C H E M I C A L S T U D I E S O N

E S C H E R I C H I A C O L I D N A - D E P E N D E N T

R N A P O L Y M E R A S E

by

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To my parents.

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Abbreviations.

The abbreviations used are those recommended by the Biochemical Journal (Biochem. J. (1975) 145, 1 - 20). Other abbreviations are:

BSA	:	bovine serum albumin,
Buffers A,G,C:		defined in Chapter 3,
CD	:	circular dichroism,
DNA ase:		deoxyribonuclease,
DNP	:	dinitrophenyl,
DTNB	:	5, 5'- dithiobis(2-nitrobenzoic acid),
DTT	:	dithiothreitol,
E. coli:		*Escherichia coli*,
NBS	:	N- bromosuccinimide,
NTP	:	nucleoside triphosphate,
PPO	:	2,5- diphenyloxazole,
RNA ase:		ribonuclease,
SDS	:	sodium dodecyl sulphate,
TNB-	:	Thionitrobenzoyl- .

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SUMMARY

The interaction of E. coli DNA dependent RNA polymerase with nucleic acids was studied by chemical modification of both the enzyme and DNA. Chemical modification requires large quantities of a pure homogeneous preparation of a protein. Core enzyme was purified from up to 1 kg of E. coli, in yields of about 10 mg per 100 g cells, and to 95 % purity. Core-enzyme was used because it is the simplest active form, with most of the properties, of the complete enzyme.

Work on the binding of synthetic peptides and proteins to nucleic acids suggests that tryptophan and tyrosine form stacking interactions with nucleic acid bases, and that lysine interacts ionically. N-bromosuccinimide was used to modify the tryptophan and tyrosine groups of RNA polymerase. A 30 fold molar excess of reagent rapidly and completely inactivated the enzyme. The modification resulted in oxidation of 1 - 2 tryptophans and 8 ($\pm$2) cysteines, loss of 9 % of the protein fluorescence, and no loss of overall structure (as judged by the accessibility of thiols to reaction with 5, 5′- dithiobis(2-nitrobenzoic acid). Substrates did not protect against inactivation. The oxidation of 8 thiols, which accounted for up to 40 % of the inactivation, was limited to 4 thiols and 10 % inactivation by reversibly protecting the surface SH groups with 5, 5′-dithiobis(2-nitrobenzoic acid). Inactivation of protected enzyme required a 30 fold molar excess of N-bromosuccinimide, and led to a loss of 11 % of the protein fluorescence, and no change ($<2\%$) in the overall conformation (as judged by far ultraviolet circular dichroism). Amino acid analysis showed that between 3 and 6 tyrosines, and no methionines ($\pm$2) or histidines ($\pm$5) were oxidised. The error in determining tryptophan ($\pm$2) was too large to detect the oxidation previously observed by extinction changes.

Stacking of aromatic amino acids with nucleic acid bases would contribute to the binding energy to DNA and nucleotides, and would help unwind the double helix. N-bromosuccinimide inactivation of thiol protected RNA polymerase resulted in no decrease in DNA binding activity (as judged by salt elution from DNA agarose), and no loss in ATP binding. No difference in inactivation was observed when assayed with native and denatured DNA, and inactivation was also independent of the initiation circumventing dinucleotide G-A, suggesting that the major cause of inactivation was not loss of unwinding or initiating activity. Further work is required to resolve the cause of inactivation.

Methyl- [^{14}C] acetimidate modification of 50 lysines resulted in complete inactivation of core enzyme. Both purine nucleoside triphosphates and denatured DNA protected against inactivation. ATP, GTP, CTP, and denatured DNA together resulted in maximal protection against inactivation, and protection of about 8 lysines against modification. Since amidination should not disrupt ionic interactions greatly, lysine may have another role in RNA polymerase. Protection against inactivation was used to obtain a dissociation constant of 80 $\pm$ 20 μM for denatured DNA from core enzyme.

The kinetics of formaldehyde melting of DNA was studied as a probe of protein-DNA interactions. The kinetic analysis of Trifonov et al. (1968) did not adequately describe the unwinding of the small, double-helical, DNA from phage T7, despite taking precautions in the experimental techniques. Calf thymus DNA, and calf thymus DNA with nucleoside triphosphates and low concentrations of holoenzyme, gave interpretable kinetics. However the shortcomings of calf thymus DNA as a template, and the difficulties with explaining the kinetics with larger concentrations of enzyme, and with T7 DNA, detract from the method as a probe of RNA polymerase - DNA interactions.

CHAPTER 1

INTRODUCTION.

