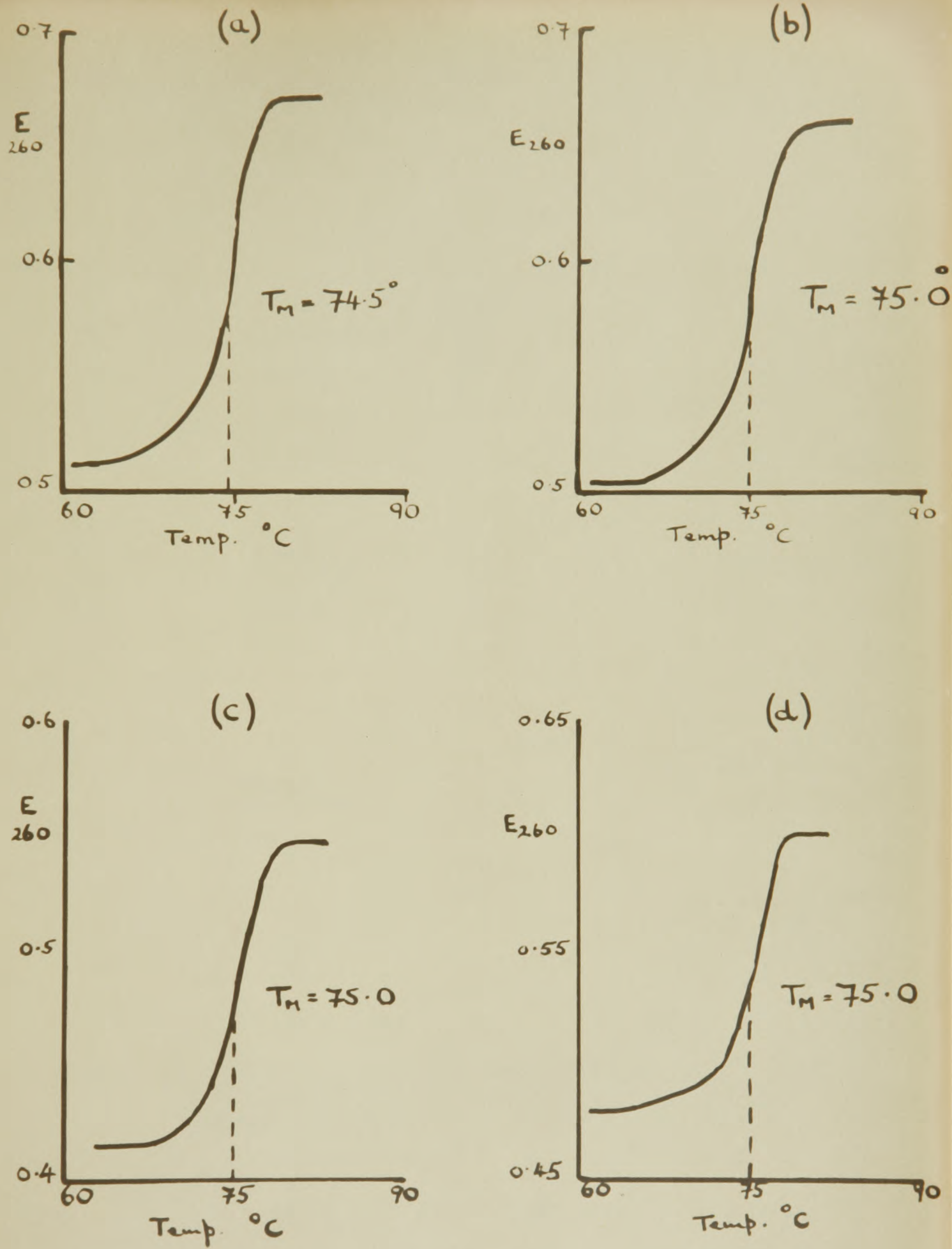


Fig. 6. Melting curves of DNAs from $15T^-$ before and after death (Preparation II)

(a) Before death DNA, deproteinised with $CHCl_3$.
(b) After death DNA, deproteinised with $CHCl_3$.
(c) Before death DNA, deproteinised with $CHCl_3$ and with phenol.
(d) After death DNA, deproteinised with $CHCl_3$ and with phenol.

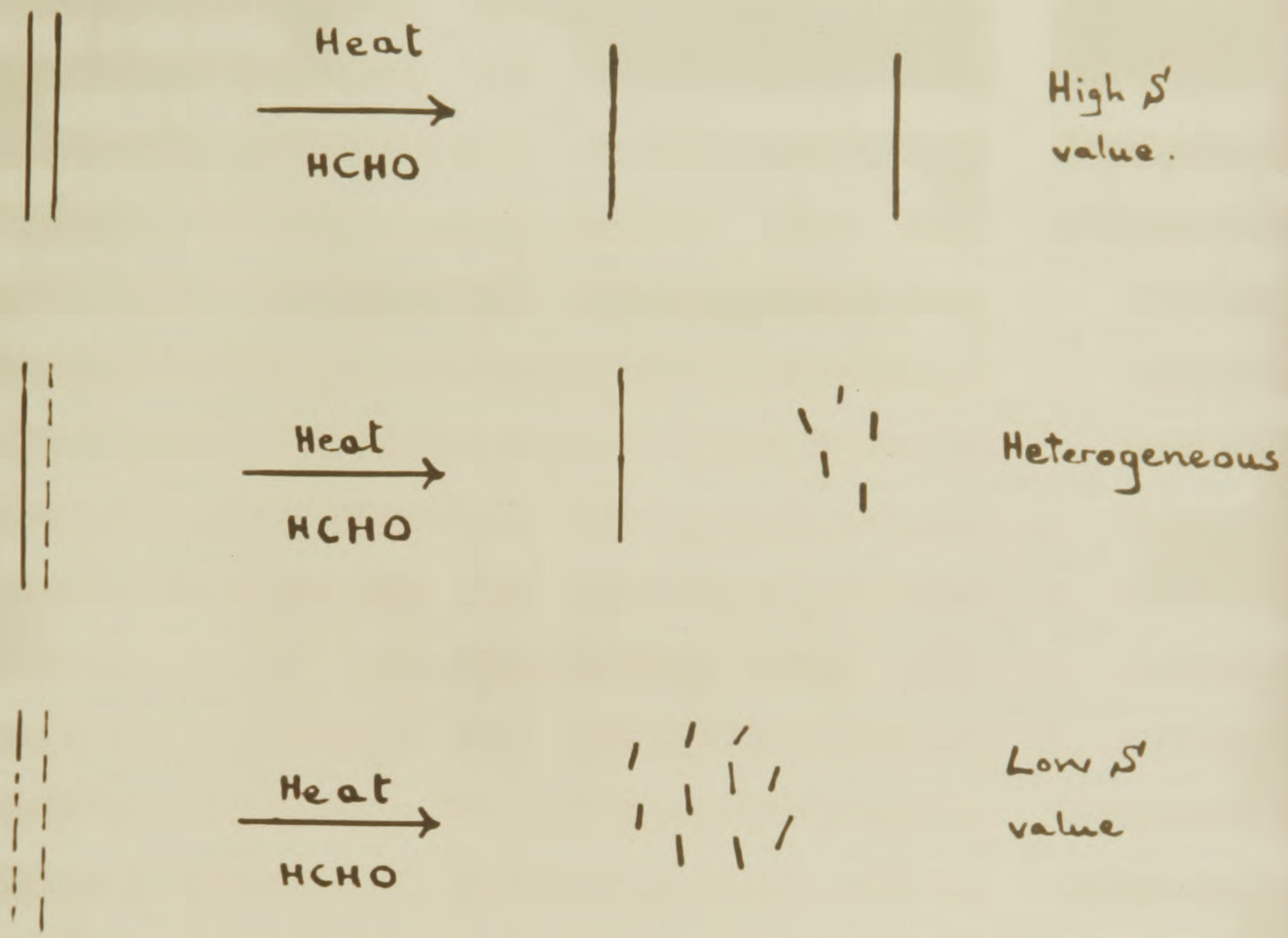


Fig. 7. Possible Effects of Heat Denaturation in 1% Formaldehyde on the sedimentation coefficients of DNA containing single-strand breaks.

15T⁻ (Preparation II, $CHCl_3$ and phenol deproteinised) with and without heating in the presence of 1% formaldehyde gave the following results at 15 µg/ml expressed as $S_{w,20}$: The solvent was 14mM phosphate buffer, pH 7.2 and 8 mM NaCl. See Table 8.

Single traces of the sedimenting boundaries of the DNAs before and after heating in 1% formaldehyde are shown in Fig. 8. It can be seen that only one sedimenting component is present in each case and rules out the possibility of single-strand breaks in one strand only. The fact that the same decrease of sedimentation coefficient occurs with DNA from before and after death on heating in 1% formaldehyde demonstrates the absence of many single-strand breaks in the DNA after thymineless death.

Calculations of the molecular weight, M_w, of the DNAs used in these experiments can be made from a knowledge of the sedimentation constant alone (Burgi & Hershey, 1963), or using the S value and the intrinsic viscosity (Mandelkern & Flory, 1952) or a modification of this derived by Eigner (1960) designed to correct for the fact that the sedimentation constants are not measured at zero concentration.

Using the equation of Burgi and Hershey:

$$S_{w,20} = 0.080 \times M_w^{0.35}$$

$$\text{therefore } \log M_w = \frac{\log 21 - \log 0.080}{0.35}$$

$$M_w = 9.5 \times 10^6$$

Using the Mandelkern-Flory equation:

Table 8

	Heated at 93° for 3 minutes		Unheated	
	Before Death DNA	After Death DNA	Before Death DNA	After Death DNA
Determination 1	16-18S	18S	22S	21S
Determination 2	17S	16-18S	23S	21S

Sedimentation constants of 15T⁻ DNAs before and after death, before and after heating in 1% formaldehyde

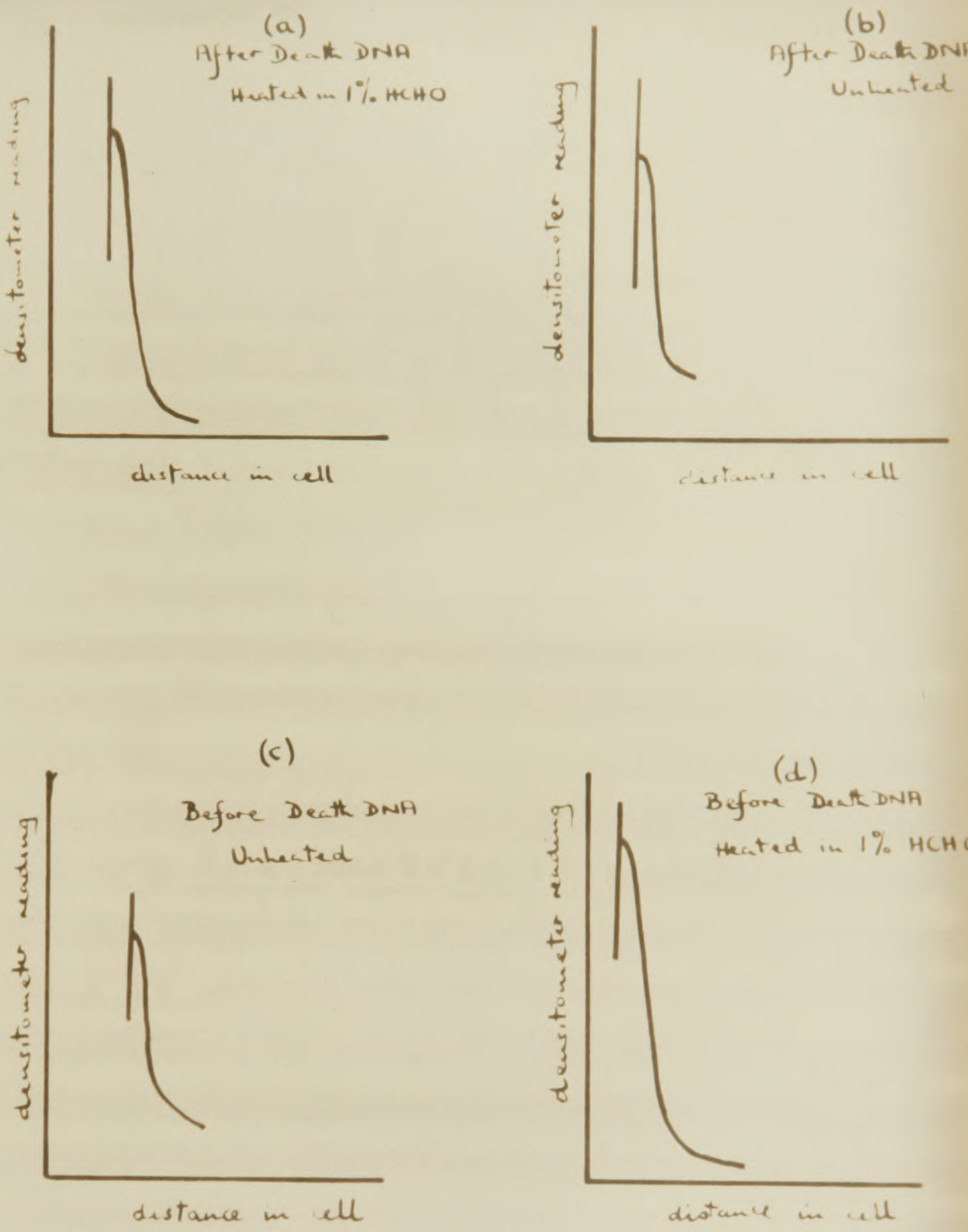


Fig. 8. Sedimenting boundaries of DNA from before and after thymineless death, before and after heating in 1% formaldehyde. The DNA concentration was 15μg/ml dissolved in 14mM phosphate, pH 7.2 plus 8mM NaCl. The DNA was sedimented in a Spinco Model E ultracentrifuge at 20° at 39,460 r.p.m. The densitometer tracings were taken from photographs taken after 10 min. at full speed. The vertical bar is parallel to the meniscus of the solution.

$$M_w = \left[\frac{s^o [\eta]^{1/3} \eta_o N}{\beta (1 - \bar{v}\rho)} \right]^{3/2}$$

where s^o = sedimentation constant at zero concentration

$[\eta]$ = intrinsic viscosity at zero shear gradient

η_o = solvent viscosity

N = Avogadro's number

$(1 - \bar{v}\rho)$ = buoyancy factor = 0.45

$\bar{v}$ = partial specific volume

ρ = buoyant density

$\beta = 2.5 \times 10^6$ found empirically

therefore

$$M_w = \frac{21.8 \times 135^{1/3} \times 1 \times 6.03 \times 10^{23}}{2.5 \times 10^6 \times 0.45}^{3/2}$$

$$= 12 \times 10^6$$

Doty, Marmur, Eigner & Schildkraut, (1960), describe a preparation of E. coli DNA with S = 22.9, an intrinsic viscosity of 120dl/gm and $M_w = 10^7$. Thus the DNA as prepared above has characteristics which are in good agreement with values in the literature.

Using Eigner's method for correcting the sedimentation constant to zero concentration:

$$s^{o} = S\,(1 + 0.8[\eta]c)$$

$$s^{o} = 22S\,(1 + 0.8 \times 135 \times 0.0015)$$

$$= 25S$$

whence since $M_w = 1.86 \times 10^4\,(s^{o}[\eta]^{1/3})^{1.33}$

then $M_w = 11.8 \times 10^6$

As suggested in the Methods section, this calculation shows that the correction to zero concentration does not have a very great effect on the calculated M_w.

Caesium Chloride Gradient Equilibrium Centrifugation Studies

To confirm the absence of many single-strand breaks in the DNA after death, the DNAs were now heated in 1% formaldehyde and rapidly cooled in ice-water and the widths of the bands formed in a CsCl density gradient measured. Since the band-width is an inverse measure of the molecular weight of the DNA (Thomas & Berns, 1961a,b; Thomas & Pinkerton, 1963) the band-width could be expected to be broader for the after death DNA than for the before death DNA.

DNA was therefore prepared by phenol deproteinisation from E. coli 15T⁻ before and after 3 hours thymine deficiency (0.5% survival) (Preparation III). A DNA concentration of 4 µg/ml was used and was buffered in 0.01 M phosphate at pH 7.2 before heating for 3 minutes at 93° in 1% formaldehyde followed by rapid cooling in ice-water. An unheated sample of Micrococcus lysodeikticus DNA was included as a density marker in all runs. See Plate 2. The density of the native E. coli was identical for before and

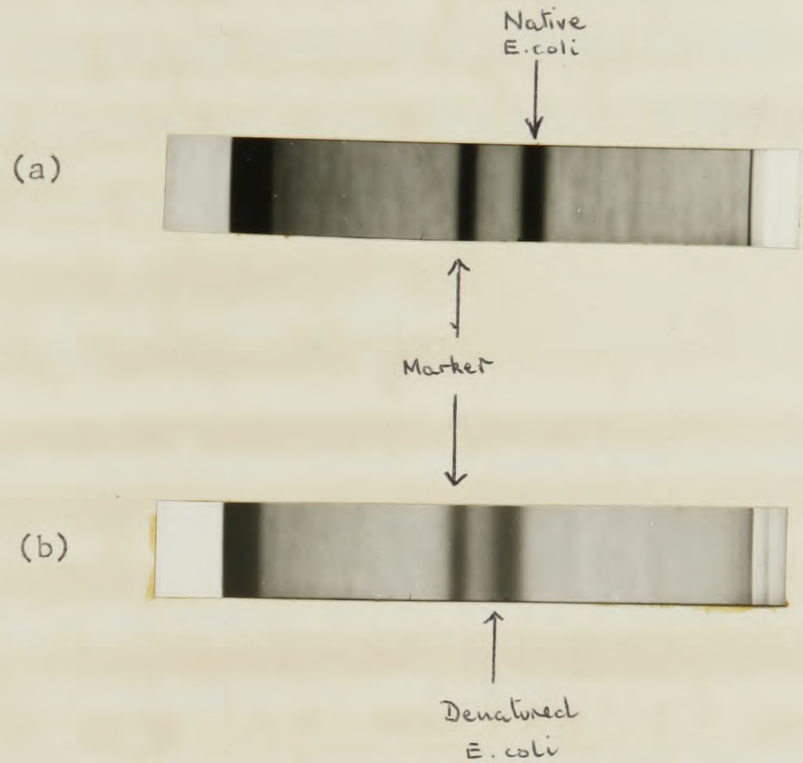


Plate 2. 15T⁻ DNA bands in a caesium chloride gradient (a) Before heating; (b) After heating in 1% formaldehyde. The marker DNA is that of Micrococcus lysodeikticus.

after death DNAs and corresponded to the published value (Schildkraut, Marmur & Doty, 1961) of 1.710 g.cm^{-3}. This confirmed the observations of Luzzatti and Revel (1962). The density of the DNA after heating in formaldehyde was found to be 1.719 g.cm^{-3}. This is 0.005 g.cm^{-3} lower than the published value for denatured E. coli DNA, but the band-width changed and the DNA was certainly no longer banding at the density characteristic of native E. coli DNA. See Figs. 9 & 10. The distribution of DNA in the bands can be expressed in terms of the following equation:

$$\frac{h}{h_o} = \exp\left[\frac{-W^2M}{8\beta}\right]$$

where h = the height above the base line of a particular point on the trace,

h_o = the maximum height of the band-trace above the base line,

W = band-width at this point,

β = a constant which is related to the variance σ^2 of the band by the equation $\sigma^2 = \beta/M$

M = molecular weight for a uniform population of molecules

Thus, a plot of log h/h_o v. W^2 gives a straight line whose slope is proportional to the molecular weight of the DNA. See Fig. 11.

The slopes for the appropriate DNA samples expressed in arbitrary units is given in Table 9.

The exact value of the slope depends greatly on the choice of base line which is made difficult by the unevenness of the densitometer tracing.

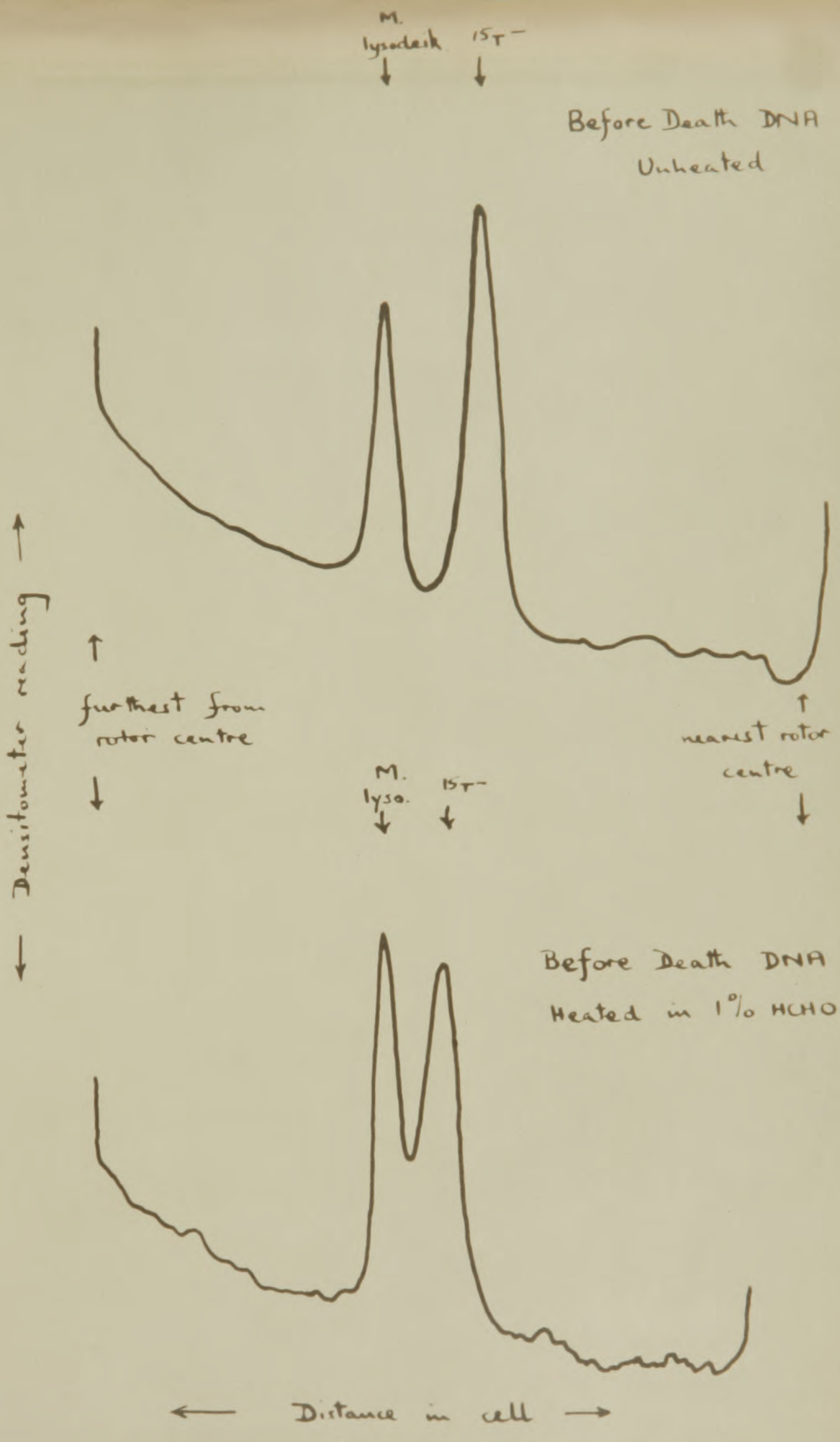


Fig. 9. Densitometer tracings of $15T^-$ DNA before thymineless death in a caesium chloride gradient, before and after heating in 1% formaldehyde. The DNA concentration was 4µg/ml dissolved in 0.01M phosphate, pH 7.2. Photographs were taken after 30hr. at 44,770 r.p.m. at 25°.