1.1 GENERAL CONSIDERATIONS.

The accurate inheritance of the genetic message, and its correct interpretation, results from recognition, on the one hand between nucleic acids, and on the other between nucleic acids and proteins. Although it is twenty-two years since it was realised that accurate recognition between nucleic acids results from pairing between its monomer units (Watson & Crick, 1953), no such principles have yet emerged for the recognition between proteins and nucleic acids. This is no doubt due to the great range of such interactions, at all levels of their organisation, and with varying degrees of specificity.

Nucleic acid structure can be classified according to its level of organisation, in a manner analogous to that used for proteins (Mahler & Cordes, 1966). The primary structure is defined as its sequence, and the secondary structure as the Watson-Crick helices. The Watson-Crick helices are a repetitive packing of monomer units, and are in turn a packing unit for the three dimensional structure. The tertiary structure would then be the three dimensional organisation of secondary structure, e.g. the folding of the arms of t-RNA (Kim et al., 1974; Robertus et al., 1974), the supercoiling resulting from a fixed unwinding of the Watson-Crick helices (which may result in a repetitive or more complex structure), and the "nucleosome" of the chromosome (Oudet et al., 1975). A mechanism by which the Watson-Crick helix may be folded has been described by Crick & Klug (1975). A higher structural order (quaternary structure) results from a packing of tertiary structure units, as for example the two RNA subunits of the ribosome, and the packing of nucleosomes in the chromosome. A zero order structure can be defined as the monomer unit, the nucleotide.

Interactions between nucleic acids and proteins can be distinguished at all levels of their organisation, from interactions between their

monomer units, to interactions between their quaternary structures. The primary and secondary structures of nucleic acids may have directed the primary and secondary structures of proteins in evolution (Black & Orgel, 1975; Carter & Kraut, 1974), whilst in nucleosomes and ribosomes proteins and nucleic acids interact to generate their tertiary structure. Proteins can recognise all levels of organisation of a nucleic acid. Glutamate dehydrogenase and DNA polymerase recognise nucleotides, whereas lac repressor and H_1 restriction endonuclease can recognise a specific sequence. RNA ase III, pancreatic RNA ase and T4 gene 32 protein can recognise the presence of secondary structure (i.e. helix or coil forms). Amino-acyl tRNA synthetases recognise the common tertiary structure which appears to be present in all tRNAs (Kim et al., 1974), although discrimination between tRNAs is a poorly understood process. DNA-dependent RNA polymerase recognises all levels of organisation of a nucleic acid. This enzyme binds nucleotides, locates sequences to start and stop RNA chain growth, catalyses the helix-coil transition, recognises prominent structural features of DNA in locating promoter regions, and its activity is controlled by the tertiary structure of supercoiled DNA and quaternary structure of the chromosome.

Various degrees of specificity can be distinguished in protein-nucleic acid interactions. At two extremes are T4 gene 32 protein and eucaryotic sperm cell protamines which are sequence non-specific, and repressor molecules which are sequence specific. RNA polymerase shows little specificity in its DNA binding activity, but probably requires a specific sequence for the correct initiation and termination of an RNA chain (sections 1.2.2.1, 1.2.2.4). Sequence independent interactions involve mainly ionic forces, whilst the molecular basis of the accurate recognition of nucleotide sequence is one of the major puzzles of molecular biology. Aromatic amino acids may have an

important role in this recognition (section 1.3). In this study the nature of the interactions between nucleic acids and E. coli DNA-dependent RNA polymerase has been investigated by chemical modification (section 1.4) of both the protein and the DNA.

1.2 E. COLI DNA-DEPENDENT RNA POLYMERASE.

1.2.1 Introduction.

Transcription of DNA in bacteria is catalysed by DNA-dependent RNA polymerases (systematic name = nucleoside triphosphate: RNA nucleotidyl transferase (DNA dependent); E.C. 2.7.7.6). They are large molecules (molecular weights from 400,000 - 500,000), and have a complex subunit structure. Two forms have been identified in pure preparations, holoenzyme and core enzyme. Core enzyme lacks a single subunit, σ, which is required for correct initiation of RNA formation.

The normal reaction is: pppPu + npppX $\xrightarrow{DNA + Mg^{++}}$ pppPu(pX)n + nppi, where Pu = purine, pppX = nucleoside triphosphate, and ppi = inorganic pyrophosphate. With an intact double helical DNA and holoenzyme, the enzyme binds to the DNA and eventually locates an initiation site. Initiation of RNA formation occurs by coupling ATP or GTP to a second nucleoside triphosphate, with the elimination of inorganic pyrophosphate. Elongation occurs by the addition of nucleoside monophosphates, from the nucleoside triphosphate substrates, to the 3′ OH end of the growing RNA chain. Eventually chain growth terminates, and both enzyme and RNA are released from the template. The enzyme initiates and terminates at specific sites, and accurately copies the DNA template. Transcription in the cell is carefully controlled, both by interactions of genetic sequences, metabolites, and accessory factors with RNA polymerase, and by the interaction of accessory factors with DNA.

A number of reviews of bacterial RNA polymerases are available: Chamberlin (1974, a,b), Travers (1974 a, b, c; 1971), Bautz (1973, 1972), von Hippel & McGee (1972), Losick (1972), Reznikoff (1972), Burgess (1971), Sagar-Sethi (1971). Some of the more recent developments, and properties relevant to this thesis will be discussed.

1.2.2 Catalytic properties.

1.2.2.1 DNA Binding.

At low ionic strength RNA polymerase has a sequence independent affinity for DNA. The number of molecules which can bind is limited only by the size of the enzyme. Increasing the ionic strength lowers the binding affinity (Pettijohn & Kamiya, 1969). With an intact, double helical, linear DNA, and at low ionic strength, two types of binding site are present, the "A" and "B" sites (Hinkle & Chamberlin, 1972a). There is a large number of B sites (1300 on T7 DNA) and dissociation is very rapid ($t_{\frac{1}{2}} \sim 1$ s). There are fewer A sites (8 on T7 DNA) and the complexes are very stable ($t_{\frac{1}{2}} = 30 - 60$ h). Location of A sites by holoenzyme is slow ($t_{\frac{1}{2}} \sim 22$ s), and is independent of DNA concentration (Hinkle & Chamberlin, 1972, b). Since the overall reaction in a simple bimolecular process should be second order, it appears that the first order release of enzyme from B sites is rate limiting, and that the rate of A site location is governed by the relative sizes of the A and B regions. The A sites occur in the promoter regions of the DNA, and the 8 sites on T7 DNA appear to be in a storage region for enzyme molecules able to initiate rapidly at the 3 start points for transcription (Chamberlin & Ring, 1972; Dunn & Studier, 1973; Schäfer et al., 1973).

DNA in the tight complexes at A sites is nuclease resistant, and this has been used to isolate the holoenzyme binding sites. The DNAase resistant fragment is 40 base pairs long (Le Talaer et al., 1973), but regions as short as 14 base pairs have been isolated (Giacomoni et al., 1974). Recently improved techniques for DNA sequencing have allowed the polymerase binding sites to be sequenced, and sequences are known for fd DNA (Schaller et al., 1975; Heyden et al., 1975), T7 A3 promoter (Pribnow, 1975), SV 40 (Dhar et al., 1974), lambda P_L (Maniatis et al., 1974), tyr tRNA promoter (Sekiya & Khorana, 1974; Sekiya et al., 1975),

and lac DNA (Dickson et al.,1975).

The sequences of the protected fragments are different, and this is also true for the T4 and T7 binding sites, which do not cross hybridise (Niyogi & Underwood, 1975). The start point for RNA synthesis is in the middle of the 40 base pair protected region, and 5 base pairs to the left of it there is the following common 7 base-pair sequence:

5′ T - A - T - Pu - A - T - G 3′

3′ A - T - A - Py - T - A - C 5′

(Pribnow, 1975; Heyden et al., 1975). The promoter region (the region controlling initiation of RNA synthesis) seems to extend beyond the protected region because the fragments will not reform complexes able to start mRNA synthesis correctly. Fragments extending 30 base pairs to the left of the initiation point cannot reform tight complexes (Maurer et al., 1974) whereas those extending 90 base pairs to the left can (Zain et al., 1974). Since promoter mutations have been located 35 base pairs to the left of the initiation point (Gottesman & Weisberg et al., 1971; Hopkins, 1974) it seems likely that holoenzyme initially recognises some feature of the DNA at least 35 base pairs to the left of the start point. A clue to the type of structure which might be initially recognised by holoenzyme comes from the observation of Dhar et al. (1974) that RNA polymerase protects against Hin endonuclease cleavage. Since this area also contains the "entry" site for holoenzyme, the initial recognition may involve the palindromic sequence recognised by Hin endonuclease.