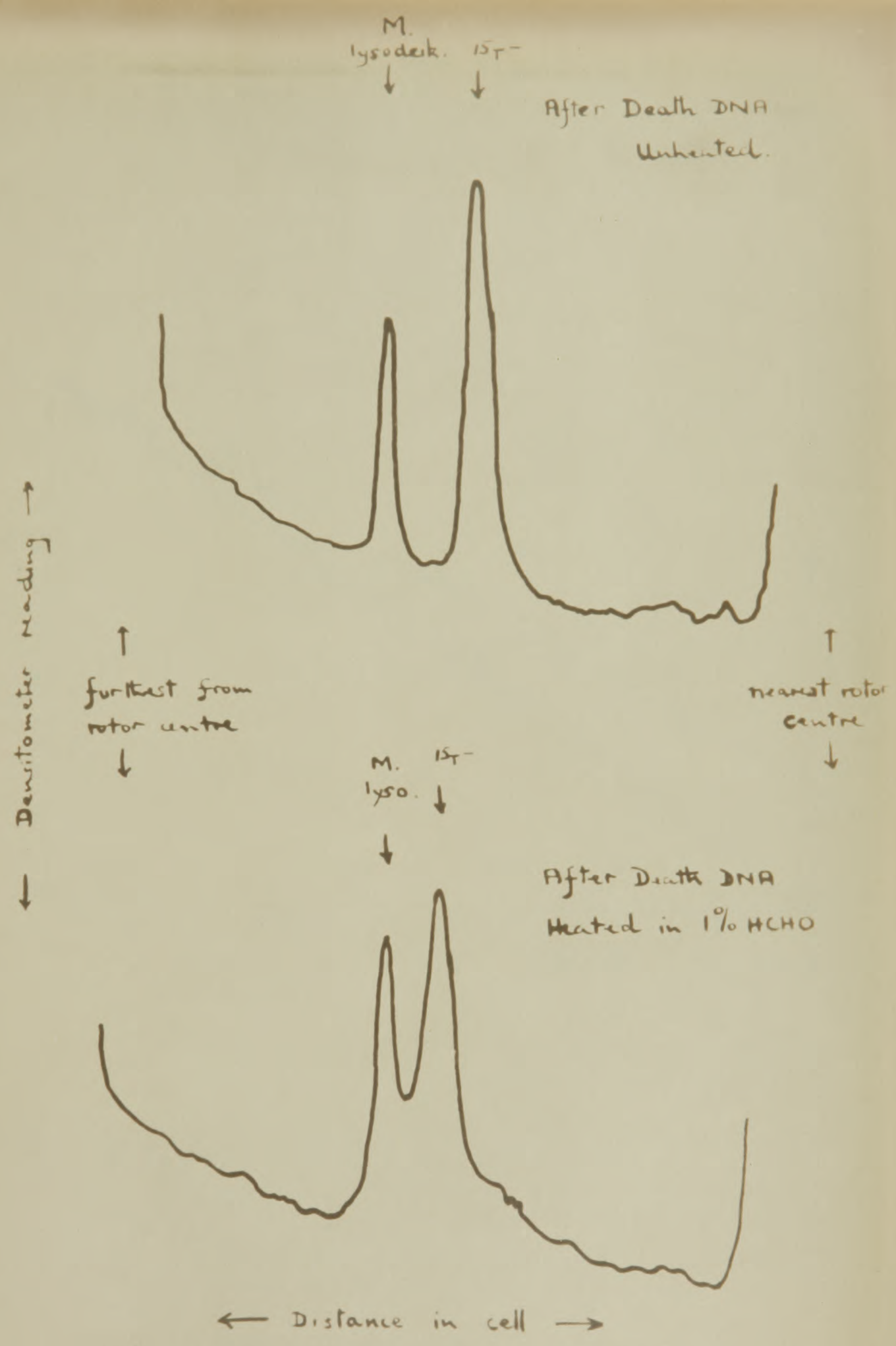


Fig. 10. Densitometer tracings of $15T^-$ DNA after thymineless death in a caesium chloride gradient, before and after heating in 1% formaldehyde. The DNA concentration was 4µg/ml in 0.01M phosphate, pH 7.2. Photographs were taken after 30 hours at 44,770 r.p.m. at 25°.

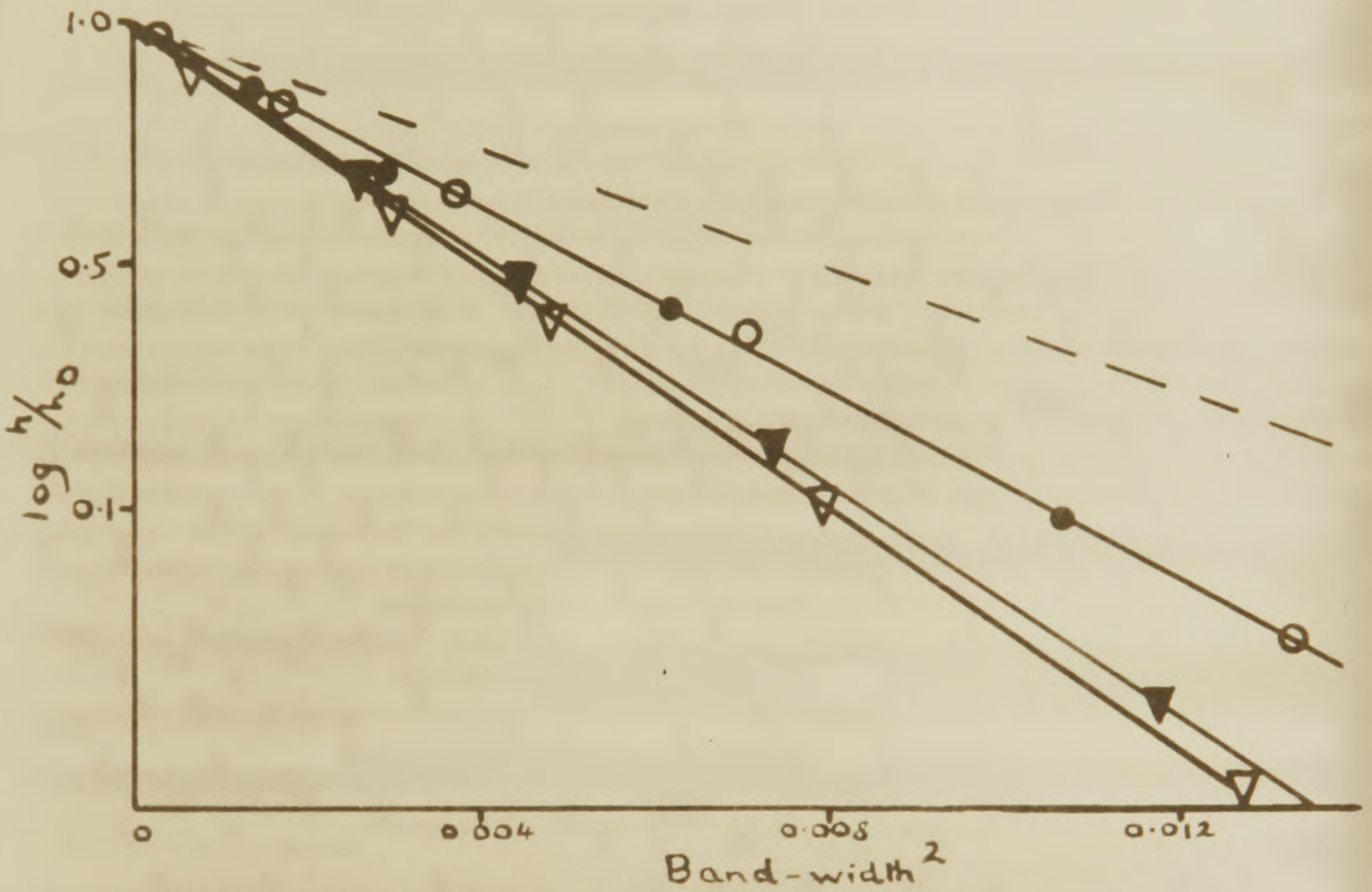


Fig. 11. Plot of W^2 v. log h/h_0. The values were obtained by direct measurement of the densitometer traces shown in Figs. 9 & 10. The dotted line indicates the position expected for the heat-denatured DNA if it had half the molecular weight of native DNA. Before death DNA before heating = (▽); before death DNA after heating = (○); after death DNA before heating = (▼); after death DNA after heating = (●).

Table 9

DNA sample	Slope: log $h/h_o/W^2$
After death Unheated	740
Before death Unheated	770
After death Heated	546
Before death Heated	530

Slope $h/h_o/W^2$ for band-width determinations before and after death, before and after heating in 1% formaldehyde

These values are interpreted as showing that there is no difference between the band-widths of before and after death DNAs, and hence that there are no single strand breaks in the DNA after thymineless death.

It will be observed that the band-width molecular weights as expressed by the slope of the lines are not halved on heat denaturation as would be expected if the DNA strands were completely separated. This is probably because some cross-linking of the DNA by impurities in the formaldehyde has taken place even though care was taken to redistill the formaldehyde immediately before use. A small degree of cross-linking might explain the discrepancy in the buoyant densities. However, since no difference in behaviour of the two DNAs was observed these observations do not affect the conclusions.

Thus, there is no great degree of single-strand breaks in the DNA induced by thymineless death.

The Possibility of Cross-linking in 15T⁻ DNA after Thymineless Death

UV irradiation, Mitomycin C and nitrogen mustards all form inter-chain (cross) links in DNA, and these agents and thymineless death also induce lysogenic phage. This fact suggested that the lethal event in thymineless death might be a cross-linking of the DNA. To investigate this possibility DNA was prepared from 15T⁻ before and after 1½ hrs and 3 hrs thymine deficiency (Preparation IV). The *E. coli* DNA was then heated in dilute saline-citrate for 6 mins at 100° followed by rapid cooling in ice-water. The denatured DNA at 5 μg/ml final concentration

was then buffered with phosphate at pH 7.2 as described in Methods, and subjected to caesium chloride density centrifugation (Iyer & Szybalski, 1963). If cross-links exist in the DNA these would not be broken on heating the DNA and therefore renaturation would be expected to occur on rapid cooling. The densitometer tracings of the DNA bands are shown in Fig. 12.

It is clear that after 1½ hours thymine deficiency (~ 2% survival) all the DNA bands at a density characteristic of denatured E. coli DNA. If cross-linking had occurred, some DNA would be banding at a density characteristic of native E. coli DNA. After 3 hr. deficiency the picture is probably the same, the unevenness of the base line making it impossible to rule out 1% (the area under the unevenness on the base line expressed as a percentage of the area under the denatured DNA band) of the DNA molecules being cross-linked.

Sedimentation analysis of these DNAs shows that their sedimentation constants are in the region of 30S. This represents a molecular weight of about 30-40 millions (Rubinstein et al., 1961) and therefore suggests that the E. coli chromosome has been broken into about 100 pieces. One cross-link per chromosome would thus mean that 1% of the broken DNA would have a cross-link. This is just about the level of resolution of the method, and it is concluded that thymine deficiency does not induce more than one cross-link per chromosome in the DNA.

Autoradiography of $15T^-$ before and after thymineless death

Since all the above experiments have been carried out with DNA of

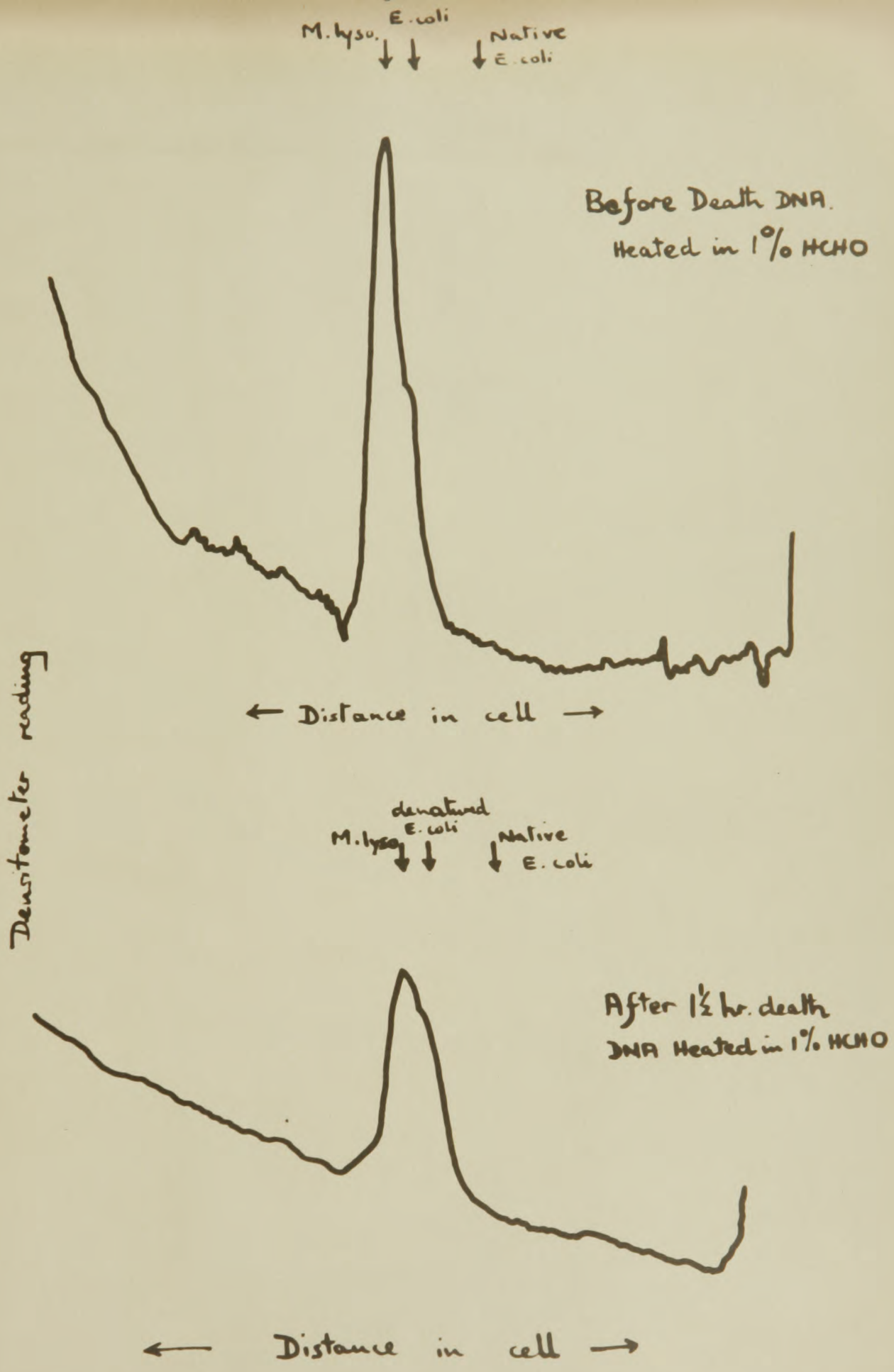


Fig. 12. Densitometer tracings of 15T⁻ DNA in a caesium chloride gradient after heating in 1% formaldehyde followed by rapid cooling. DNA concentration = 5µg/ml. The solvent is 0.6mM NaCl plus 0.06mM trisodium citrate, pH 7.0. Photographs were taken after 30 hours at 44,770 r.p.m. at 25°.

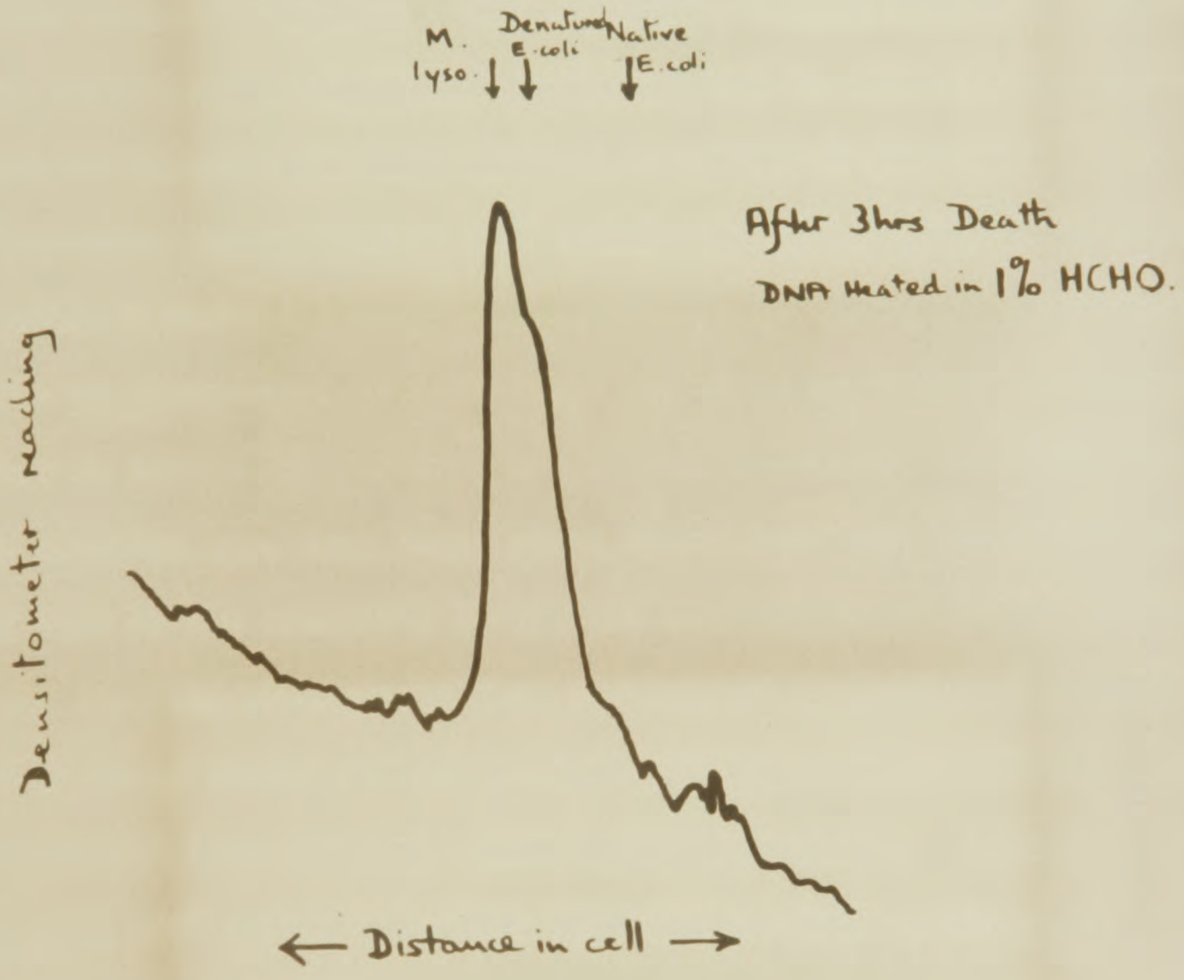


Fig. 12. (continued).

molecular weight between 10 and 40 millions, any effects on the structure of the DNA at a level less than say 100 times per chromosome would not have been picked up except in the cross-linking experiment. At the present time the only way of studying whole E. coli chromosomes is by the autoradiographic method of Cairns. This enables a visualisation of the whole chromosome labelled with tritiated thymidine, and in order to see whether any effects of thymineless death could be observed at this level, Cairns' technique was applied to 15T⁻ before and after thymineless death and also after a further ½ hour's DNA synthesis following 1½ hours death. Photographs of chromosomes so visualised are shown in Plates 5,6 and 7.

Whilst no single example of a completely unravelled chromosome has been obtained, examples have been found where 50-60% has been unravelled and where the bulk of the remainder is visible as a tangle of silver grains. It is clear from the photographs that chromosomal fragments of similar sizes can be found before and after death and also under conditions where DNA synthesis has been restored after thymineless death. Plates 3 and 4 show E. coli 15T⁻ before and after thymineless death on the same scale as the autoradiographs.

Since no obvious differences were found between autoradiographs of chromosomes before and after death, it is concluded that no massive fragmentation of the E. coli chromosome occurs during thymineless death, and that the fragility of the DNA with respect to the isolation procedure used in this technique is the same after thymineless death as before.



Plate 3. E.coli 15T⁻ grown with 16 µg/ml thymine. Magnification = 860x. The line represents 25 µ.