The region of the promoter between the entry and start site may have a storage function. The T7 promoter region can store 8 holoenzyme molecules in a form able to initiate RNA chain formation rapidly (Schäfer et al., 1973). There are 150 base pairs between the first and second, and 80 base pairs between the second and third initiation sites(Darlix & Dausse, 1975), enough space for 7 closely packed polymerase molecules. Holoenzyme, at the promoter site, can exist in

two conformational states, the transition between them involves the local opening of 4 - 8 base pairs of the DNA (Saucier & Wang, 1972 ; Mangel & Chamberlin, 1974a,b). This opening region may be related to the 7 base pair A-T rich common sequence in the nuclease resistant fragments, since opening is more sensitive to glycerol denaturation than expected from the overall GC content of the DNA, indicating it is an A-T rich region (Travers, 1974 a), and the UV 5 lac mutation, which enhances opening, alters the sequence to match the 7 base pair region (Pribnow, 1975). However Dickson et al., (1975) have found that the entry region is A-T rich, and they suggest that this is the opening site.

In summary holoenzyme binding to DNA involves the following steps:

1) random binding of holoenzyme to DNA until it encounters a promoter site
2) recognition of a promoter entry site.
3) transition from entry to initiation site without dissociation from the DNA
4) opening of the DNA helix in preparation for RNA chain initiation and elongation.

Many aspects of this model need to be clarified. It would be interesting to obtain more sequence information so that the exact relationship of storage, entry, and initiation sites may be defined, and to see if there are common structural features which may provide a recognition site for holoenzyme.

Soon after initiation holoenzyme loses the σ subunit, and the remaining core enzyme continues to elongate the RNA chain (Krakow & von de Helm, 1970). σ factor resolves the two conflicting requirements of RNA polymerase of having an initial selectivity for part of the DNA, but once transcription has started, having an overall affinity for the DNA (Yarus, 1969). Holoenzyme binds more weakly than core enzyme to the B sites of T7 DNA (the dissociation half times are 1 s and 60 min

respectively), and this facilitates greatly its search for promoter sites (Hinkle & Chamberlin, 1972 a). Core enzyme initiates preferentially from nicks on linear helical templates (Vogt, 1969), whereas holoenzyme binds tightly but will not initiate from them (Boule-Charest & Memet-Bratley, 1972; Hinkle & Chamberlin, 1972 b). This suggests that holoenzyme has a more rigid specificity for initiation sequences, and that core enzyme is unable to catalyse promoter opening. However, core enzyme retains some ability to open DNA because it opens intact poly d(AT) (Hinkle & Chamberlin, 1970), and asymmetrically transcribes supercoiled DNA (Domingo et al., 1975).

1.2.2.2 Initiation.

Initiation involves the formation of the first phosphodiester bond between two nucleoside triphosphates. This step is different from subsequent steps in that the acceptor which bears the 3′ OH group is a nucleoside triphosphate, rather than a polynucleotide. In vivo studies show that RNA chains are initiated preferentially with a purine nucleotide (Maitra & Hurwitz, 1965; Bremer et al., 1965). Studies with synthetic copolymers, such as poly d(AT), show that initiation occurs almost exclusively with ATP or GTP at the 5′ end (Maitra et al., 1967; Kleppe & Khorana, 1972; Teras et al., 1972). Transcription of template strands containing exclusively purines is greatly reduced (Straat & Ts'o, 1969). These results suggest that RNA polymerase has two sites for binding nucleoside triphosphates, the first (the initiation site) binds purine nucleotides specifically, whilst the second site binds all four nucleoside triphosphates. Wu & Goldthwait (1969 a,b) have obtained direct evidence for a distinct purine nucleotide binding site on the enzyme. They showed that ATP and GTP, but not CTP and UTP, quench the protein fluorescence. ATP and GTP binding at this site is inhibited by rifamycin, a specific inhibitor of initiation (section 1.2.3.3).

The initiation site appears to have a rigid specificity for nucleotides. Purine analogues with minor modifications in the 5 membered ring, away from the portion involved in normal Ĥ bonding, have been found to decrease greatly the rate of chain initiation (Nishimura et al., 1966; Darlix et al., 1971; Ward & Reich, 1972). This rigid specificity is not displayed by the second site, which accepts a greater variety of analogues in the elongation reaction (Chamberlin, 1974 a; Darlix & Fromageot, 1974; Steitz & Scheit, 1975).

1.2.2.3 Elongation.

After formation of the first phosphodiester bond the dinucleotide moves to the product site (which may be related to the initiation site), and subsequent nucleoside triphosphates can bind to allow elongation to continue. σ factor is released during elongation (Gerard et al., 1972; Krakow & von de Helm, 1970). The product site can bind 12 nucleotides of an RNA chain and its position relative to the DNA binding site may help helix reformation (Kumar & Krakow, 1975).