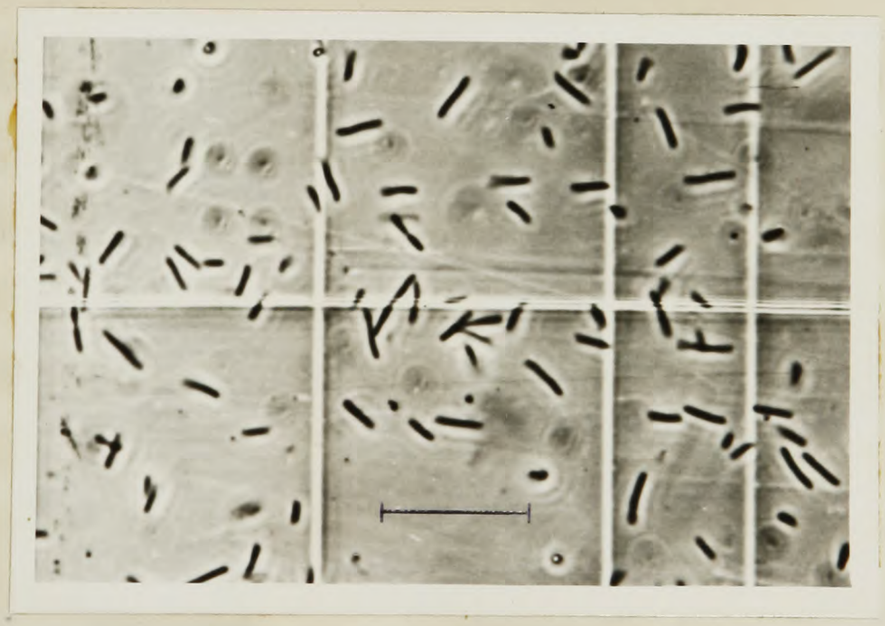


Plate 4. E.coli 15T⁻ after 2½ hours thymine deficiency Magnification = 860x. The line represents 25 µ.

These photographs are on the same scale as Plates 5,6 & 7.

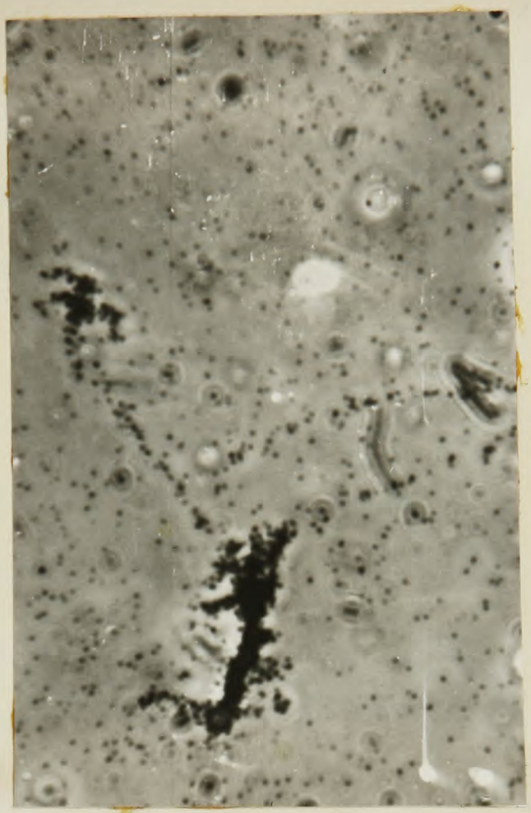


Plate 5. Chromosomal fragment isolated from 15T⁻ before thymineless death. Magn.= 860x. Excluding the tangle, 250 μ of chromosome are visible in this photograph.

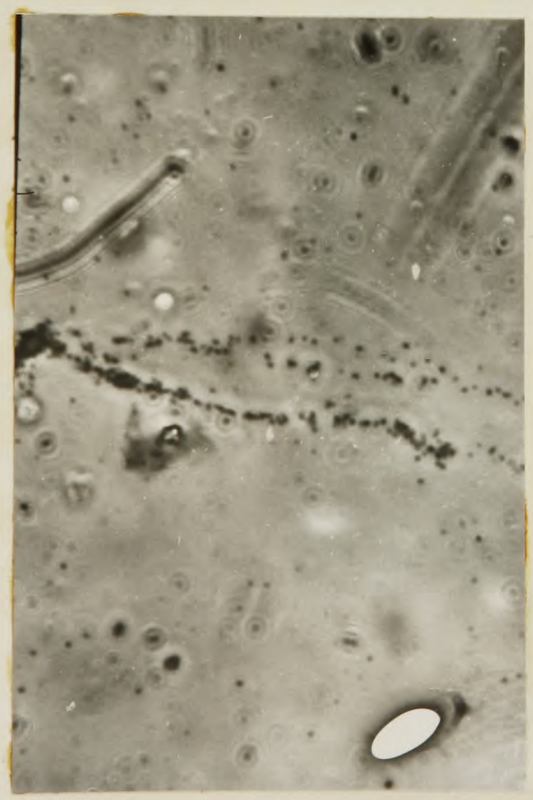


Plate 6. Chromosomal fragment isolated from 15T⁻ after 1½ hr. thymine deficiency. Magn. = 860x. Excluding the tangle at the far end, 250 μ of chromosome are visible here.

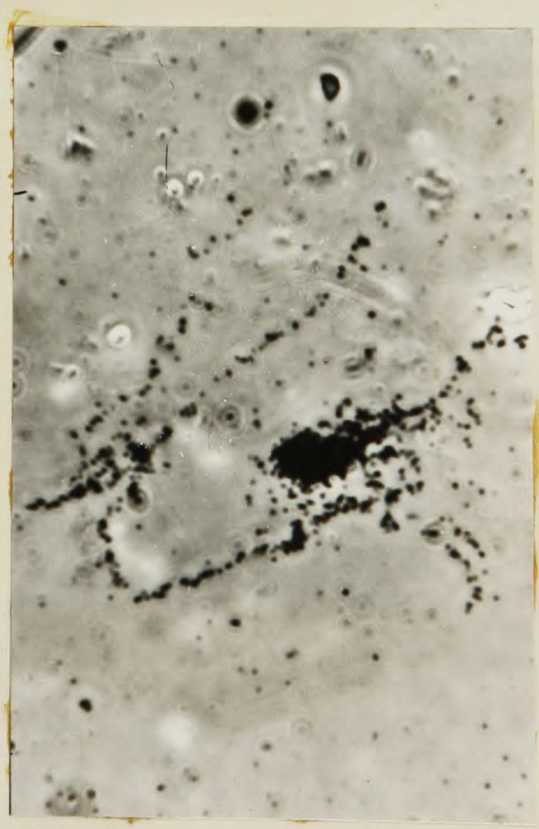


Plate 7. Chromosomal fragment isolated from 15T⁻ after 1½ hr. thymine deficiency plus a further ½ hr. with tritiated thymidine. Magn.=860x. Excl. tangle, 450 μ are visible.

DNA Synthesis in 15T⁻ during Thymine Deficiency

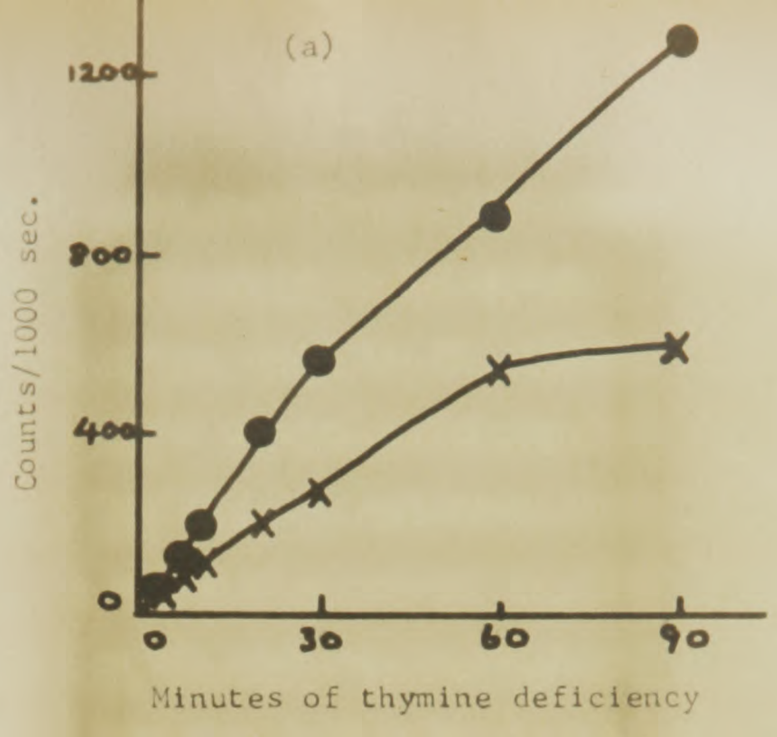
As discussed in the Introduction there is little firm evidence for DNA synthesis in 15T⁻ during thymine starvation. In order to investigate this the incorporation of ^{32}P into DNA during thymine deficiency was measured as described in Methods. The use of diphenylamine and formic acid should result in the diphenylamine catalysing the release of inter-purine inorganic phosphate (Burton & Petersen, 1960). The difference in the radioactivity released as inorganic phosphate between the formic acid treated and the diphenylamine/formic acid treated samples should give a measure of the ^{32}P incorporated into the DNA, and hence of the DNA synthesis during thymine deficiency. The results obtained in this experiment are shown in Fig. 13.

It is clear that ^{32}P incorporation has occurred into DNA-like material and that this has continued throughout the 1½ hours of thymine deficiency (< 3% survival). A later experiment, designed to follow label into particular pyrimidine sequences, confirmed this view. The specific activity of the phosphate in the medium at the time of thymine deprivation was 13.3 curies/mole (1mC ^{32}P in 250 ml culture with phosphate concentration = 0.3 μmoles/ml), the specific activity of the DNA-P after ½ hr was 0.18 curies/mole

$$\left[\frac{\text{counts/min/sample}}{\text{moles P in sample} \times \text{counting efficiency} \times \text{no. disintegrations/min/curie}} \right.$$

$$\left. = \frac{3 \times 10^{4} \times 60 \times 10^{-10}}{0.75 \times 3.7 \times 6.5} \quad \text{curies/ mole} \right]$$

and similarly after 1½ hours was about 0.36 curies/mole.



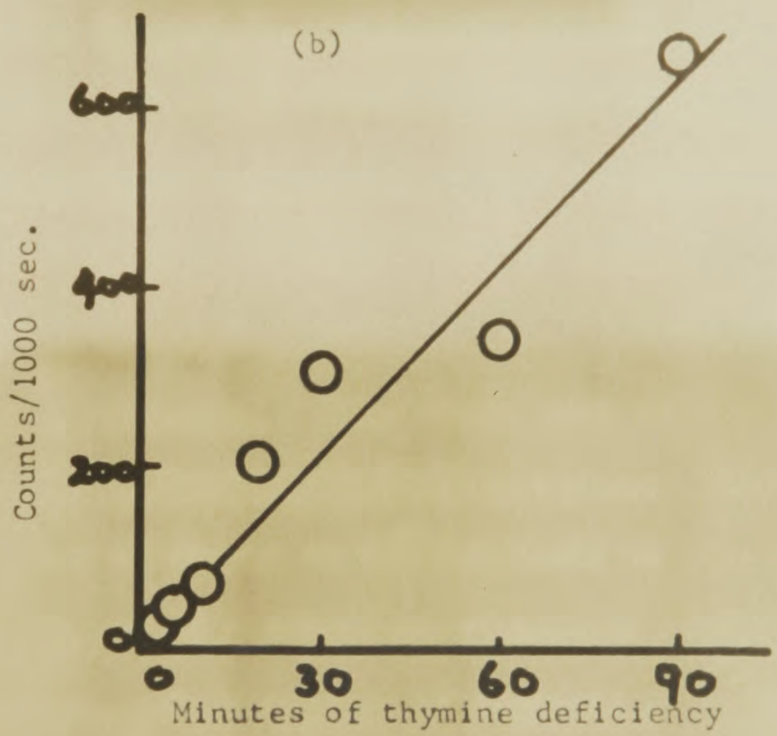


Fig. 13. Incorporation of ^{32}P into DNA during thymineless death in E.coli 15T⁻.

(a) Fractions from bacteria during thymine deficiency were treated with 66% formic acid (X); and 2% diphenylamine in 66% formic acid (●) and the ^{32}P in the liberated inorganic phosphate was measured as described in Methods. Background = 55 counts/1000 sec.

(b) The DNA synthesis during thymineless death: points were obtained from the above graph by subtracting the counts released in 66% formic acid from the counts released in diphenylamine/formic acid.

Assuming that there is good equilibration between the phosphate pools within the cells and the phosphate in the medium, the ratio of the specific activities should give a measure of the DNA synthesised in the absence of thymine. Thus after ½ hour thymine deficiency there is about 1.25% increase in DNA, and after 1½ hours deficiency there is about 2.5% increase in DNA. The number of revertants to wild-type in the population is less than 0.1% and so DNA synthesis by the revertants would account for an increase in DNA of less than 0.2% in 90 minutes. In view of the mutagenicity of thymine deficiency however, it is conceivable that bacteria may become leaky with respect to thymidylate synthetase, even though the enzyme levels attained are not sufficient to prevent thymineless death. For this to be the explanation of the observed DNA synthesis, many more than 1% of the bacterial population would have to be leaky and the evidence is not available at the present time to rule this out.

Morphological Effects of Thymineless Death

Since no evidence of damage to the DNA could be found at the biochemical level, it was of interest to examine E. coli 15TAU by electron microscopy. Preparations of bacteria which had undergone the following treatments were cut into ultrathin sections (80 mμ):-

(a) $T^+(AU)^+$ in log phase.

(b) $T^+(AU)^+ \longrightarrow T^-(AU)^-$ for 4 hrs.

(c) $T^+(AU)^+ \longrightarrow Bu^+(AU)^-$ for 90 min. $\longrightarrow T^-(AU)^-$ for 3 hrs.

(d) $T^+(AU)^+ \longrightarrow T^+(AU)^-$ for 90 min. $\longrightarrow T^-(AU)^-$ for 3 hrs.

(e) $T^+(AU)^+ \longrightarrow T^+(AU)^-$ for 90 min. $\longrightarrow T^-(AU)^+$ for 3 hrs.

The pictures obtained are shown in Plates 8-12 and represent sections which were typical of the specimens viewed in the microscope.

It is interesting to note that after thymineless death the bacteria do not look vastly different. The osmium-tetroxide fixing procedure stains lipoprotein (Kellenberger, 1959) but does not affect the nucleic acid. Thus the white areas in the photographs are either empty spaces or nucleic acid. Most noticeably, the cells have elongated during thymineless death (Plates 9 and 12). Since this is true of cells which have died in $T^-(AU)^-$, although this is not very obvious from the photograph shown, as well as in $T^-(AU)^+$ it is clear that filament formation and unbalanced growth are not necessarily related. Elongation appeared to be far more extensive however when $(AU)^+$ rather than $(AU)^-$ conditions obtained. It is clear from Plate 11 that incubation in $T^-(AU)^-$ does not necessarily produce filaments since prior immunisation with $T^+(AU)^-$ before resuspension in $T^-(AU)^-$ did not give filaments. Thus there does seem to be a correlation between filament formation and traumatic conditions; thymineless death being one of them.

It is evident from Plate 9 that the cytoplasm of cells which have undergone death in $T^-(AU)^-$ has shrunk away from the bacterial cell-walls. This could be due to an artifact in the preparation, but since cells which have died in $T^-(AU)^+$ are capable of RNA and protein synthesis, the newly synthesised polymers can fill the expanded volume of the filaments. With death in $T^-(AU)^-$ this is not the case and it would seem reasonable to

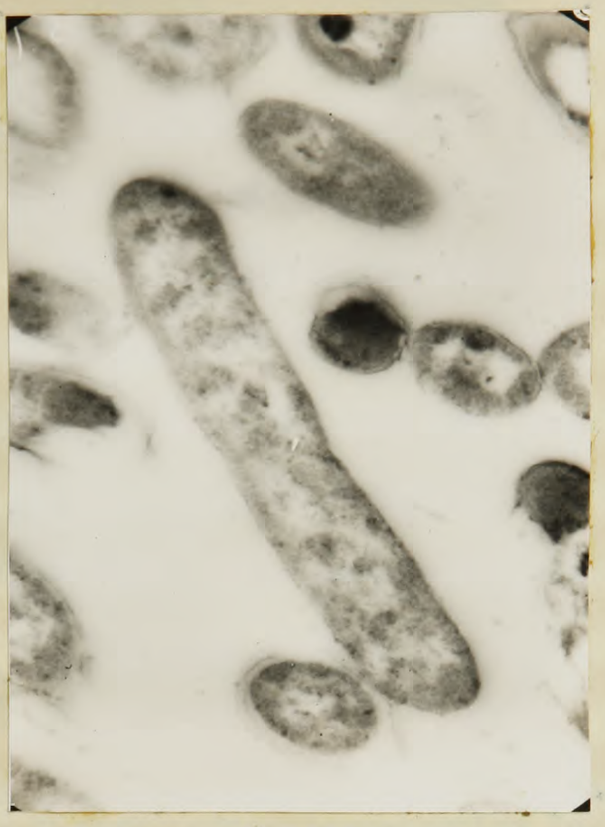


Plate 8. Electron micrograph of 15TAU grown in $T^{+}(AU)^{+}$.
Magnification = 25,800x

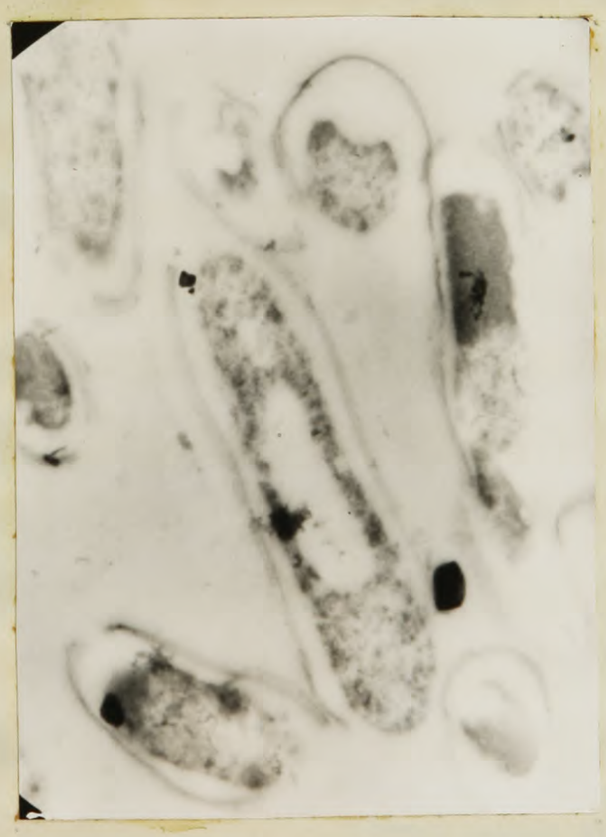


Plate 9. Electron micrograph of 15TAU grown in $T^{-}(AU)^{-}$ for 4 hr.
Magnification = 25,800x

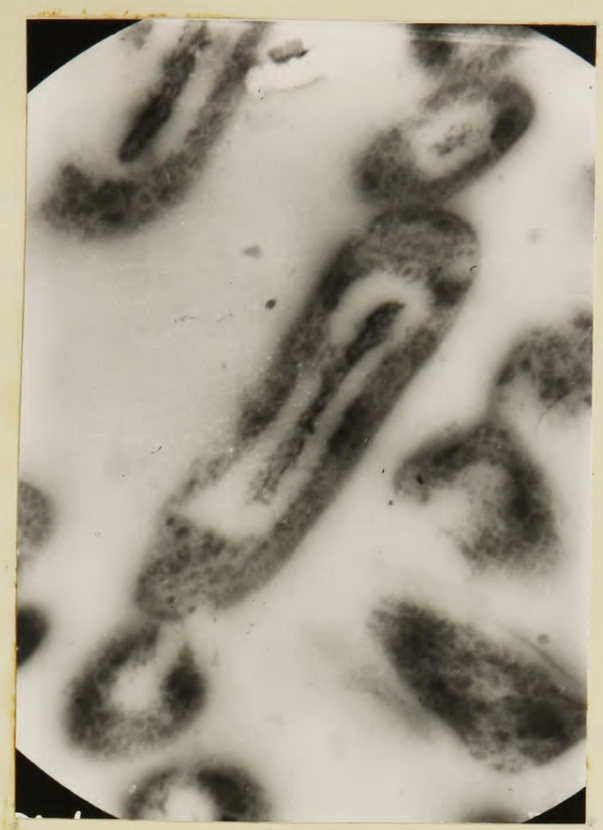


Plate 10. Electron micrograph of 15TAU grown in $T^{-}(AU)^{-}$ after immunisation in $Bu^{+}(AU)^{-}$. Magnification = 25,800x.