The ternary complex of protein-DNA-RNA is stable to high ionic strength (Naito & Ishihama, 1973). The enzyme has increased resistance to inhibitors (Zillig et al., 1970b), proteolysis (Lowe, 1974), inactivation by thiol reagents (Nicholson & King, 1973), and heat denaturation (Steade & Jones, 1967). DNA base pairs in the complex are "open" (Saucier & Wang, 1972; Malcolm et al., 1975), with a CD spectrum characteristic of the A rather than the B form (Beabealashvilly et al., 1972), and are sensitive to reaction with formaldehyde (Kosaganov et al., 1971). The DNA-RNA interactions are transient, and can be disrupted with protein denaturants (Bremer & Konrad, 1964). The elongation complex can accurately select the correct nucleotide from the normal substrates. The error frequency is less than 1 in 1700, or an average error of 0·6 nucleotides in an E. coli mRNA (Bass &

Polonsky, 1974). Under optimum conditions, at 37°C, chain growth is 20 - 50 nucleotides per second. (Richardson, 1970). The rate varies with base sequence (Darlix & Fromageot, 1972), and the slow sequences may be related to chain termination.

1.2.2.4 Termination.

Correct termination involves three distinct events, recognition of a termination sequence, release of RNA, and release of enzyme. Termination seems to involve both specific sequences and termination factors. Termination is sequence specific, factor independent, on phage fd DNA (Takanami et al., 1971), T7 DNA (Schweiger et al., 1971), T5 DNA (Schafer & Zillig, 1973b), λ DNA (Rosenberg et al., 1975), and E. coli mRNA DNA (Pettijohn et al., 1971). Specific sequences have been found at 3′ end of a number of in vitro synthesised RNA's, and they may form part of the recognition site for termination. The sequence $Pu(U)_6Pu$ has been found at the 3′ end of λ 6S RNA (Lebowitz et al., 1971; Larsen et al., 1970), λ 4S RNA (Blattner & Dahlberg, 1972; Dahlberg & Blattner, 1973), an E. coli RNA (Rosenberg et al., 1975), and a B. subtilis RNA (Rosenberg et al., 1975). Similarly phi 80 phage RNA terminates in $G(U)_6AA$ (Pieczenik et al., 1972). However T7 early mRNA appears to terminate with CCC (Peters & Haywood, 1974 a, b).

A number of termination factors have been isolated (Schäfer & Zillig, 1973 a, c; Yang & Zubay, 1974), of which ρ factor has been most extensively studied (Roberts, 1969). ρ causes preferential termination at a limited number of sites. With λ DNA it increases the proportion of 4S to 6S RNA synthesised, and alters the position of termination by increasing termination at $Pu(U)_6AU$ as opposed to $Pu(U)_6A$ (Rosenberg et al., 1975). With T7 DNA ρ stimulates termination at the ends of gene 0.3, 0.7, and in the middle of gene 1, and together with RNA ase III, generates the correct molar ratios of the early mRNA's (Darlix, 1974 a).

Several mechanisms have been proposed to account for the activity of ρ factor. It might block elongation by binding to DNA at specific sites. Oda & Takanami (1972) found by electron microscopy that ρ assumes a hexagonal structure, and forms rings around the helix at specific sites on RF fd DNA. A similar ring structure has been found with the lac repressor (Ohshima et al., 1975). Alternatively ρ may bind to RNA polymerase when it is in a specific conformation at "hesitation" sites on DNA (Darlix & Horaist, 1975). Hesitation sites are either $(dA)_n.(dT)_n$ or $(dG)_n.(dC)_n$ rich, and are preceeded by the specific sequence GCCUUUAU and followed by GAU (Rosenberg et al., 1974). Since the RNA transcribed from hesitation regions is cleaved by double-strand specific RNA ase III, the RNA sequence must have two fold symmetry (Darlix, 1975; Darlix, 1974 a, b). ρ factor alone cannot account for specific termination because it does not lead to release of RNA polymerase from DNA (Goldberg & Hurwitz, 1972).

An analogy has been drawn between initiation and termination since they both occur at specific sites, they both use catalytic factors which bind to the enzyme, and they both have sites on the enzyme with a more rigid specificity for nucleotides (and analogues) than the elongation site (Darlix, 1974 b).

1.2.2.5 Other catalysed reactions.

Besides the normal template directed formation of a complementary RNA chain, RNA polymerase can form, both in the presence and absence of templates, polymers with repeated sequences. Template directed homopolymer formation occurs when RNA polymerase is incubated with DNA or RNA, and one or two of the nucleoside triphosphates (Nishimura et al., 1964; Chamberlin & Berg, 1964). The reaction results from repeated copying of a short sequence of the template, and is completely supressed when a substrate complementary to the template base distal to the

reiterative sequence is included in the reaction. Synthesis of poly A from TTTTTC is supressed when GTP is included, even at micromolar concentrations (Chamberlin & Berg, 1964).