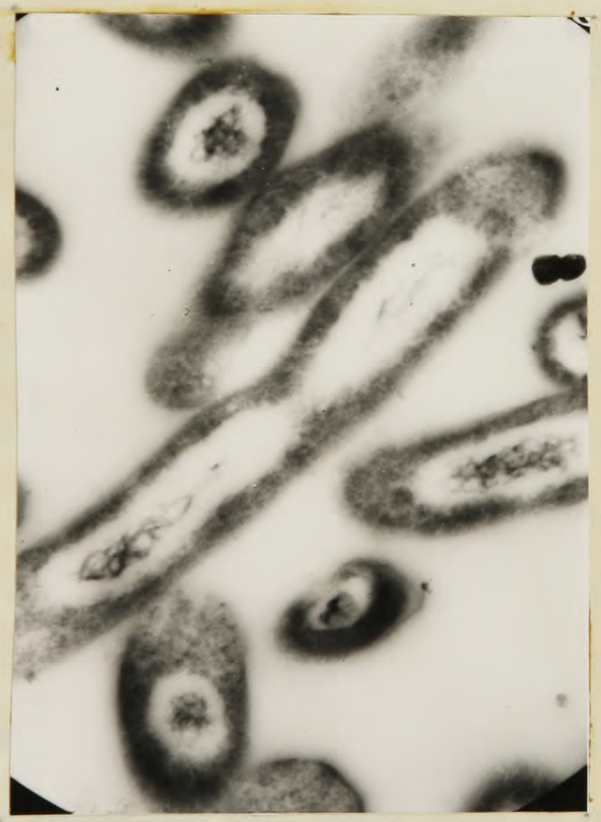


Plate 11. Electron micrograph of 15TAU grown in $T^{-}(AU)^{-}$ after immunisation in $T^{+}(AU)^{-}$. Magnification = 25,800x

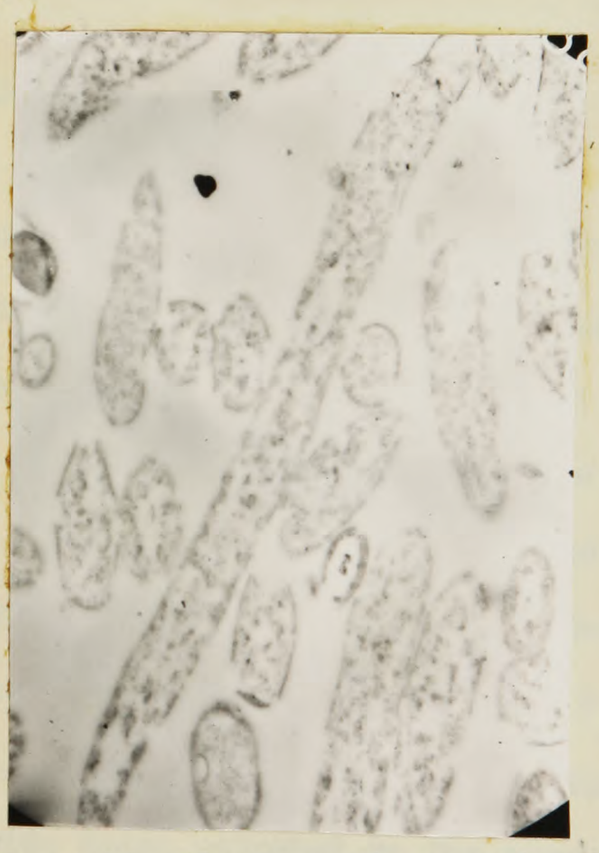


Plate 12. Electron Micrograph of 15TAU grown in $T^{-}(AU)^{+}$ after immunisation in $T^{+}(AU)^{-}$.

Magnification = 13,000x

assume that the filament formation in the absence of polymer synthesis would give rise to bacteria having a lower density, and that these might contract during the embedding in the polymethacrylate.

Immunisation of 15TAU in $T^+(AU)^-$ and in $Bu^+(AU)^-$ followed by incubation for 3 hr. in $T^-(AU)^-$ (Plates 10 and 11) shows no apparent morphological differences. This is consistent with the observation that $Bu^+(AU)^-$ is just as efficient at immunising 15TAU as is $T^+(AU)^-$ (see a later section).

It is not obvious from any of the photographs that there has been a massive fragmentation of the DNA, and this is consistent with previous morphological observations (Maaløe & Hanawalt, 1961), and the results described in this thesis.

Investigations on the Deoxyribose Compounds Appearing in the Medium during Thymine Deficiency.

Riley (1962) had shown that a compound appeared in the culture medium of both $15T^-$ and B_3 during thymine deficiency which reacted in the Burton diphenylamine reaction to give a colour whose spectrum was identical to that given by deoxyribose under the same conditions. Considerations of the specificity of this reaction made it highly likely that the compound was indeed deoxyribose or some close derivative of it. Riley had further shown that the deoxyribose compound in the supernatant of a culture of B_3 after 5 hr. thymine deficiency would not stick on a charcoal-Celite column. This suggested that there were no nucleotide bases attached to the sugar.

It was of interest to see whether the deoxyribose compound left the cell in this form, or whether the sugar was attached to a base and subsequently hydrolysed or phosphorolysed by an extracellular enzyme.

The Nature of the Deoxyribose Compound.

A culture of 15T⁻ was therefore deprived of thymine for 3 hr. and 10 ml samples taken every ½ hr. These were spun at 2500 g for 10 min. to remove the bacteria and then washed through charcoal-Celite columns with water, dried down in a rotary-film evaporator and made up to 10 ml with water. The column was eluted with 10 ml of ethanolic-ammonia, the eluate dried down in a rotary-film evaporator and taken up in 1 ml water. Assay of these solutions showed that no diphenylamine-reacting material appeared in the ethanolic-ammonia fraction at all. The recovery of the fraction of deoxyribose which did not stick to the column is shown in Fig. 14.

It is therefore apparent that the deoxyribose in the medium either leaves the cell unattached to a base or that hydrolysis takes place immediately the compound enters the medium.

Absence of a Phosphate Group on the Deoxyribose Compound.

The acidic conditions of the diphenylamine reaction are such that deoxyribose-1-phosphate, deoxyribose-5-phosphate as well as deoxyribose would react (Overend and Stacey, 1955). To distinguish between these possibilities the culture supernatant was obtained from a culture of 15T⁻

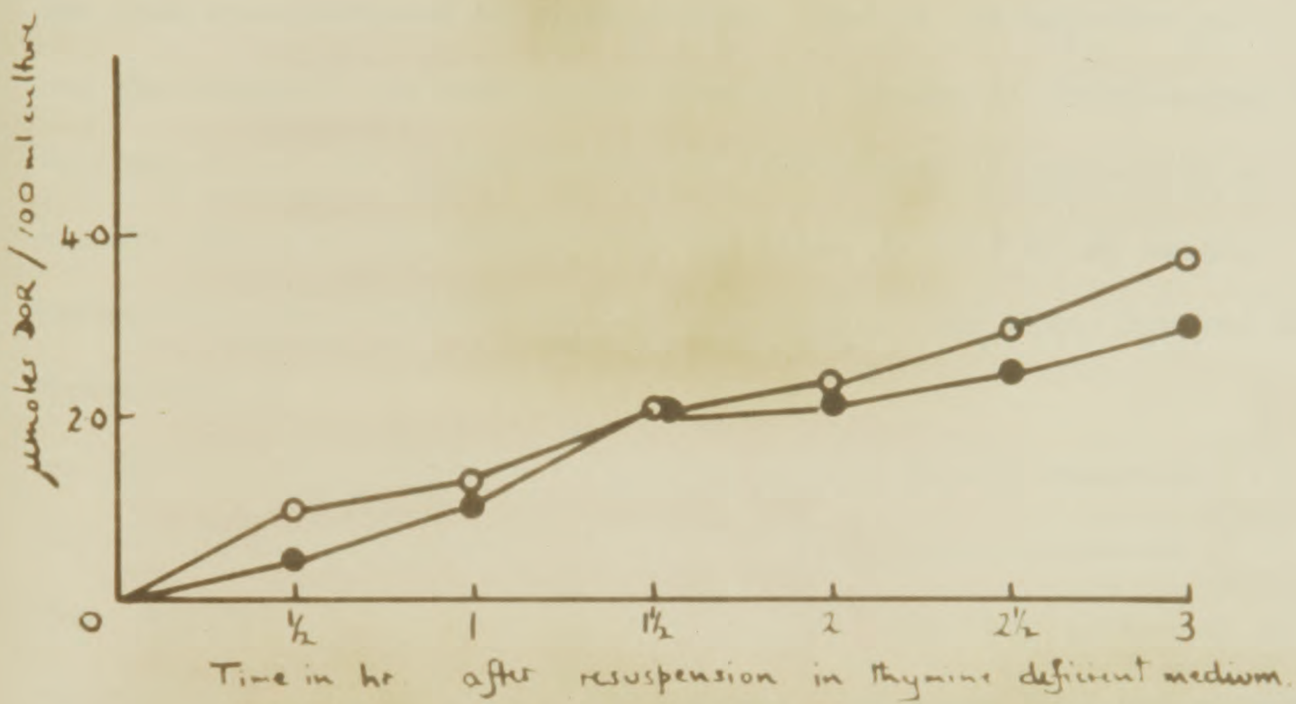


Fig. 14. Recovery from a charcoal-Celite column of deoxyribose compounds excreted by $15T^-$ during thymine starvation. Samples of the culture medium, diluted 10-fold with water, were adjusted to pH 5 and washed through charcoal-Celite columns. Assay of the deoxyribose in the samples before (O) and after (●) passage through the columns shows that the majority of the deoxyribose is not retained by the columns.

which had been deprived of thymine for 3 hr. The majority of the bacteria were spun off at 5420 g for 5 min. and the supernatant was filtered through a Millipore filter of pore-size 0.45 μ to remove the remaining bacteria. 5 ml of this culture fluid was then diluted 10-fold with water to reduce the salt concentration to about 0.01M. The pH was adjusted to 9 with NaOH and the solution was then passed through a column of Dowex-1-formate (0.7 cm. x 4.5 cm). The column was washed with a further 120 ml of water, and the effluent was freeze-dried and taken up in 5 ml of water. This solution and the original culture supernatant were then assayed for deoxyribose:

No. mμmoles deoxyribose/ml of culture before Dowex-1 treatment = 84.6

No. mμmoles deoxyribose/ml of culture after Dowex-1 treatment = 79.4

This represents a 94% recovery and suggests that very little of the deoxyribose was held back by the column. It seems that the excreted compound is free deoxyribose.

Effect of the Absence of Protein and RNA Synthesis on Deoxyribose Excretion during Thymine Deficiency

(a) The effect of 200 μg/ml chloramphenicol.

A culture of 15T⁻ was grown to an extinction of 0.42 at 650 mμ, harvested, washed and resuspended in thymine deficient medium. The culture was immediately divided into 3 aliquots. To one was added 16 μg/ml thymine to the second was added 200 μg/ml chloramphenicol, and no addition was made

to the third. The cultures were then incubated for 3 hr. with aeration and samples were taken every ½ hr. The bacteria-free culture medium was assayed for deoxyribose and the results are shown in Fig. 15.

It is clear that chloramphenicol markedly inhibits the output of deoxyribose though not as effectively as does 16 μg/ml of thymine.

(b) The Effect of the Absence of Arginine and/or Uracil on the Deoxyribose output of 15TAU

15TAU was grown up to an extinction of 0.3 at 650 mμ, harvested, washed and resuspended in $T^-(AU)^-$ and split into 8 aliquots. Supplements were added to these cultures to give the following compositions:

1. $T^-A^+U^+$
2. $T^-A^+U^-$
3. $T^-A^-U^+$
4. $T^-A^-U^-$
5. $T^+U^-A^-$
6. $T^+U^+A^-$
7. $T^+U^+A^+$
8. $T^+U^-A^+$

The cultures were incubated with aeration at 37° for 3 hr. and samples were taken at ½ hr. intervals and the bacteria-free supernatant assayed for deoxyribose. The results are shown in Fig. 16.

It is clear from this experiment that both the presence of uracil and arginine are necessary for the maximum output of deoxyribose, and this confirms the observations made on $15T^-$ with 200 μg/ml chloramphenicol. The small amount of deoxyribose output which does occur in some of the other cases could be due to a small amount of lysis, or due to the known leakiness of 15TAU (2% U, 5% A). In spite of the fact that cells in

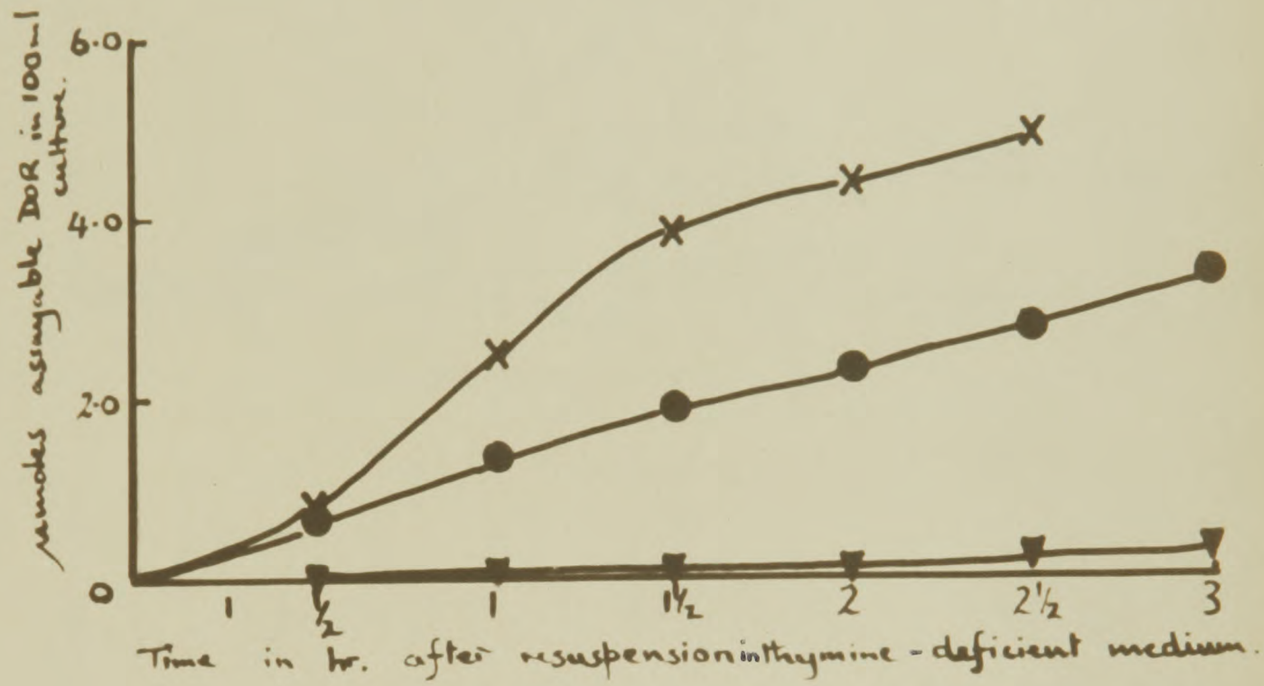


Fig. 15. Effect of chloramphenicol on the excretion of deoxyribose by 15T⁻ during thymine starvation. A culture of 15T⁻ in thymine deficient medium was split into 3 aliquots and to one was added 200μg/ml chloramphenicol (●); to another was added 16μg/ml thymine (▼); and to the third no additions were made (×). The three cultures were then assayed at various times for deoxyribose excreted into the media.

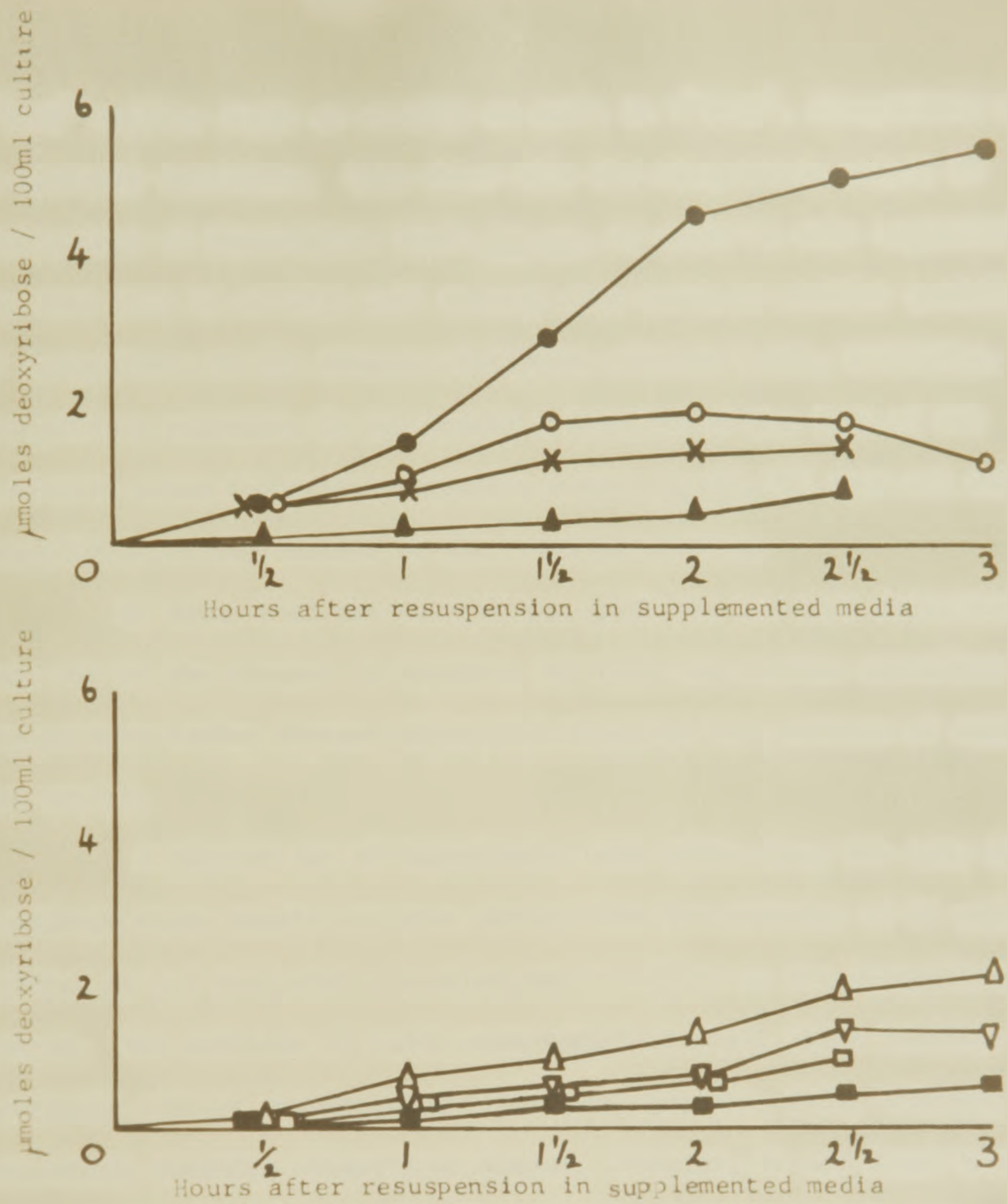


Fig. 16. Effect of the absence of arginine and uracil on the excretion of deoxyribose by 15TAU during thymine deficiency. A culture of 15TAU was split into 8 aliquots and supplemented as follows: $T^+A^+U^+$ (■); $T^+A^-U^-$ (□); $T^+A^-U^-$ (▽); $T^-A^-U^+$ (△); $T^-A^+U^-$ (▲); $T^-A^-U^-$ (✕); $T^-A^-U^+$ (○); $T^-A^+U^+$ (●). the cultures were then incubated and samples of the culture medium were assayed for deoxyribose.

$T^-(AU)^-$ undergo death as readily as cells in $T^-(AU)^+$, deoxyribose output is markedly inhibited. This shows that the deoxyribose output is independent of thymineless death per se, as would be also suggested by the fact that B3 at high cell density also excretes similar quantities of deoxyribose in the absence of thymine without losing viability. Deoxyribose excretion is not merely a function of the lack of DNA synthesis since the purine-requiring mutant E.coli B96 does not excrete deoxyribose in the absence of its required adenine.

It seems that the deoxyribose synthesis has been derepressed in some way in the absence of thymine, but that the maximum excretion of deoxyribose during thymine deficiency requires the presence of arginine and uracil.

A further set of experiments with 15TAU along the same lines confirmed these reults and also showed that the addition of 80 µg/ml uridine or 0.3 µmoles/ml adenosine had no effect on the deoxyribose output. The addition of 0.34% phenylethyl alcohol, which inhibits DNA synthesis without causing death (Berrah & Konetzka, 1962; Folsome, 1963) completely inhibits deoxyribose output. However, it was found that under these conditions this concentration of phenylethyl alcohol also inhibited RNA and protein synthesis, and the drop in deoxyribose output could be attributed to this. Addition of 0.34% phenylethyl alcohol after 1½ hr. thymine deficiency gives only a slight inhibition of the final deoxyribose output. See Fig.17. This shows that inhibition of macromolecular syntheses after 1½ hr. does not grossly affect the

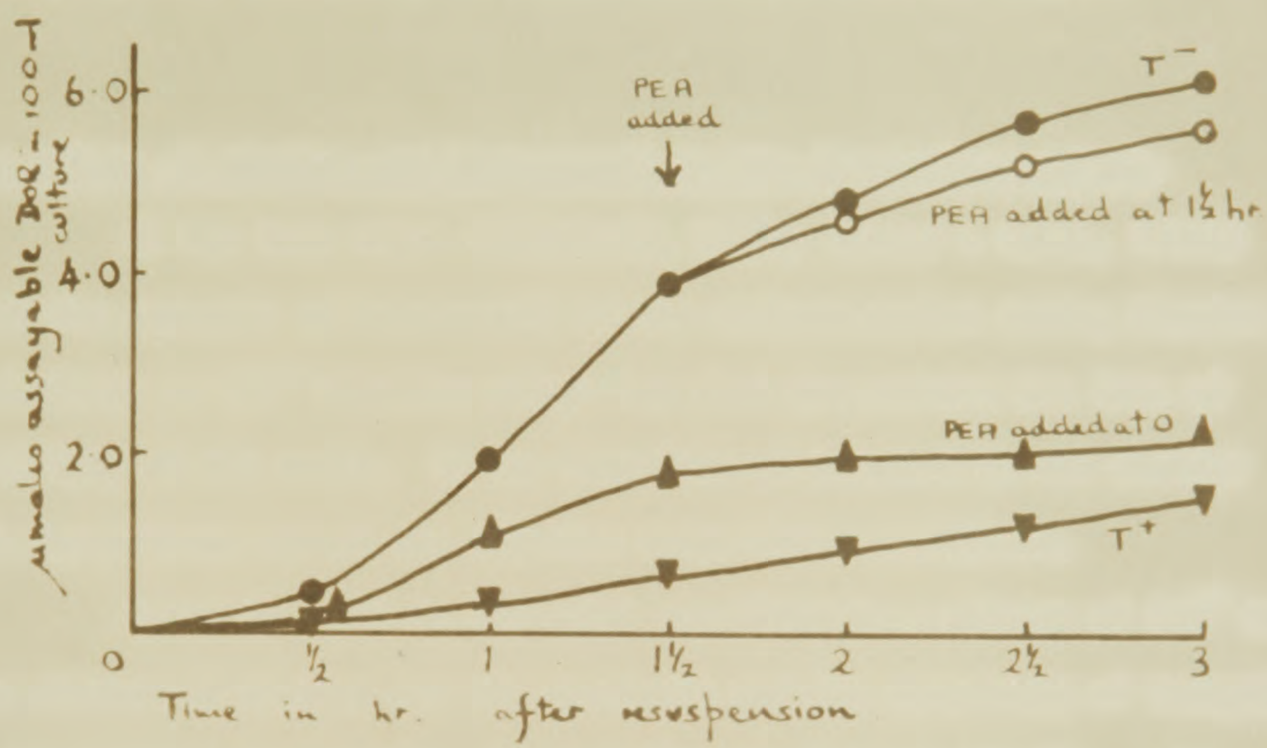


Fig. 17. Effect of phenylethyl alcohol on the deoxyribose excretion of 15T⁻ during thymine deficiency. At zero time (▲) and after 1½ hours (○) phenylethyl alcohol (0.34% final concentration) was added to aliquots of a culture of 15T⁻ growing in the absence of thymine (●). The culture media were then assayed for deoxyribose. The curve obtained when 16μg/ml thymine were added to an aliquot of the same culture at zero time (▼) is also shown.

deoxyribose output and would suggest that the deoxyribose made after this time is made or excreted by enzymes already synthesised. The effect of the addition of 200 µg/ml chloramphenicol at various times during thymine deficiency is consistent with this. See Fig.18.

The effect of thymine and bromouracil on the deoxyribose output.

It has been shown (Strelzoff, 1962a; Barner & Cohen, 1954b) that the presence of bromouracil in the culture medium during thymine deficiency in 15T⁻ slightly delays but does not prevent the loss of viability of the cells. It was therefore of interest to see whether the deoxyribose output was affected under these conditions.

Bu at 20 µg/ml or 200 µg/ml was added to a culture of 15T⁻ in the absence of thymine, and DNA synthesis and the appearance of deoxyribose in the medium was assayed. The results are shown in Fig.19. As was observed by Strelzoff, the Bu allows DNA synthesis to occur, and it is clear from Fig.19 that 200 µg/ml Bu is as efficient at repressing deoxyribose excretion as is 16 µg/ml thymine.

Although there is as yet no evidence that "bromouracil death" is the same phenomenon as thymineless death, it is clear that death in the absence of thymine is not necessarily accompanied by the appearance of deoxyribose in the medium; rather, under these conditions, deoxyribose excretion depends on the presence or absence of DNA synthesis.

Consistent with this is the fact that restoration of thymine to a

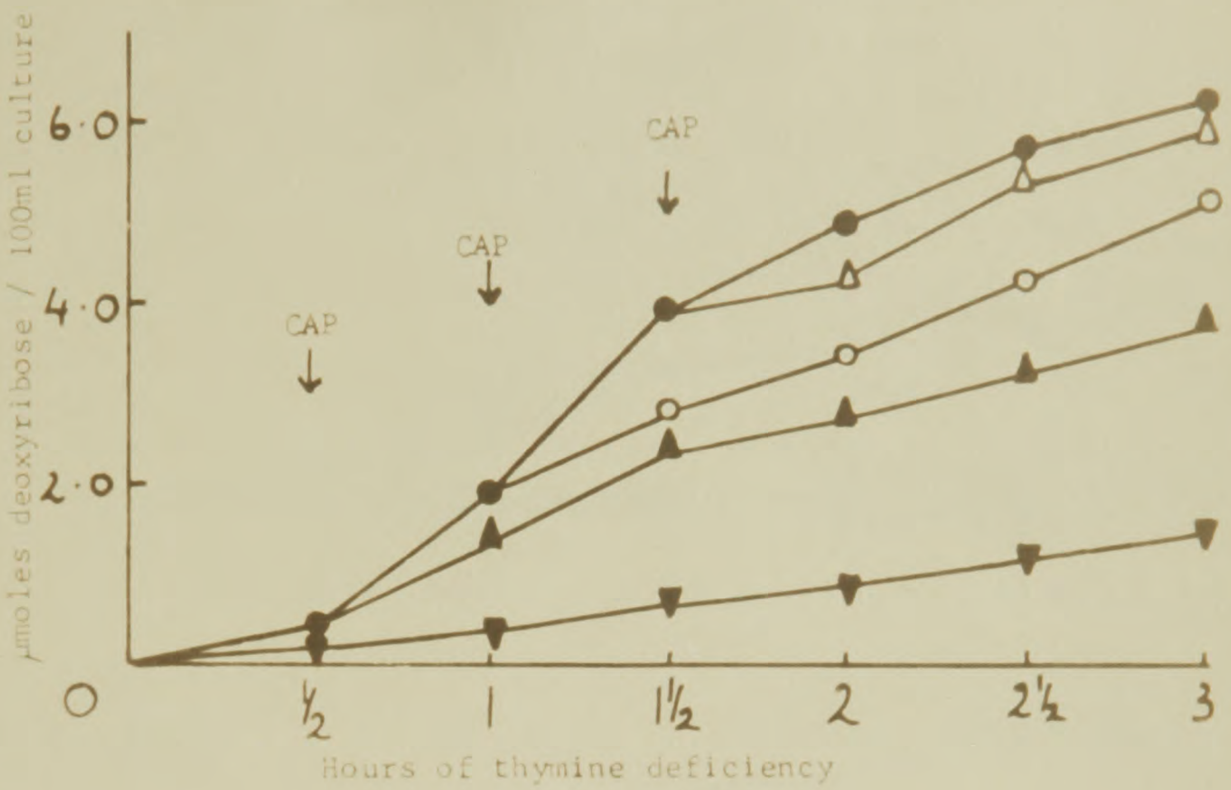


Fig. 18. Effect of the addition of chloramphenicol to 15T⁻ at various times during thymine starvation on the excretion of deoxyribose. The curves show the effect of the addition of 200µg/ml of chloramphenicol at ½hr. (▲); 1 hr. (○); and 1½ hr. (△) to a thymine deficient culture of 15T⁻ (●), on the excretion of deoxyribose.

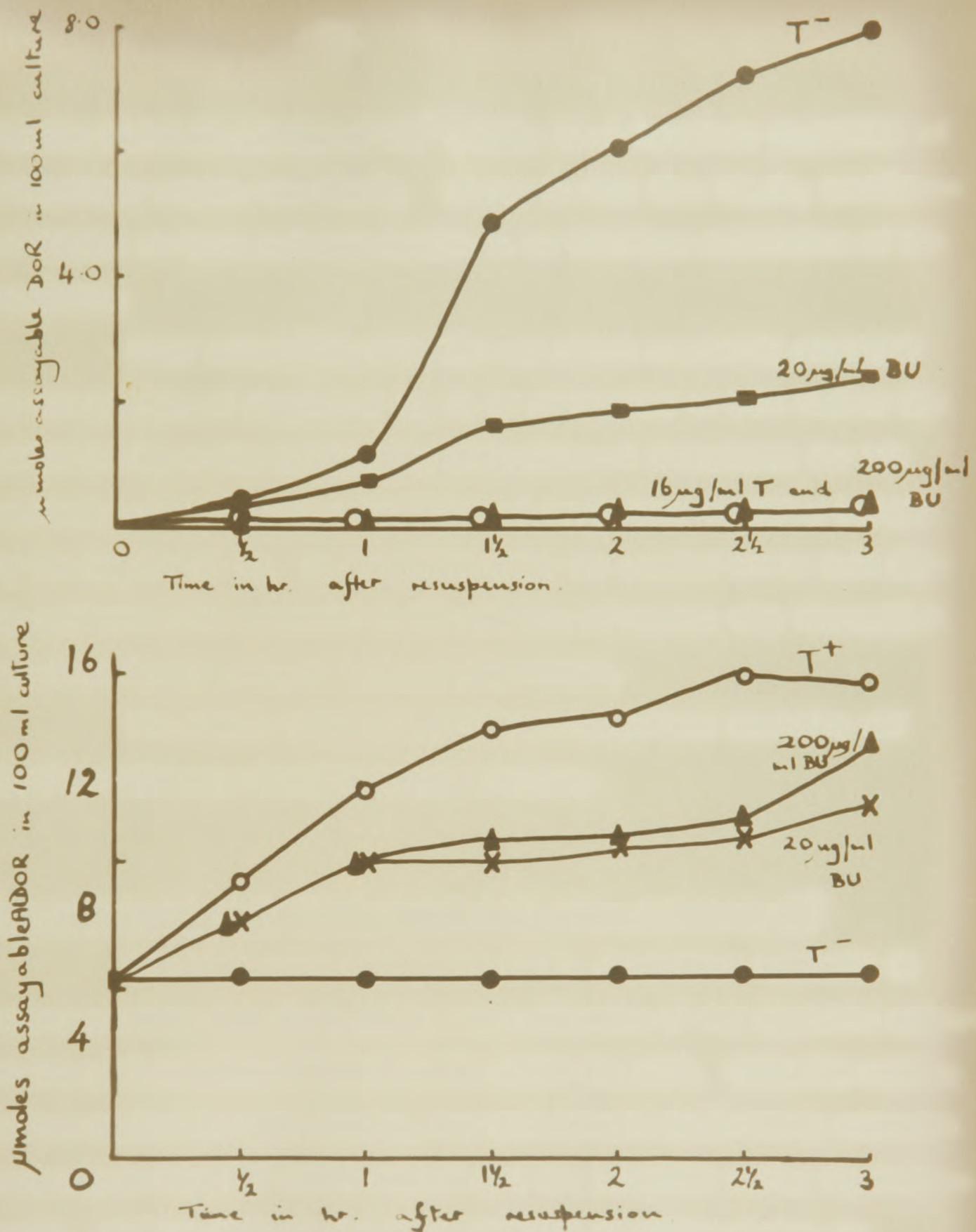


Fig. 19. The Effect of bromouracil on the excretion of deoxyribose during thymine deficiency in 15T⁻. In the upper diagram is shown the effect of adding 200μg/ml (▲) and 20μg/ml (■) of Bu, and 16μg/ml of thymine (○) to a thymine deficient culture of 15T⁻ (●) on the excretion of deoxyribose. The lower diagram shows the DNA synthesis occurring in the same cultures: 16μg/ml thymine (○); 20μg/ml Bu (×); 200μg/ml Bu (▲); no addition (●).

thymine deficient culture of 15T⁻ results in an immediate repression of deoxyribose output. See Fig.20. As can be seen from Fig.21, readdition of thymine to these cultures results in an immediate restoration of DNA synthesis.

Since deoxyribose excretion was almost completely prevented by the presence of thymine or Bu, it is of interest to know whether the effect is due to the accompanying utilisation of deoxyribose for DNA synthesis, or perhaps to a repression of deoxyribose formation induced by the thymine or Bu. An experiment which was carried out to test this by inhibiting DNA synthesis in the presence of thymine with Mitomycin C was inconclusive owing to lysis of the culture.

The precise relationship between deoxyribose excretion and thymine deficiency therefore remains unclear.

The Acid-soluble Nucleotides Inside the Thymine deficient 15T⁻.

With such gross derangement of the deoxyribose synthesis it seemed possible that there may be some reflection of its metabolic control in the acid-soluble nucleotide pools within the cell. Using the mapping method devised for this purpose, the acid-soluble UV-absorbing compounds were mapped from 15T⁻ cells sampled at ½ hr. intervals during thymineless death. Whether grown on glucose or mannitol as carbon source, no obviously gross changes occurred in the relative levels of ribonucleotides, although there was an all round increase in all spots. The most obvious changes were an accumulation of uracil, xanthine, hypoxanthine and a



Fig. 20.

The effect of the addition of thymine at various times during thymine deficiency on the excretion of deoxyribose by 15T⁻. 16μg/ml of thymine was added to a culture of 15T⁻ during thymine starvation at various stages of deficiency: zero time (○); ¼ hr. (✕); ½ hr. (▲); ¾ hr. (△); 1 hr. (▼); 1¼ hr. (▽); 1½ hr. (□); no addition (●). The excreted deoxyribose was then assayed for 2½ hours.

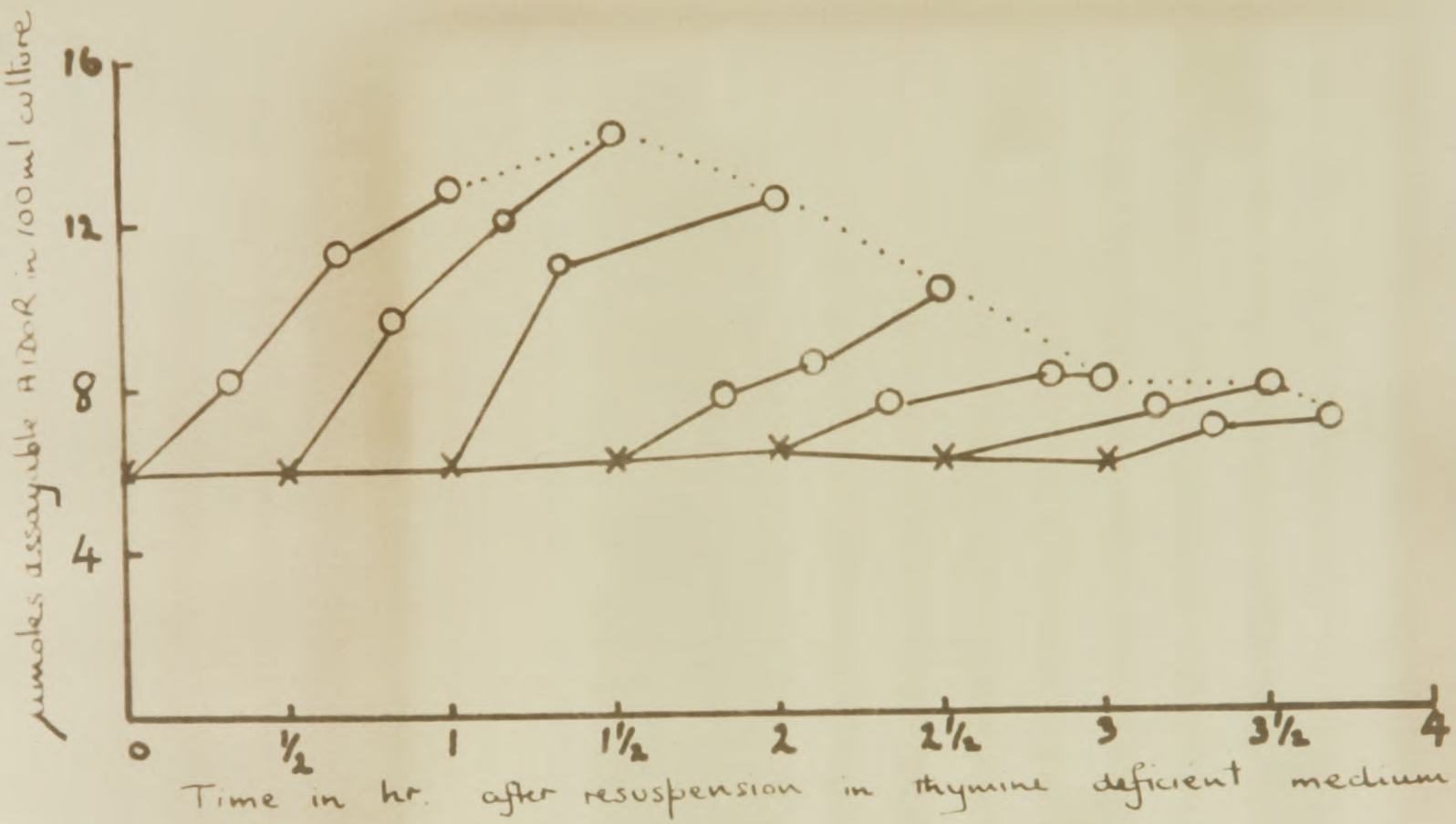


Fig. 21. The DNA synthesising ability of $15T^-$ during thymineless death. 16μg/ml of thymine (final concentration) was added to $15T^-$ at various stages of thymine starvation and the increase in acid-insoluble deoxyribose was measured. No thymine added (X); after addition of thymine (O). The dotted line shows the DNA that can be synthesised after 2 hours with thymine, throughout thymine deficiency.

spot containing ribose, labelled Y in the Plates, which remains unidentified. In general the amount of each ribonucleotide increases with time during thymine deficiency, but all nucleotides increase to about the same extent. See Plates 13-15.

The level of deoxyribonucleotides in E.coli is about 1% of that of the ribonucleotides, and it would not be expected to detect this level of material on the paper chromatogram.

Thus it seems that apart from the accumulation of uracil, hypoxanthine and xanthine, the first two of which are excreted by cells undergoing thymineless death (Barner & Cohen, 1954b), there is no great change in the ribonucleotide pattern of the cell during thymineless death.

Effects of Preincubation of 15T⁻ with deoxyribonucleosides on the Kinetics of Thymineless Death

Although no great change occurs in the ribonucleotide pools occurs during thymineless death it has been suggested that the accumulation of dATP may be the cause of death (Maaløe, 1963). It was therefore of interest to see whether prior incubation for one generation with dA, dU, dG or dC had any effects on the kinetics of thymineless death. From Fig.22 it can be seen that no differences were observed compared with the control.



Plate 13. Acid-soluble nucleotides from 15T⁻ before thymineless death.

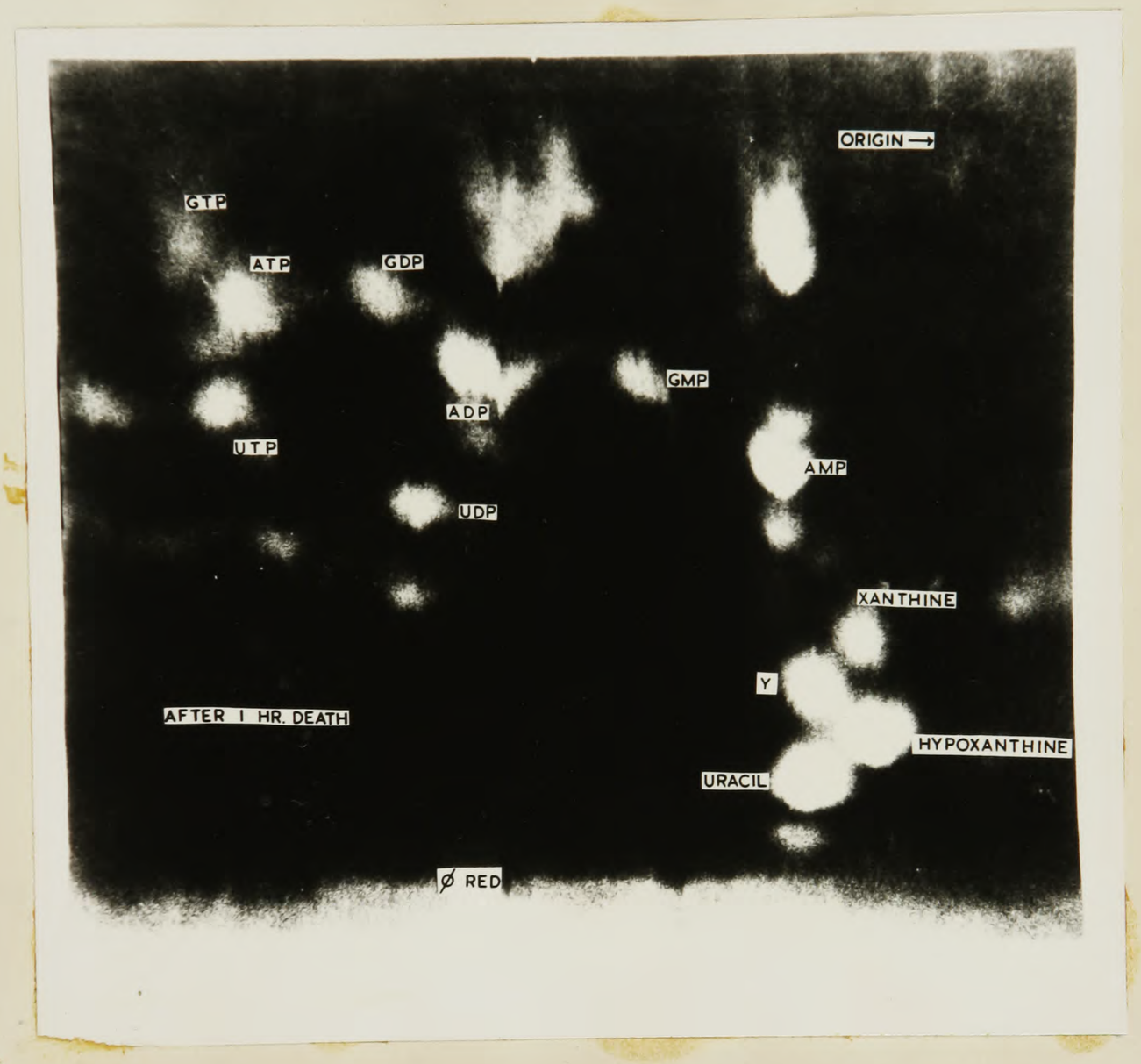


Plate 14. Acid-soluble nucleotides from 15T⁻ after 1 hour thymine deficiency.



Plate 15. Acid-soluble nucleotides from 15T⁻ after 2½ hours thymine deficiency.