In the absence of template poly(rA). poly(rU) and poly(rIC) are formed when ATP and GTP (Mehrota & Khorana, 1965; Smith et al., 1967) and ITP and CTP (Krakow & Karstadt, 1967 ; Krakow & von de Helm, 1970) respectively are incubated with RNA polymerase. This is an intrinsic property of the enzyme, and does not result from contamination with poly A polymerase (August et al., 1962) or polynucleotide phosphorylase (Kimhi & Littauer, 1968), which are often present in impure preparations of the enzyme. Unprimed synthesis requires Mn^{++} and high concentrations of enzyme, and shows a lag of about 30 min (Smith et al., 1967).

1.2.2.6 Control of RNA polymerase activity.

Two types of transcriptional control can be distinguished, the reversible controls associated with rapid adaptation to environmental changes, and the irreversible controls accompanying programmed development (Gros, 1974). There are both fine and coarse reversible controls. Fine controls are exerted by regulatory proteins such as repressors and activators, which control the availability of the promoter to RNA polymerase (Lewin, 1974). This control occurs by either an affect on the open and closed states of the promoter (e.g. catabolite activator protein + cAMP, Nakanishi, 1974), or by physical exclusion of RNA polymerase (λ repressor, Chadwick et al., 1970; trp repressor, Squires et al., 1975).

Coarse control is manifest as an overall control of the transcriptional capacity of the cell, and may be mediated by two distinct conformational states of holoenzyme, the equilibrium between them being controlled by regulatory factors such as ppGpp (Travers, 1974). Two minor species of holoenzyme have been isolated from E. coli (Fukuda et al., 1974;

Wickner & Kornberg, 1974; Iwakura et al., 1974), and various catalytic heterogeneities have been observed (Chao & Speyer, 1973; Travers & Buckland, 1973; Chelala et al., 1971), but these have not been correlated with known in vivo properties of the enzyme.

Control during programmed development is associated with structural changes in the transcriptional apparatus, including replacement of σ factor with other proteins, modification of the core enzyme structure, and complete replacement with a phage specified RNA polymerase. Phage infection often leads to the production of new polypeptides which alter the properties of the host RNA polymerase (Duffy & Geiduschek, 1975; Spiegelman & Whiteley, 1974). During sporulation in B. subtilis a polypeptide is produced which inhibits the association between core enzyme and σ (Tjian & Losick, 1974), whilst the core structure is not altered (Rexer et al., 1975; Kerjan & Szulmaster, 1974). T4 infection of E. coli results in a modification of core enzyme (Zillig et al., 1974; Goff, 1974). An entirely different RNA polymerase molecule is synthesised during infection with T7 (Golomb & Chamberlin, 1974; Niles, 1974), T3 (Chakraborty et al., 1973) P4 (Barrett et al., 1972) and PBS 2 (Clark et al., 1974). These RNA polymerase molecules are smaller than the host polymerase, probably reflecting the smaller number of controls exerted on their function. Studies of these polymerases may provide information on the molecular properties of the host enzyme which allow such multifarious controls to be exerted on it.

1.2.3 Molecular Properties.

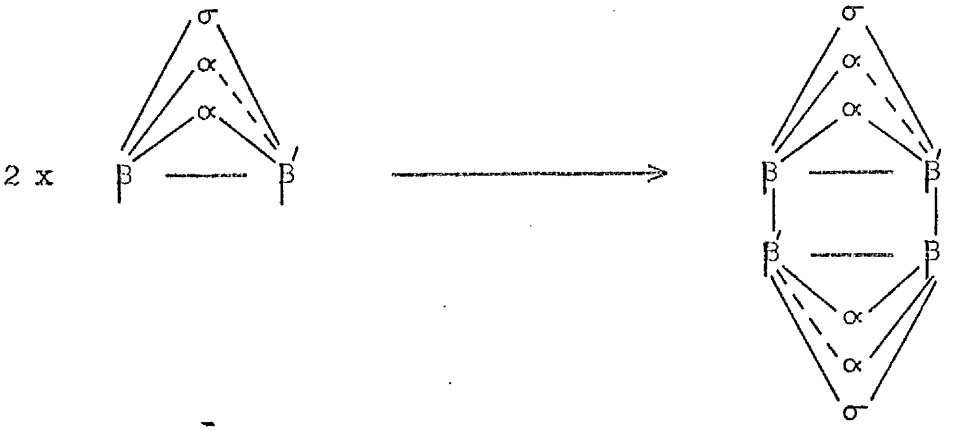
1.2.3.1 Structural properties.