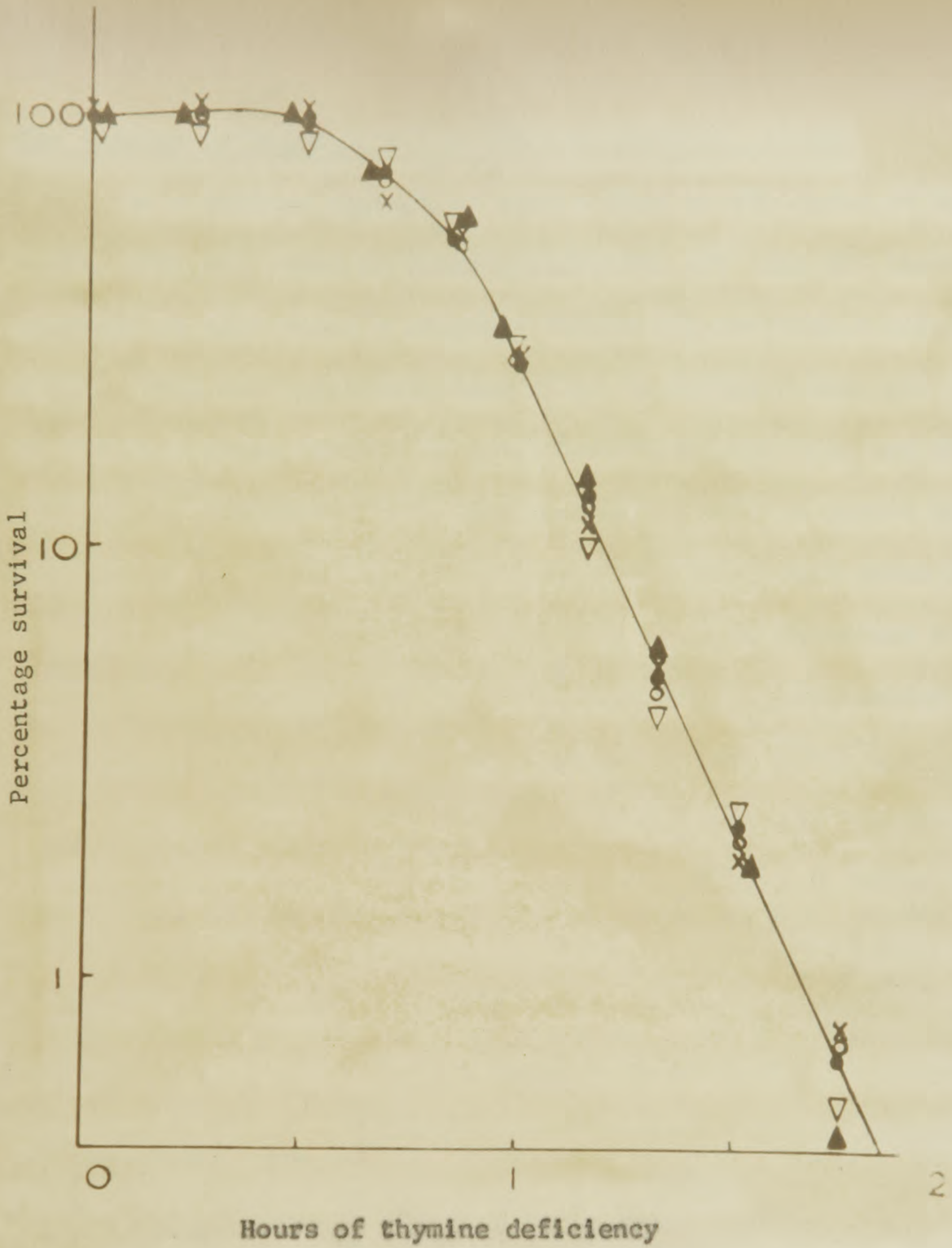


Fig. 22. Effect of prior incubation with deoxynucleosides on the kinetics of thymineless death in 15T⁻. 15T⁻ was grown to an extinction of 0.15 at 650 mμ and split into five aliquots. 1.2×10^{-3} M dC=(●); dU=(▲); dA=(▽); dG=(○); and nothing =(X), were added to the cultures and growth was continued for another 55 minutes (E_{650}=0.3). The cultures were then harvested, washed and resuspended in thymine-deficient media and the kinetics of death were followed by performing viable counts at suitable times.

The Effects of Bromouracil on Thymine-deficient E.coli

In a further attempt to find a clue to the cause of thymineless death, BudR was added to thymine-deficient 15T⁻ and the viability was observed to fall after a slightly longer lag and with a slightly slower rate. This had previously been shown by Maaløe & Hanawalt (1961) and by Barner & Cohen (1956b) in non-synchronous cultures, and by Strelzoff (1962a) in cultures of 15T⁻ synchronised by half an hour's thymine deficiency. Maaløe has also shown that 15TAU, when grown in $Bu^{+}(AU)^{-}$, do not lose their viability. It was of interest therefore to see whether incubation in $Bu^{+}(AU)^{-}$ also resulted in immunisation against thymineless death on subsequent resuspension in $T^{-}(AU)^{-}$.

It was first of importance to verify that the apparent state of immunisation observed in $T^{-}(AU)^{-}$ after incubation in $T^{+}(AU)^{-}$ for 90 minutes was indeed a state of immunisation rather than just a condition in which the cells were completely "shut off" and hence unable to use the energy known to be required for thymineless death. The respiration was therefore measured using Warburg manometry and is compared with that of 15TAU under other conditions in Fig.23.

Cells in $T^{-}(AU)^{-}$ after 90 minutes preincubation in $T^{+}(AU)^{-}$ have an almost identical respiration rate to that of cells in $T^{-}(AU)^{-}$ which have not been immunised and are undergoing thymineless death. Immunisation is not therefore due to a general inhibition of metabolic processes.

As can be seen in Fig.24, incubation in $Bu^{+}(AU)^{-}$ for 90 minutes

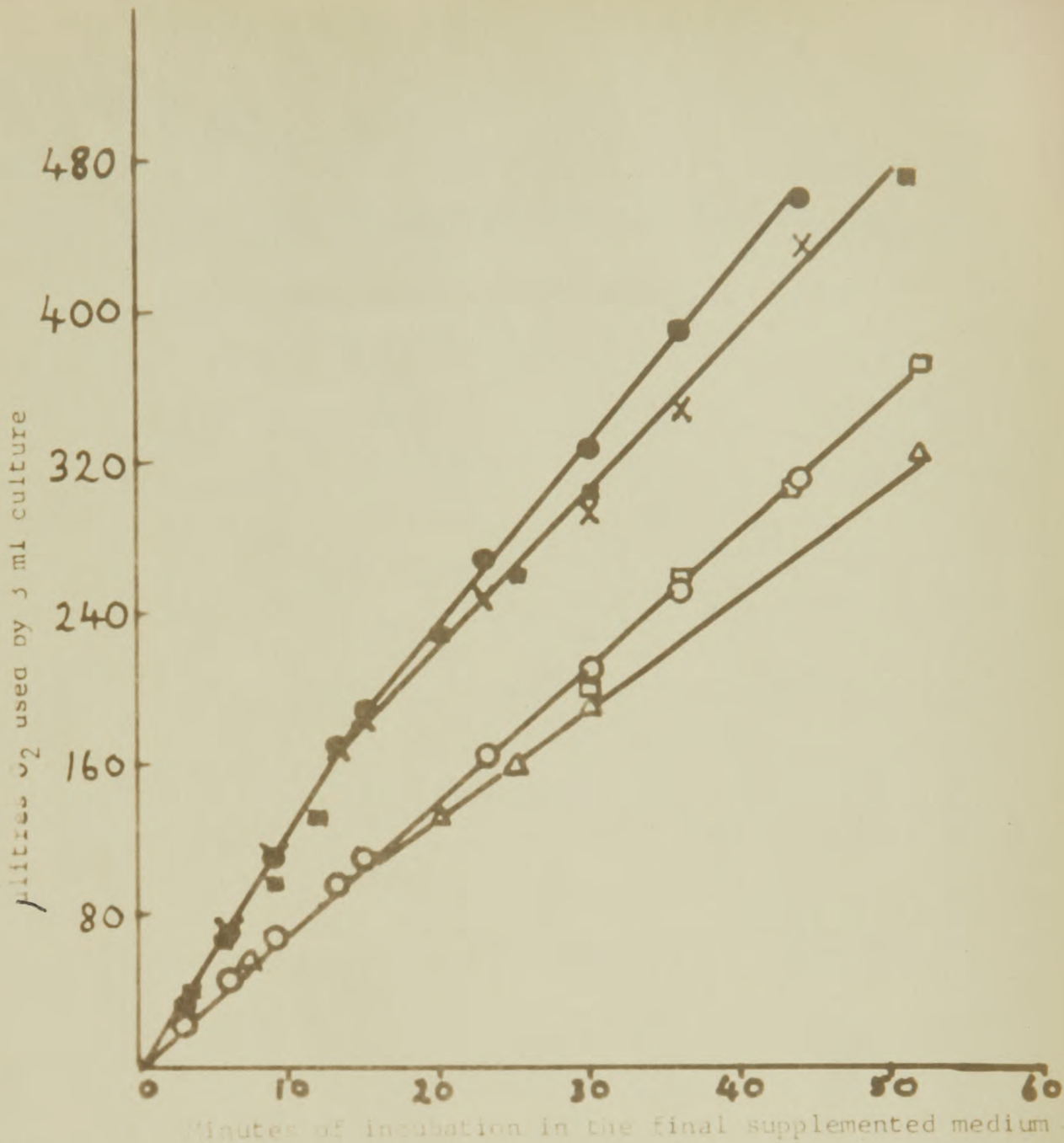


Fig. 23. The effect of immunisation of 15TAU in $T^{+}(AU)^{-}$ on the respiration rate in $T^{-}(AU)^{-}$. 3 ml of cultures treated in various ways were placed in supplemented media in Warburg flasks and the oxygen uptake was measured: all bacteria were grown in $T^{+}(AU)^{+}$ and then transferred to the following media; respiration was measured only in the last of each media after a sequence of treatments. $T^{+}(AU)^{+}$ (●); $T^{-}(AU)^{+}$ (✕); $T^{+}(AU)^{-}$ for 90min.→$T^{-}(AU)^{-}$ (■); $T^{-}(AU)^{-}$ (○); $T^{+}(AU)\rightarrow T^{-}(AU)^{-}$ (△); $T^{-}(AU)^{-}$ (□).

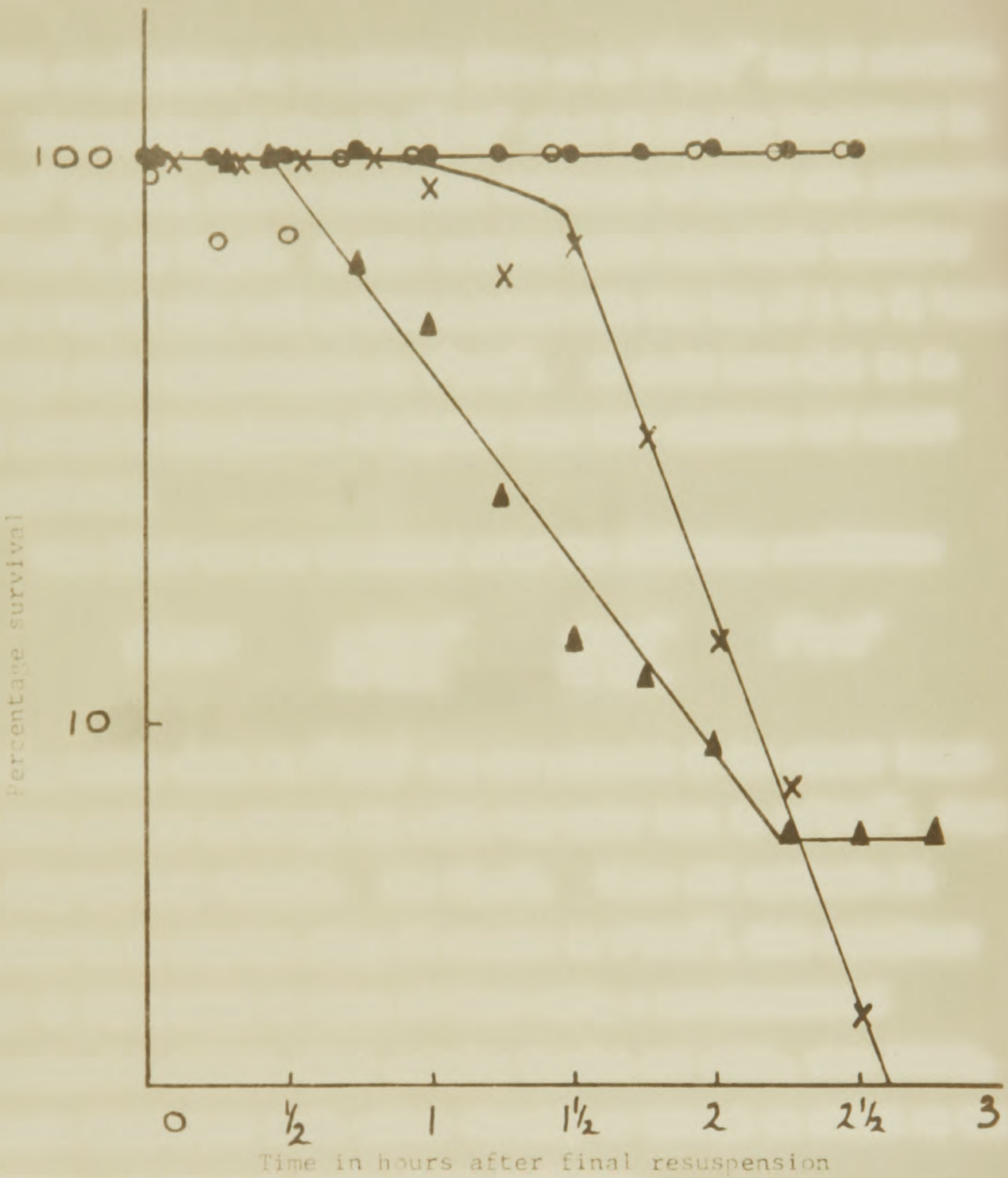


Fig. 24. The efficacy of $Su^{+}(AU)^{-}$ in immunising 15TAU against thymineless death in $T^{-}(AU)^{-}$. A culture of 15TAU growing in $T^{+}(AU)^{+}$ was harvested and split into 4 aliquots. One was resuspended in $T^{-}(AU)^{-}$=(▲); another was resuspended in $T^{+}(AU)^{-}$ for 90 minutes followed by resuspension in $T^{-}(AU)^{+}$=(x); another was resuspended in $T^{+}(AU)^{-}$ for 90 minutes and then resuspended in $T^{-}(AU)^{-}$=(●); and the last was resuspended in $Su^{+}(AU)^{-}$ for 90 minutes followed by resuspension in $T^{-}(AU)^{-}$=(○). Viable counts were then measured at suitable times.

is just as effective at immunising 15TAU as is $T^+(AU)^-$ for 90 minutes.

Resuspension of 15TAU in $T^-(AU)^+$ after 90 minutes incubation in $Bu^+(AU)^-$ however, shows a remarkable difference compared with preincubation in $T^+(AU)^-$. See Fig.25. In this case the cells lose their viability after a lag of only half an hour compared with the lag of 1 to 1½ hours observed after preincubation in $T^+(AU)^-$. It would seem that Bu is only lethal in the absence of thymine when both arginine and uracil are available.

When 15TAU was now incubated under the following conditions:

$$T^+(AU)^+ \longrightarrow \underset{90\ \text{min.}}{Bu^+(AU)^-} \longrightarrow \underset{20\ \text{min.}}{T^+(AU)^-} \longrightarrow T^-(AU)^+$$

the lag before death in $T^-(AU)^+$ was now extended to its normal length of about an hour as though the cells had never been in $Bu^+(AU)^-$. That is, they had been "cured". See Fig.26. Since DNA synthesis is expected to be complete after 90 minutes in $T^+(AU)^-$, this experiment might be interpreted in terms of there being a non-chromosomal (cytoplasmic) molecule which contains thymine and which, when intact, prevents thymineless death either directly or through an effect on the initiation of the next cycle of DNA synthesis.

Further support for the existence of such a cytoplasmic molecule containing thymine was obtained by incubating 15TAU under the following conditions:

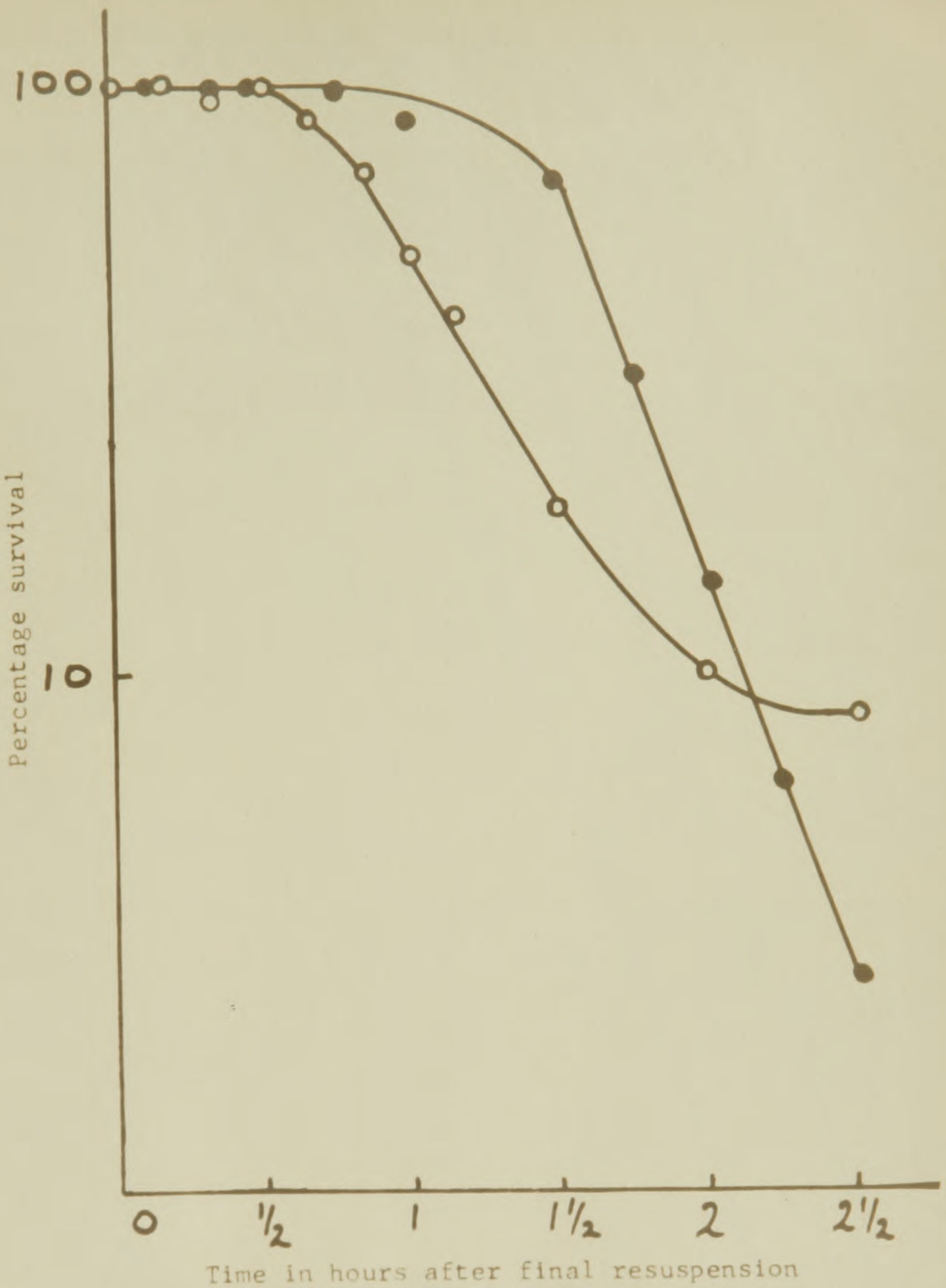


Fig. 25. The effect of incubation in $Bu^{+}(AU)^{-}$ in reducing the lag phase before death for 15TAU in $T-(AU)^{+}$. A culture of 15TAU growing in $T^{+}(AU)^{+}$ was harvested and split into two aliquots. One was incubated for 90 min. in $Bu^{+}(AU)^{-}$ (O); and the other for 90 min. in $T^{+}(AU)^{-}$ (●); both were then resuspended in $T^{-}(AU)^{+}$ and their viable counts were measured at suitable times.

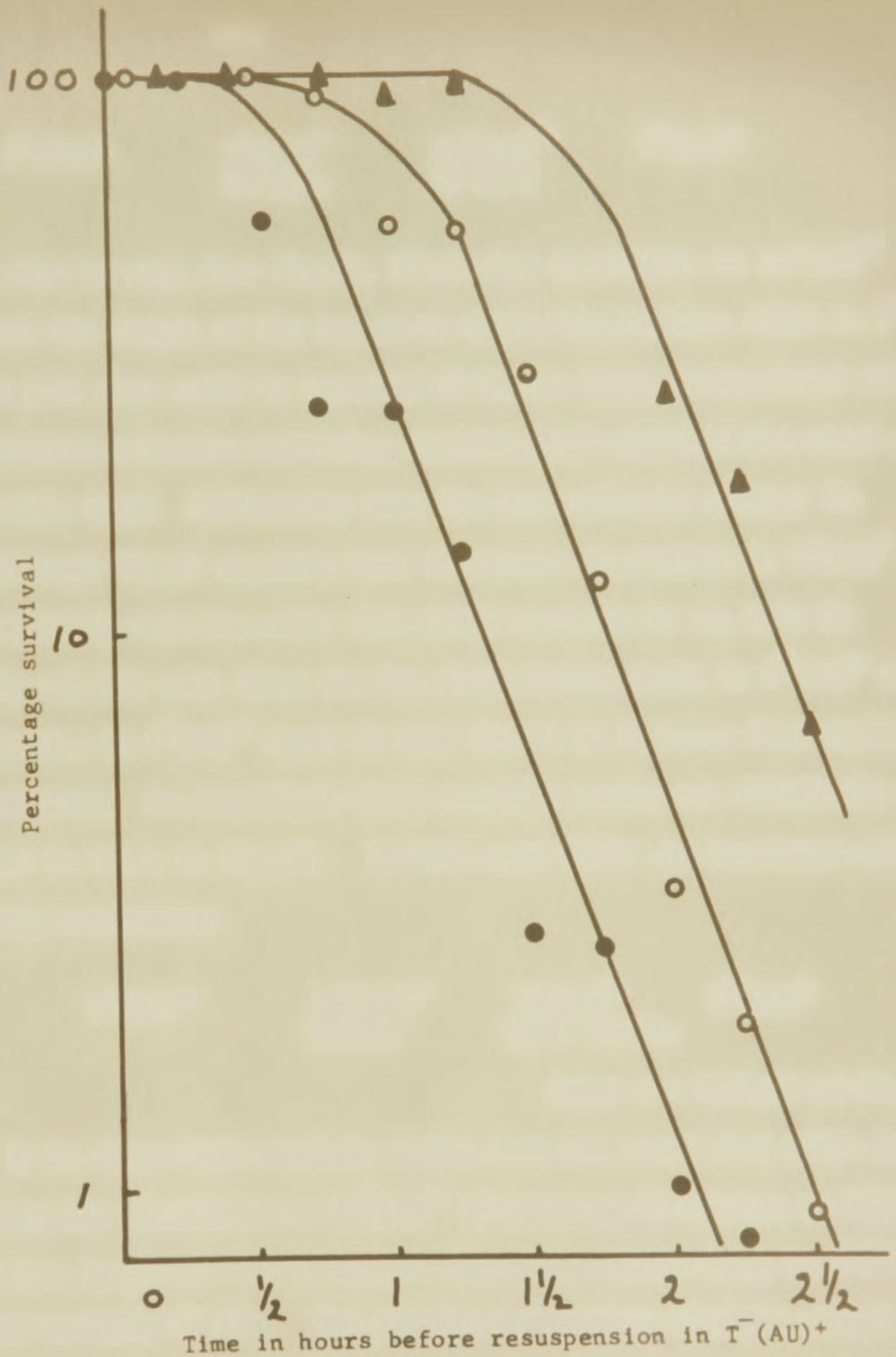


Fig. 26. The "curing" phenomenon. A culture of 15TAU growing in $T^+(AU)^+$ was harvested and split into 3 aliquots. One was incubated for 90 min. in $Bu^+(AU)^-$ before resuspension in $T^-(AU)^+$ =(●); another was incubated for 90 min. in $Bu^+(AU)^-$ and "cured" for 20 min. in $T^-(AU)^-$ before resuspension in $T^-(AU)^+$ =(▲); and the third was incubated for 90 min. in $T^+(AU)^-$ and for a further 20 min. in $Bu^+(AU)^-$ before resuspension in $T^-(AU)^+$ =(○). The viable counts of the 3 cultures were then measured.

$$T^+(AU)^+ \longrightarrow \underset{90\ min.}{T^+(AU)^-} \longrightarrow \underset{20\ min.}{Bu^+(AU)^-} \longrightarrow T^-(AU)^+$$

As can also be seen from Fig.26, 20 minutes incubation in $Bu^+(AU)^-$ decreases the length of the lag phase before thymineless death in $T^-(AU)^+$. This is just what would be expected if Bu was being incorporated into a cytoplasmic molecule containing thymine which affected the onset of thymineless death differently from the same molecule without Bu.