The protomeric active unit of E. coli RNA polymerase consists of 5 subunits, β', β, 2 x α, σ, with molecular weights of about 165,000, 155,000, 2 x 40,000, and 95,000 (Chamberlin, 1974b; section 3.4.4). Another small subunit, ω (about 10,000), is occasionally found

associated with the purified enzyme, but it is thought to be a tight binding impurity because it is removed by high salt gel-filtration and does not affect the known activities of the enzyme (Burgess & Travers, 1971). The σ subunit is catalytic (Travers, 1975) and can readily be dissociated from holoenzyme to give core-enzyme (Burgess et al., 1969). β', β, and α are distinct polypeptides, as shown by peptide mapping (Schachner & Zillig, 1971), amino acid composition, and N terminal sequence (Fujiki & Zurek, 1975). They are tightly bound to each other, and are all essential for the reconstitution of active enzyme (Ishihama, 1972; Yarbrough, 1973). The enzyme has a strong tendency to aggregate at low salt concentrations. Below an ionic strength of 0·1M holoenzyme aggregates to a dimer, whilst below 0·27M core enzyme forms a hexamer (Berg & Chamberlin, 1970).

The overall shape of the molecule has been studied by small angle X-ray scattering (Pilz et al., 1972) and electron microscopy (Kitano & Kameyama, 1969; Lubin, 1969, Davis & Hyman, 1970). X-ray scattering shows that core enzyme has an elongated shape, with an axial ratio of 1 : 2. σ has little effect on the overall structure. The shapes observed by electron microscopy are highly variable and are much smaller than those observed by X-ray scattering. This may result from the staining procedure and the "loosely knit" structure of the protein.

The subunit arrangement has been studied by chemical cross-linking (Lowe, 1974) and proteolysis (Lowe, 1974; King et al., 1974b; Lill & Hartman, 1975). Cross-linking experiments are complicated by the large and similar molecular weights of β and β'. Proteolysis with a variety of proteases, under various conditions, gives information on the relative exposure of the subunits. Combining these data with the subassemblies which have been identified, the following tentative arrangement can be deduced:

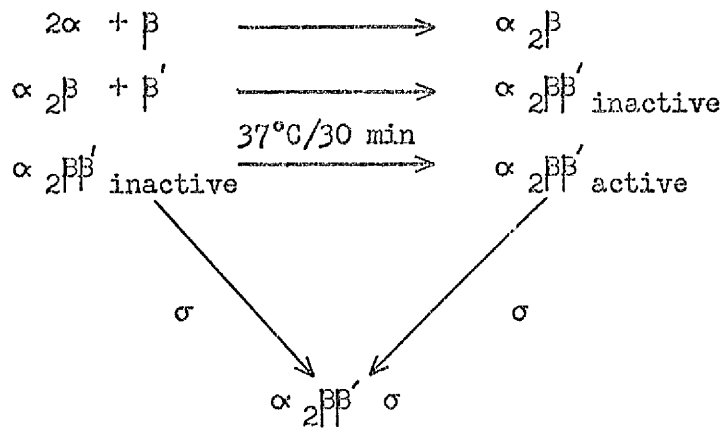


Discontinuous lines mean uncertain contacts.

Features of the model:

α — β — α	:	$\alpha_2\beta$ subassembly (Fukuda & Ishihama, 1974).
α — β, α — β′	:	crosslinking (Lowe, 1974); subassembly (Burgess, 1971).
σ — β	:	subassembly (Sagar-Sethi, 1971).
σ — β′	:	subassembly (Stetter & Zillig, 1974).
β — σ — β′	:	equal protection of β & β′ by σ against proteolysis (Lill & Hartman, 1975).
β — β′	:	exposure of α to proteolysis (Lill & Hartman, 1975) and relative sizes of α and β, β′.
β — β′ β′ — β	:	symmetry of dimer formation, and protection against proteolysis (Lill & Hartman, 1975).
α — σ	:	unknown
α — α	:	unknown
α — β′ — α	:	unknown

The subunits can be isolated and used to reconstitute active enzyme (Ishihama & Ito, 1972; Ito & Ishihama, 1973; Ishihama et al., 1973; Fukuda & Ishihama, 1974; Harding & Beychok, 1974; Yarbrough & Hurwitz, 1974; Palm et al., 1974). Reassociation occurs in the following sequence:



The inactive complex of $\alpha_2\beta\beta'$ has many of the structural features of the original enzyme, but has a reduced sedimentation coefficient and Stokes radius. It undergoes a temperature sensitive conformational change to active enzyme (Harding & Beychok, 1974). Intergenic hybrids have been obtained between Micrococcus luteus and E. coli core enzymes. The hybrids are active despite differences in size (Micrococcus luteus β' = 150,000, β = 145,000, α = 44,000) and charge, and indicate that each subunit has distinct functions which are closely similar in the two bacteria (Lill et al., 1975). A study of subunit assemblies, and reconstitution of modified subunits, should prove useful in elucidating the structure and activities of the subunits.