Although Fig.26 suggests that 20 minutes incubation in $T^+(AU)^-$ may completely cure the cells from their exposure to Bu, this is probably not the case since the lag phase before death in $T^-(AU)^+$ after immunisation in $T^+(AU)^-$ seems to vary from 1 to 1½ hours from experiment to experiment. An experiment where complete curing was definitely not obtained is shown in Fig.27. In this case 15TAU was incubated under the following conditions:

$$T^+(AU)^+ \longrightarrow \underset{x\ min.}{Bu^+(AU)^-} \longrightarrow \underset{(90 - x)min.}{T^+(AU)^-} \longrightarrow T^-(AU)^+$$

where $x = 5, 10, 15$ or 90.

It is clear that complete curing does not occur when cells are incubated for 15 minutes in $Bu^+(AU)^-$ followed by 75 minutes in $T^+(AU)^-$, indeed the length of the subsequent lag phase before death in $T^-(AU)^+$ is some function of $1/x$. The precise explanation of these observations is not known yet, but the observations are consistent with the hypothesis of a cytoplasmic molecule containing thymine controlling thymineless death in some way.

It has been reported (Stahl, Crasemann, Okun, Fox and Laird, 1961) that bacteriophages containing Bu-labelled DNA are sensitive to visible

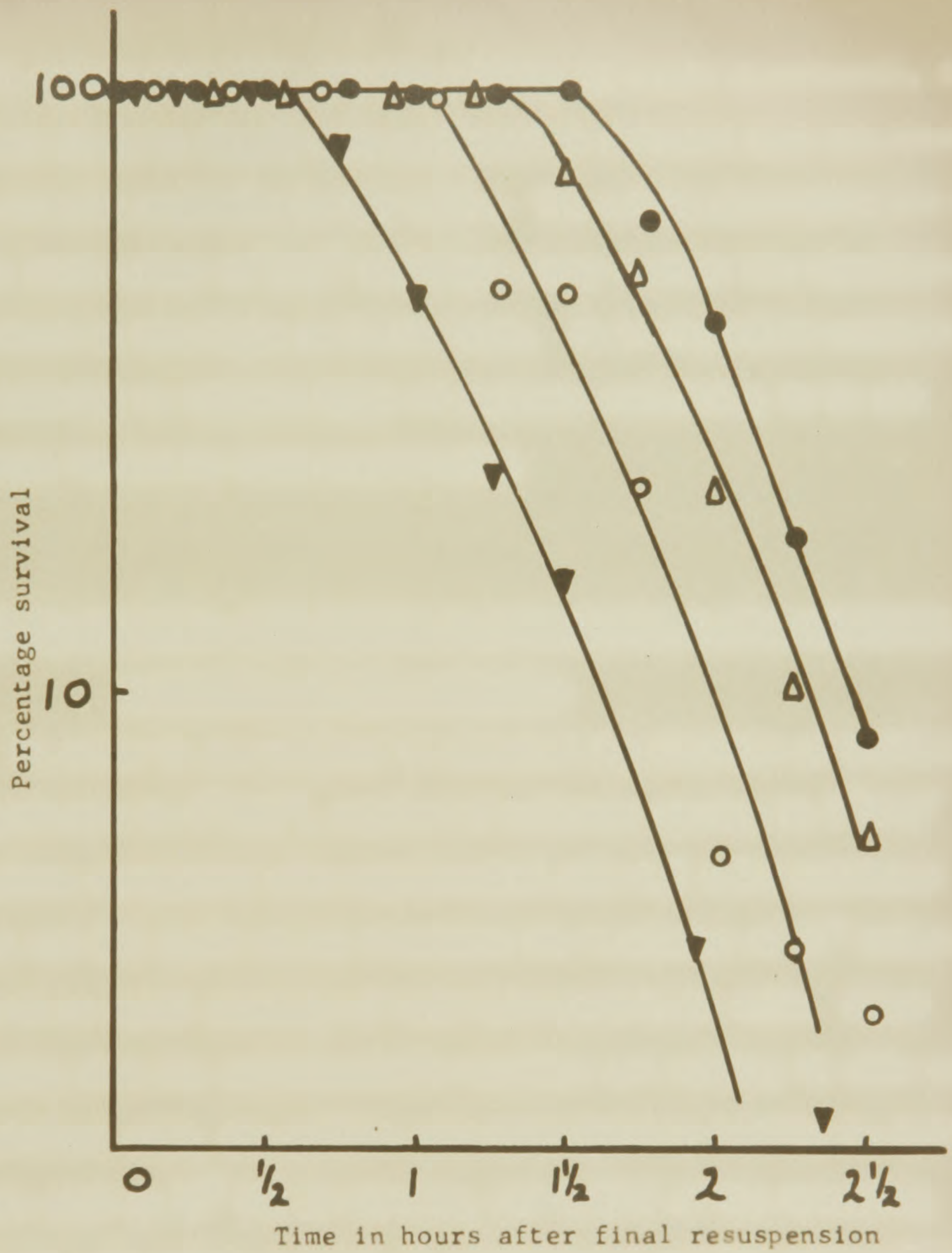


Fig. 27. The effect of various periods of $Bu^{+}(AU)^{-}$ and $T^{+}(AU)^{-}$ on the kinetics of death of 15TAU in $T^{-}(AU)^{+}$. A culture of 15TAU growing in $T^{+}(AU)^{+}$ was harvested and split into 4 aliquots. One was incubated in $Bu^{+}(AU)^{-}$ for 5 min.(●);one for 10 min.(Δ);one for 15 min.(○); and one for 90 min.(▼). The cells were then resuspended in $T^{+}(AU)^{-}$ for 85 min.,80 min.,75 min. and 0 min resply. The cells are then resuspended in $T^{-}(AU)^{+}$ and viable counts followed for 2½ hours.

light. To ensure that this was not a factor in these experiments, the experiments shown in Fig.26 were performed in darkness from the moment that Bu was made available to the cells. The curing phenomenon and the premature death after $Bu^{+}(AU)^{-}$ treatment occur whether the experiments are performed in the light or in the dark. It is therefore concluded that sensitivity to visible light of bacteria containing Bu is not an important factor in these experiments.

Effect of immunisation with thymine or bromouracil on the subsequent initiation of DNA synthesis.

In order to see whether Bu affected the kinetics of initiation of DNA synthesis in $T^{-}(AU)^{+}$, samples of cultures growing in $T^{-}(AU)^{+}$ after various treatments were supplemented with 0.5 µC/ml tritiated thymidine and 200 µg/ml dA and incubated for 2 minutes at 37° before addition of an equal quantity of cold 0.4N perchloric acid. After centrifuging down the acid-insoluble material and washing it with cold 0.2N perchloric acid, the pellets were hydrolysed by heating in 0.5N perchloric acid at 70° for 20 minutes, and 0.05ml of the resulting supernatants were added to 1.1ml of scintillator and the radioactivity incorporated into DNA at various times was measured in a scintillation counter. The results obtained are shown in Fig.28 expressed as specific activity of the DNA. The background was 219 counts/400sec. and a specific

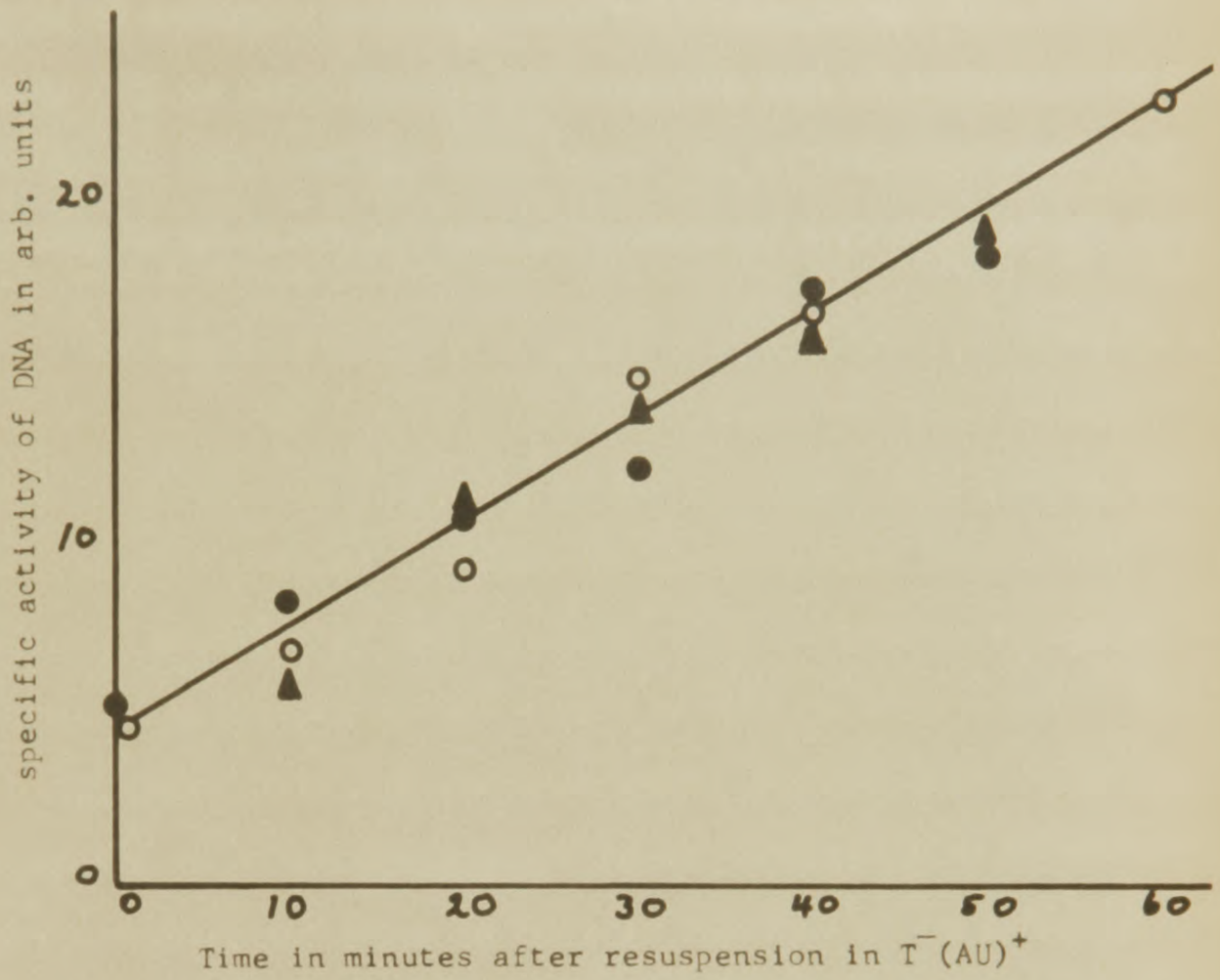


Fig. 28. The reinitiation of DNA synthesis in $T^-(AU)^+$ after immunisation in $Bu^+(AU)^-$ or $T^+(AU)^-$. A culture of 15TAU growing in $T^+(AU)^+$ was harvested and split into 3 aliquots. One was immunised for 90 minutes in $T^+(AU)^-$ before resuspension in $T^-(AU)^+$ =(●); another was immunised for 90 minutes in $Bu^+(AU)^-$ before resuspension in $T^-(AU)^+$ =(○); and the third was immunised for 90 minutes in $T^+(AU)^-$ followed by a further 20 minutes in $Bu^+(AU)^-$ before resuspension in $T^-(AU)^+$ =(▲). Samples were taken from the cultures after the final resuspension at 10 minute intervals and supplemented with tritiated thymidine for 2 minutes. The reaction was then stopped and the specific activity of the DNA of the cells was measured.

activity of 25 arbitrary units represents 2000 counts/400 sec. above background. It is clear that in all cases the rate of initiation of DNA synthesis is the same over the first hour. It is therefore concluded that the effects of Bu are not on the kinetics of initiation of DNA synthesis.

DISCUSSION

DISCUSSION

The Possibility of Damage to the DNA

The possibility of changes occurring in the DNA during thymineless death has been examined closely both with respect to physical and chemical effects. The reported evidence of alkali-lability has been shown to be due to artifacts. The inability to reprecipitate DNA after alkali-treatment is clearly not a function of the DNA since added herring sperm DNA was similarly affected. Whilst the compound which causes this phenomenon is evidently of some interest, it is irrelevant to thymineless death since it is present in 15T⁻ before as well as after death. The cause of the decrease in diphenylamine colour has been shown to be due to the production of an inhibitor of the colour development by alkali-treatment of the thymine-deficient cells. The identity of the material responsible for this is unknown. It could however be one or more proteins perhaps rich in cystine, which are produced under conditions of thymine starvation and unbalanced growth, and this would be consistent with the observed effects of alkali-treated cystine on the diphenylamine reaction.

At the physical level, the fact that alkali-treatment reduces the viscosity of after death DNA no more than of before death DNA is a strong argument for the normal stability of after death DNA. In spite of the low viscosities of the alkali-treated DNA solutions, the methods used are sufficiently accurate for an answer to be given. The results show that if there are alkali-labile sites in the DNA after death, their frequency is much less than 1 in 60,000 base pairs: the approximate size of the DNA molecules prepared by the extraction methods used in

this work.

The possibility of single-strand breaks which were not sensitive to alkali has also been ruled out to the same limits. The sedimentation patterns given by the after death DNA before and after heating in 1% formaldehyde showed no differences from those of normal DNA, and the intrinsic viscosity and the susceptibility to shear as measured by the reduction of specific viscosity on passage through a syringe needle under pressure, both showed no significant differences compared with normal DNA.

The claim of Mennigmann and Szybalski (1962b) that phenol-deproteinised DNA from thymine-deficient 15T$^-$ is more susceptible to shear, seems therefore to be unfounded. It is clear from the experiments described here that whether the DNA is deproteinised by $CHCl_3$ or by phenol, the viscosities of the DNAs extracted after and before death are identical. The method of deproteinisation is also seen to have no effect on the melting-curves of the DNAs before and after death, and the identity of these melting curves is further evidence for the integrity of the DNA.

The use of band-width measurements of the DNAs before and after heating in 1% formaldehyde have led to the same conclusions. Cross-linking due to impurities in the formaldehyde could account for the finding that the band-width of the denatured DNA was not as expected for a halving of the molecular weight of native DNA. The fact that the Denatured DNA bands at a rather lower density than that expected

for denatured E. coli DNA is consistent with this since cross-linked DNAs which were not cross-linked in register would be likely to be mostly, but not fully, denatured.

Thus the buoyant density would be between that of native and denatured E. coli DNA as observed. The fact that only one band is found suggests that all the molecules are acting similarly. The important point as far as this discussion is concerned is that the behaviour of the DNA extracted before and after thymineless death is identical.

As has already been pointed out, the literature contains many references to the difficulty of extracting DNA from bacteria which have undergone thymineless death. In this work, it has been seen that the DNA yields have always been of a similar magnitude to those from normal bacteria, and it would seem that provided the procedure used for extracting the DNA includes sufficient deproteinisation steps to remove all the extra protein produced by unbalanced growth, the DNA will spool quite normally on the addition of ethanol to an aqueous solution. Under these circumstances it is reasonable to expect that the purification methods have not selected out intact DNA which remains in the dead bacteria, but have produced just as good a cross-section as with the extraction of normal DNA.

A striking confirmation of the physical integrity of the DNA after thymineless death is seen in the results of the autoradiography experiments. Here bacterial chromosomes could be visualised from $15T^-$ after thymineless death had occurred, with just the same frequency and in

just as good a state of integrity as from normal bacteria. The fact that no circular chromosomes were seen is probably due to the fact that only a small number of films (50) were exposed during these experiments.

In spite of the fact that no damage could be detected in the DNA after thymineless death, the techniques used would have been unable to detect single-strand breaks which were occurring at a frequency of less than 50-100 times per chromosome. Since it is known that up to 14 single-strand breaks can occur without being lethal (Fuerst & Stent, 1957), this still leaves a lethal range which would have been undetectable by the present methods, although it is not clear how thymineless death could produce these breaks in the pre-existing chromosome.

The absence of more than one cross-link per chromosome has also been demonstrated.

When a thymineless bacterium is deprived of thymine, DNA synthesis is occurring at only one site in the chromosome. If the absence of thymine causes damage only at the site of DNA polymerase activity then this would occur at only one site per chromosome. This would therefore be quite compatible with the normal capacity for inducing enzymes and with the chemical and physical results reported in this thesis.

The synthesis of deoxyribose in thymineless bacteria.

The appearance of deoxyribose in the culture medium during thymine deficiency has been shown to be independent of thymineless death since it occurs with B_3 at high cell density when little death occurs, and it does not occur during death of 15TAU in $T^-(AU)^-$. This means that death

cannot be due to an accumulation of deoxyribose in the cell.

The compound appearing in the medium appears to be free deoxyribose since it is not absorbed on a charcoal column, nor does it appear to be phosphorylated. Since the formation of deoxyribose in vivo occurs at the level of the nucleoside diphosphates (Reichard et al., 1961), removal of the bases and the phosphate groups must occur before the deoxyribose is excreted. It is not known whether all or only some of the deoxynucleoside diphosphates act as precursors of the excreted deoxyribose; addition of uridine or adenosine before or during thymine starvation has no effect on the excretion of deoxyribose and suggests that the utilisation of these compounds can still be regulated normally if indeed they are precursors of the excreted deoxyribose.

The requirement for protein and RNA synthesis for the maximum excretion of deoxyribose would suggest that either the enzymes concerned in its production require resynthesis or that enzymes have to be synthesised to allow the excretion of the deoxyribose from the cell. Since DNA synthesis can continue to the completion of a round of replication in the absence of protein and RNA synthesis (15TAU in $T^{+}(AU)^{-}$) it would suggest that sufficient enzymes are present in the cells to synthesise a considerable amount of deoxyribose, and that the second alternative is therefore more likely. It is possible however that in the absence of thymine some deoxyribonucleotides accumulate in the cell and inhibit the conversion of nucleoside diphosphates to deoxynucleoside diphosphates.

This would then mean that synthesis of new enzymes for the conversion would be necessary to obtain the maximum excretion under conditions of thymine deficiency.

In this work no gross changes in the ribonucleotide pools in 15T⁻ during thymine starvation have been observed except for an all round increase. It has also been observed that changes induced in the nucleotide pools by feeding deoxynucleosides have no effect on the kinetics of death. The observations of Munch-Petersen and Neuhard (1964) however make it conceivable that the accumulation of dATP could be lethal to 15TAU. The crucial experiment is to look for dATP accumulation in 15TAU in the absence of protein and DNA synthesis before and after immunisation. This will show whether there is any connection between dATP accumulation and thymineless death.

DNA synthesis during thymine deficiency.

It has been shown that some DNA synthesis continues during thymineless death even though there is apparently no thymidylate synthetase in the cells to provide thymine (Barner & Cohen, 1959). The experiments show that the extent of this synthesis is about a 2% increase after 2 hours thymine starvation. From our knowledge of the *in vitro* activity of DNA polymerase (Lehman, Bessman, Simms & Kornberg, 1958) it is possible that a small amount of DNA synthesis may occur in the absence of thymine. This synthesis may perhaps continue because the template strands fold out (Fresco & Alberts, 1960) to compensate for the lack of thymine: thus damage to the DNA would result. In any one cell

the damage would be localised on a small part of the chromosome, but in a non-synchronised culture, damage at every site would be represented equally. This type of process might explain the induction of mutations during thymine deficiency.

Thymineless Induction

Damage to the DNA might conceivably be the cause of the thymineless induction of lysogenic phages (Korn & Weissbach, 1962) if it prevented the synthesis of the cytoplasmic repressor (Jacob & Campbell, 1959) provided of course that the repressor were unstable. Alternatively, the repressor may contain thymine as an effector (Jacob & Monod, 1961). Thymine deficiency would then result in an ineffective repressor which would be analagous to the repressor damaged by UV-light (Fisher, cited by Hayes, 1964). In both cases induction of the lysogenic phage would be expected.

Mutants of phage λ which cannot be induced by UV-light (λ ind$^-$) also cannot be induced by thymine deficiency (Sicard & Devoret, 1962). It might be argued that this is evidence that UV-light and thymine deficiency act at the level of the repressor, but it is also possible that the mutation in the DNA may be such as to give rise to repressor which is insensitive to UV-light and a repressor gene which can no longer be damaged by thymine deficiency.

Analagous arguments could apply to the induction of colicins, and indeed the induction of unidentified colicins or other lethal factors could be the cause of thymineless death. If the effect was in fact

directly on the cytoplasmic repressor of a colicin, then it might be expected that mutants of thymineless bacteria would be found that did not undergo thymineless death. Alternatively, damage in one of several "lethal sites" distributed around the chromosome would make immune mutants very rare since the amount of DNA synthesis occurring in 2-3 hours (99% death), i.e. about 2%, suggests that there must be at least 50 such "lethal sites" which can be damaged. However, it will be seen that the observations discussed in the next section can more easily be explained by direct effects in a cytoplasmic factor.

The effects of bromouracil on thymineless death.

The observations with Bu are probably the most interesting and the most difficult to interpret. Bu is known to induce mutations when it is incorporated into DNA (Zamenhof, 1959; Freese, 1959a,b; Litman & Pardee, 1960; Rudner, 1961; Strelsoff, 1962a,b). These mutations are probably caused by "errors in substitution" and "errors in replication" caused by base-pairing mistakes owing to the greater propensity for Bu to exist in the enol form under physiological conditions compared with thymine (Freese, 1959a; Brookes & Lawley, 1962). Although this does not seem to be the complete explanation (Trautner et al., 1962; Witkin & Sicurella, 1964) it appears that the DNA can function even when Bu is incorporated into both strands. Thus Litman and Pardee (1956) have shown that 100% of the DNA-thymine of phage T2 can be replaced by

Bu and still yield infective phage. Similarly, Szybalski et al. (1960) have shown that 70% of the DNA-thymine can be replaced by Bu to yield DNA which has the same specific transforming activity as unsubstituted DNA.

Why then does growth of 15TAU in $Bu^{+}(AU)^{+}$ result in a loss of viability?

The experiments reported in this thesis show quite clearly that the kinetics of death can be profoundly affected by incubation in thymine or Bu under conditions where no DNA synthesis is expected to occur. From what is known of the enzymic reactions which are possible with DNA, it is difficult to see how this can be explained in terms of exchange of Bu for thymine in the intact chromosome. Although it cannot be ruled out that a small amount of exchange in the DNA may occur which is of biological significance, it will be assumed for the purposes of this Discussion that such an exchange does not occur, and that exchange in a non-chromosomal substance is what is actually involved here. Can such a hypothesis explain the loss of viability of 15TAU in $Bu^{+}(AU)^{+}$?

In order to simplify the Discussion of this problem it will also be assumed that "bromouracil death" and thymineless death are aspects of the same phenomenon. The only evidence for this is that Bu is an analogue of thymine and that the kinetics of the two deaths are very similar.

There is good evidence (Maaløe and Hanawalt, 1961) to suggest that thymineless bacteria are only susceptible to thymineless death if a round of DNA replication has been initiated. This could explain why cells of

15TAU do not die in $T^-(AU)^-$ after immunisation in $T^+(AU)^-$ or $Bu^+(AU)^-$. What is not possible to explain on this basis alone is why the kinetics of death should differ in $T^-(AU)^+$ after immunisation in these two media; why death occurs in $Bu^+(AU)^+$; and why the curing phenomena are observed. To explain these observations the following alternative hypotheses are put forward:

Hypothesis 1. A cytoplasmic molecule is postulated to be able to incorporate Bu, thymine or uracil with affinities such that T > U > Bu. Death is assumed to occur when the molecule contains uracil, but not when it contains thymine or Bu, but Bu can be displaced by uracil in the absence of thymine. Under these circumstances no death would occur in the presence of thymine $\left[T^+(AU)^+, T^+(AU)^-\right]$ or in the presence of Bu when uracil is unavailable e.g. in $Bu^+(AU)^-$ where any available uracil is used for DNA synthesis. Death would occur however in the presence of Bu when uracil was available $\left[Bu^+(AU)^+\right]$, and also when thymine is absent and uracil available $\left[T^-(AU)^+, T^-(AU)^-\right]$. The premature death in $T^-(AU)^+$ after immunisation in $Bu^+(AU)^-$ would be due to the greater ease of exchange between uracil and Bu after removal of the exogenous Bu. This hypothesis would explain the curing phenomena.

Hypothesis 2. The cytoplasmic molecule can contain Bu or thymine and only the molecule containing thymine can prevent death. This would explain the fact that death occurs in the absence of thymine $\left[T^-(AU)^-, T^-(AU)^+\right]$ and in the presence of Bu $\left[Bu^+(AU)^+\right]$. It would also

explain why death occurs more quickly after immunising in $Bu^+(AU)^-$ than in $T^+(AU)^-$ since the cytoplasmic molecule now contains Bu rather than thymine. The curing phenomena are also explained in the same way. To explain the absence of death in $Bu^+(AU)^-$ the additional assumption would have to be made that messenger RNA synthesis was a necessary step for death to occur (Hanawalt, 1963), and that this was prevented under these conditions by DNA synthesis using all the available uracil.

Both these hypotheses appear to be consistent with the available facts, and at present there is no means of choosing between them. It is still very much an open question whether this cytoplasmic substance containing thymine is a repressor of some lethal factor, but it is clear that when it contains Bu it does not affect the normal initiation of DNA synthesis.

It is also compatible with the facts that the cytoplasmic factor reverses damage produced in the DNA (in the same way, perhaps, that the reactivating enzyme restores UV-damage (Setlow, Swenson & Carrier, 1963; Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964). The absence of thymine or the presence of Bu would then inhibit its activity.

This work has not solved the problem of thymineless death, but it has pointed to some new experimental approaches which may provide useful clues: it is clearly of importance to investigate further the nature of the DNA synthesis which occurs during thymineless death. If this DNA is thymine-deficient then this should be detectable by measuring the

specific activities of pyrimidine oligonucleotides derived from DNA which has been radioactively labelled during thymine starvation. It is also of importance to investigate with isotopically-labelled precursors whether DNA synthesis is really complete after 90 minutes in $Bu^{+}(AU)^{-}$ or $T^{+}(AU)^{-}$, and whether any exchange between Bu and thymine can occur in the intact chromosome. Lastly, if there is indeed a cytoplasmic factor which contains thymine, then incubating 15TAU in tritiated-$T^{+}(AU)^{-}$ after immunisation should specifically label this factor and allow its isolation and characterisation by chromatography or density centrifugation.

REFERENCES

References

Adams, M. (1950). Meth. Med. Res., 2, 1.

Adams, M. & Wasserman, F. (1956). Virology, 2, 96.

Barner, H. & Cohen, S. (1954a). J. Bacteriol., 68, 80.

Barner, H. & Cohen, S. (1954b). Proc. Nat. Acad. Sci., Wash., 40, 885.

Barner, H. & Cohen, S. (1955). Fed. Proc., 14, 177.

Barner, H. & Cohen, S. (1956a). J. Bacteriol., 71, 149.

Barner, H. & Cohen, S. (1956b). J. Bacteriol., 71, 588.

Barner, H. & Cohen, S. (1956c). J. Bacteriol., 72, 115.

Barner, H. & Cohen, S. (1957). J. Bacteriol., 74, 350.

Barner, H. & Cohen, S. (1958) with an addendum by Kanazir, D. Biochim. Biophys. Acta, 30, 12.

Barner, H. & Cohen, S. (1959). J. Biol. Chem., 234, 2987.

Beck, W., Hook, S. & Hurlock Barnett, B. (1962). Biochim. Biophys. Acta, 55, 455.

Berrah, G. & Konetzka, W. (1962). J. Bacteriol., 83, 738.

Billen, D. (1959). Biochim. Biophys. Acta, 34, 110.

Billen, D. (1960a). Bac. Proc., 184.

Billen, D. (1960b). J. Bacteriol., 80, 86.

Bonhoeffer, F. & Gierer, A. (1963). J. Mol. Biol., 7, 534.

Boyce, R. & Howard-Flanders, P. (1964). Proc. Nat. Acad. Sci., Wash., 51, 293.

Boyce, R. & Setlow, R. (1962). Biochim. Biophys. Acta, 61, 618.

Brabander, W. & Romig, W. (1960). Bac. Proc., 187.

Brenner, S. & Smith, J. (1959). Virology, 8, 124.

Brock, T. & Brock, M. (1960). Bac. Proc., 178.

Brookes, P. & Lawley, P. (1962). J. Mol. Biol., 4, 216.

Burgi, E. & Hershey, A. (1963). Biophys. J., 3, 309.

Burton, K. (1956). Biochem. J., 62, 315.

Burton, K. & Petersen, G. (1960). Biochem. J., 75, 17.

Burton, K. (1962). Biochim. Biophys. Acta, 55, 412.

Cairns, J. (1962). J. Mol. Biol., 4, 407.

Cairns, J. (1963). J. Mol. Biol., 6, 208.

Chen, P., Toribara, J. & Warner, H. (1956). Analyt. Chem., 28, 1756.

Cohen, M., Shaw, M. & Craig, A. (1963). Proc. Nat. Acad. Sci., Wash., 50, 16.

Cohen, S. (1956), in 'Essays in Biochemistry'. Wiley, p. 77.

Cohen, S. (1957), in 'The Chemical Basis of Heredity', Ed. by McElroy & Glass. p. 651.

Cohen, S. & Arbogast, R. (1950). J. Expt. Med., 91, 619.

Cohen, S., Flaks, J., Barner, H., Loeb, M. & Lichtenstein, J. (1958). Proc. Nat. Acad. Sci.,Wash., 44, 1004.

Cohen, S., Green, M. & Barner, H. (1956). Biochim. Biophys. Acta, 22, 210.

Cohn, W. (1963), in Biochemical Preps. Ed. Brown, G., 10, 139.

Coughlin, C. & Adelberg, E. (1956). Nature, Lond., 178, 531.

Craven, G. & Downing, M. (1963). J. Biol. Chem., 238, 1464.

Crawford, L. (1958). Biochim. Biophys. Acta, 30, 359.

Crawford, L. (1959). Virology, 7, 359.

Davison, P. & Freifelder, D. (1962). J. Mol. Biol., 5, 643.

Deibel, R., Downing, M., Niven, C & Schweigert, B. (1956). J. Bacteriol., 71, 255.

Doty, P., Marmur, J., Eigner, J. & Schildkraut, C. (1960). Proc. Nat. Acad. Sci., Wash., 46, 461.

Doty, P., McGill, B. & Rice, S. (1958). Proc. Nat. Acad. Sci., Wash., 44, 432.

Dunn, D. & Smith, J. (1955a). Nature, Lond., 175, 336.

Dunn, D. & Smith, J. (1955b). Biochem. J., 60, xvii.

Dunn, D. & Smith, J. (1957). Biochem. J., 67, 494.

Dunn, D. & Smith, J. (1958). Biochem. J., 68, 627.

Dunn, D., Smith, J. & Spahr, P. (1960). J. Mol. Biol., 2, 113.

Eigner, J. (1960). Ph.D. Thesis, Harvard University.

Eigner, J., Schildkraut, C. & Doty, P. (1962). Biochim. Biophys. Acta, 55, 13.

Fleissner, E. & Borek, E. (1962). Proc. Nat. Acad. Sci., Wash., 48, 1199.

Folsome, C. (1963). Biochem. Biophys. Res. Comm., 11, 97.

Fredericq, P. (1957). Ann. Rev. Microbiol., 11, 7.

Freese, E. (1959a). J. Mol. Biol., 1, 87.

Freese, E. (1959b). Proc. Nat. Acad. Sci., Wash., 45, 622.

Fresco, J. & Alberts, B. (1960). Proc. Nat. Acad. Sci., Wash., 46, 311.

Friedkin, M., Crawford, E., Donovan, E. & Pastore, E. (1962). J. Biol. Chem., 237, 3811.

Fuerst, C. & Stent, G. (1957). J. Gen. Physiol., 40, 73.

Gallant, J. (1962). Biochim. Biophys. Acta, 61, 302.

Gallant, J. & Suskind, S. (1960). Bac. Proc., 178.

Gallant, J. & Suskind, S. (1961a). J. Bacteriol., 82, 187.

Gallant, J. & Suskind, S. (1961b). Bac. Proc., 181.

Gallant, J. & Suskind, S. (1962). Biochim. Biophys. Acta, 55, 627.

Ginoza, W. & Guild, W. (1961). Proc. Nat. Acad. Sci., Wash., 47, 633.

Gots, J. (1953). Nature, Lond., 172, 256.

Hanawalt, P. (1963). Nature, Lond., 198, 286.

Hanawalt, P., Maaløe, O., Cummings, D. & Schaechter, M. (1961). J. Mol. Biol., 3, 156.

Harold, F. & Ziporin (1958). Biochim. Biophys. Acta, 28, 482.

Hayes, W. (1964). The Genetics of Bacteria and their Viruses. p. 414. Blackwells, Oxford.

Hughes, W. (1956), in 6th Symp. Soc. Gen. Microbiol. on Bacterial Anatomy, p. 341.

Hurlbert, R., Schmitz, H., Brumm, A. & Potter, V. (1954). J. Biol. Chem., 209, 23.

Iyer, V. & Szybalski, W. (1963). Proc. Nat. Acad. Sci., Wash., 50, 355.

Jacob, F. & Campbell, A. (1959). Comptes rendues Acad. Sci., Paris, 248, 3219.

Jacob, F. & Monod, J. (1961). J. Mol. Biol., 3, 318.

Kanazir, D. (1954). Biochim. Biophys. Acta, 13, 589.

Kanazir, D., Barner, H., Flaks, J. & Cohen, S. (1959). Biochim. Biophys. Acta, 34, 341.

Kanazir, D. & Errera, M. (1954). Biochim. Biophys. Acta, 14, 62.

Kaplan, H. & Howsden, F. (1964). Proc. Nat. Acad. Sci., Wash., 51, 181.

Kay, E., Simmons, N. & Dounce, A. (1952). J. Am. Chem. Soc., 74, 1724.

Kellenberger, E. (1959), in Ninth Symp. Soc. Gen. Microbiol., p.11.

Korn, D. & Weissbach, A. (1962). Biochim. Biophys. Acta, 61, 775.

Lange, N. (1956). Ed. 'Handbook of Chemistry'. Handbook Publishers Inc., Sandusky, Ohio p. 1669.

Langer, L. & Klenow, H. (1960). Biochim. Biophys. Acta, 37, 33.

Lark, K. (1960). Biochim. Biophys. Acta, 45, 121.

Lark, K. (1961). Biochim. Biophys. Acta, 51, 107.

Lark, K. (1963). In Molecular Genetics, Ed. by Taylor, J., Acad. Press. p. 153.

Lark, K. & Pritchard, R. (1963). J. Cell Comp. Physiol., 19, 43A.

Lark. K., Repko, T. & Hofmann, E. (1963). Biochim. Biophys. Acta, 76, 9.

Lehman, I., Bessman, M., Simms, E. & Kornberg, A. (1958). J. Biol. Chem., 233, 171.

LePage, G. (1957). J. Biol. Chem., 226, 135.

Levine, M. & Berthwick, M. (1963). Virology, 21, 568.

Litman, R. & Pardee, A. (1960). Biochim. Biophys. Acta, 42, 117.

Litman, R. & Pardee, A. (1956). Nature, Lond., 178, 529.

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951). J. Biol. Chem., 193, 265.

Luzzatti, D. & Revel, C. (1962). Biochim. Biophys. Acta, 61, 305.

Mealóe, O. (1961). Cold Spring Harbor Symp. Quant Biol., 26, 45.

Mealóe, O. (1963). J. Cell Comp. Physiol., 62, Suppl. 1, 341.

Mealóe, O. & Hanawalt, P. (1961). J. Mol. Biol., 3, 144.

Mandelkern, L. & Flory, P. (1952). J. Chem. Phys., 20, 1392.

Markham, R. & Smith, J. (1949). Biochem. J., 45, 294.

Marmur, J. (1961). J. Mol. Biol., 3, 208.

Martin, J. & Doty, D. (1949). Anal. Chem., 21, 965.

Maruyama, Y. & Lark, K. (1961). Exptal. Cell. Res., 25, 161.

Matthews, C. & Cohen, S. (1963). J. Biol. Chem., 238, 367.

McFall, E. & Magasanik, B. (1960). Biochim. Biophys. Acta, 45, 610.

McFall, E. & Magasanik, B. (1962a). Biochim. Biophys. Acta, 55, 909.

McFall, E. & Magasanik, B. (1962b). Biochim. Biophys. Acta, 55, 920.

Melechen, N. & Skaar, P. (1962). Virology, 16, 21.

Mennigmann, H. & Szybalski, W. (1962a). Bac. Proc., 42.

Mennigmann, H. & Szybalski, W. (1962b). Biochem. Biophys. Res. Comm., 9, 398.

Michaelson, I. & Mandel, H. (1962). Biochem. Pharmacol., 11, 243.

Moore, S. & Stein, W. (1951). J. Biol. Chem., 192, 663.

Morris, N. & Fischer, G. (1960). Biochim. Biophys. Acta, 42, 183.

Munch-Petersen, A. (1960). Biochem. Biophys. Res. Comm., 3, 392.

Munch-Petersen, A. & Neuhard, J. (1964). Biochim. Biophys. Acta, 80, 542.

Nagata, T. (1963). Proc. Nat. Acad. Sci., Wash., 49, 551.

Nakada, D. (1960). Biochim. Biophys. Acta, 44, 241.

Nakada, D. (1962a). Nature, Lond., 195, 1303.

Nakada, D. (1962b). Biochim. Biophys. Acta, 55, 505.

Nielsen, S. & Lehninger, A. (1955). J. Biol. Chem., 215, 555.

O'Donnell, J., Mackal, R. & Evans, E. (1958). J. Biol. Chem, 233, 1523.

Ogur, M. & Rosen, G. (1950). Arch. Biochem., 25, 262.

Okada, T., Honma, J. & Sonohara, H. (1962). J. Bacteriol., 84, 602.

Okagaki, H., Tsubota, Y. & Sibatani, A. (1960). J. Bacteriol., 80, 762.

Okasaki, T., Strominger, J. & Okasaki, R. (1963). J. Bacteriol., 86, 118.

Overend, W. & Stacey, M. (1955), in Nucleic Acids Vol. I Ed. by Chargaff, E. & Davidson, J. Academic Press. p. 58.

Pardee, A. (1961). Symp. Soc. Gen. Microbiol., 11, 19.

Pastore, E. & Friedkin, M. (1962). J. Biol. Chem., 237, 3802.

Pauling, C. & Gallant, J. (1963). Genetics, 48, 905.

Petersen, G. (1963). Biochem. J., 87, 495.

Prussoff, W. (1959). Biochem. Pharmacol., 2, 221.

Reichard, P. (1960). Biochim. Biophys. Acta, 41, 368.

Reichard, P., Canellakis, Z. & Canellakis, E. (1961). J. Biol. Chem., 236, 2514.

Riley, W. (1962). D. Phil. Thesis. Univ. Oxford.

Roepke, R. & Mercer, F. (1947). J. Bacteriol., 54, 731.

Roberts, R. (1960). Ann. N.Y. Acad. Sci., 88, 752.

Rubinstein, I., Thomas, C. & Hershey, A. (1961). Proc. Nat. Acad. Sci., Wash., 47, 1113.

Rudner, R. (1961). Z.fur Vererbungs., 92, 361.

Rushizky, G. & Knight, C. (1960). Virology, 11, 236.

Schildkraut, C.,Marmur, J. & Doty, P. (1961). J. Mol. Biol., 3, 595.

Setlow, R. & Carrier, W. (1964). Proc. Nat. Acad. Sci., Wash., 51, 226.

Setlow, R., Swenson, P. & Carrier, W. (1963). Science, 142, 1464.

Shooter, K. & Butler, J. (1956). Trans. Far. Soc., 52, 734.

Sicard, N. (1961). Comptes Rendues Acad. Sci., Paris, 252, 617.

Sicard, N. & Devoret, R. (1962). Comptes Rendues Acad. Sci. Paris, 255, 1417.

Sinsheimer, R., Starman, B., Nagler, C. & Guthrie, S. (1962). J. Mol. Biol., 4, 142.

Smith, K. (1962). Biochem. Biophys. Res. Comm., 8, 157.

Stahl, F., Crasemann, I., Okun, L., Fox, F. & Laird, C. (1961). Virology, 13, 98.

Strelzoff, E. (1962a). Z. für Vererbungs., 93, 287.

Strelzoff, E. (1962b). Z. für Vererbungs, 93, 301.

Stuy, J. (1958). J. Bacteriol., 76, 668.

Sueoka, N. (1961). J. Mol. Biol., 3, 31.

Szybalski, W. Opara-Kubinska, Z., Loridiewicz, Z., Ephrati-Elizur, E. & Zamenhof, S. (1960). Nature, Lond., 188, 743.

Theil, E. & Zamenhof, S. (1962). Fed. Proc., 21, 373.

Thomas, C. & Berns, K. (1961a). J. Mol. Biol., 3, 277.

Thomas, C. & Berns, K. (1961b). J. Mol. Biol., 3, 289.

Thomas, C. & Pinkerton, T. (1962). J. Mol. Biol., 5, 356.

Tomasz, A. & Borek, E. (1962). Biochemistry, 1, 543.

Trautner, T., Swartz, M. & Kornberg, A. (1962). Proc. Nat. Acad. Sci., Wash., 48, 449.

Vinograd, J. & Hearst, J. (1962). Forschritte der Chemie Org. Naturstoffen, 20, 372.

Vogel, A. (1958), in 'Elementary Practical Organic Chemistry' p. 828. Longmans Green & Co., Lond.

Wacker, A., Dellweg, H. & Jacherts, D. (1962). J. Mol. Biol., 4, 410.

Wahba, A. & Friedkin, M. (1962). J. Biol. Chem., 237, 3794.

Wainwright, S. & Nevill, A. (1956). J. Bacteriol., 71, 254.

Weinberg, R. & Latham, A. (1956). J. Bacteriol., 72, 570.

Weygand, R., Wacker, A. & Dellweg, H. (1952). Z. Naturforsch., 7b, 19.

Yang, J. (1961). Adv. Prot. Chem., 16, 323.

Yoshikawa, H. & Suecka, N. (1963). Proc. Nat. Acad. Sci., Wash., 49, 559.

Zamenhof, S. (1959). Ann. N.Y. Acad. Sci., 81, 784.

Zamenhof, S. & Chargaff, E. (1951). Nature, Lond., 168, 609.

Zamenhof, S., De Giovanni, R. & Rich, K. (1956a). J. Bacteriol., 71, 60.

Zamenhof, S., Reiner, B., De Giovanni, R. & Rich, K. (1956b). J. Biol. Chem., 219, 165.

Zimm, B. & Crothers, D. (1962). Proc. Nat. Acad. Sci., Wash., 48, 905.