1.2.3.2 Chemical and structural modification.

Studies of modified RNA polymerase have yielded information on the functions of the amino acids and subunits of the enzyme. Modified enzyme has been obtained by purification from phage infected and mutant E. coli, by isolation of enzyme subassemblies, and by chemical modification with group specific and affinity reagents.

Mutants resistant to the inhibitors streptolydigin, rifamycin, and streptovaricin have been shown, by reconstitution experiments, to be modified in the β subunit (Rabussay & Zillig, 1969; Heil & Zillig, 1970; Iwakura et al., 1973; Boyd et al., 1974). Since rifamycin and

streptolydigin are specific inhibitors of initiation and elongation respectively, it appears that both functions reside on β (Iwakura et al., 1973). However they might act at a distance (Wu & Wu, 1974). A mutant in β′ has a decreased affinity for DNA (Panny et al., 1974). The enzyme isolated from a large number of mutants is insignificantly different in the normal activity assays (Khesin et al., 1971; Babinet, 1970; Jacobsen & Gillespie, 1971; Yura et al., 1970; Bautz, E.K.F., personal communications), and suggests that a large part of the enzyme is involved in other activities, such as control. A similar conclusion is reached from the observation that proteolysis of DNA protected RNA polymerase leads to extensive degradation, yet little loss of activity (King et al., 1974 a).

T4 infection of E. coli results in a number of modification of the host enzyme. (Walter et al., 1968; Bautz & Dunn, 1969; Seifert et al., 1969; Goff & Weber, 1970; Seifert et al., 1971). The first changes occur within 4 min of infection, and involve:

1) a fast introduction of 1 adenosine and 2 phosphates onto an α subunit, which does not require phage specified protein synthesis
2) modification of a second α subunit with ADP-ribose, which requires phage protein synthesis

Both modifications occur on a common sequence, but there is a disagreement about what the actual sequence and amino acid modified are (Goff, 1974; Zillig et al., 1974). This is the first observation of a specific α modification, and indicates that it may be involved in promoter site selection.

Affinity reagents resembling nucleoside triphosphates, DNA, and inhibitors have been used. An alkylating derivative of rifamycin (Fig 1.1) 3 - (2- bromo [1- ^{14}C] acetamidoethyl) — thiorifamycin SV covalently attaches to β in core enzyme, and β and σ in holoenzyme. (Stender et al., 1975). These results suggest that the inhibitor binding site is on

β, and that σ binds close to the inhibitor site. α confers rifamycin binding capacity to β in the subassembly $\alpha_2\beta$ (Stetter & Zillig, 1974), but the lack of α - rifamycin links suggests that α does not form part of the binding site.

Affinity labelling with purine analogues leads to specific attachment to the initiation site on β (Spoor et al., 1970; Nixon et al., 1972; Wu & Wu, 1974). The pyrimidine analogues $[^{35}S]$ - 4 - thio - UTP (Frischauf & Scheit, 1973) and 5 - formyl - UTP (Armstrong et al., 1974) react with both the β and β′ subunits, which suggests that the β′ subunit has the elongation site. The lack of specificity may result from the weak association constant for pyrimidine nucleotides and RNA polymerase (Wu & Goldthwait, 1969b). Affinity labelling with the template analogue polydeoxy - 4 - thiothymidylic acid (Frischauf & Scheit, 1973) suggests the DNA binding site is on the β′ subunit.

Group specific reagents, which modify thiol, amino, imidazole, and indole side chains, have been used. Unfortunately, in most cases, the specificity of the reaction and the extent of reaction have not been established. This has probably arisen because of the large size of the enzyme, and the difficulties associated with obtaining it in large quantities.

Thiols are the easiest to modify specifically, and hence the effects of their modification has been studied extensively (Krakow, 1966; Ishihama & Hurwitz, 1969; Smith et al., 1971; Lillehaug et al., 1973; Harding & Beychok, 1974; Mikulski et al., 1973; Nicholson & King, 1973; Yarbrough & Wu, 1974). Although some of the results are contradictory (often well defined conditions have not been used), several conclusions can be reached. There are three classes of thiols, containing 7 reactive, 12 less reactive, and 15 unreactive SH groups, the exact number in each group depending upon the reagent used. The reactive group is not essential for activity, although some inhibition

can be obtained depending on the assay used. Partial modification of the less reactive group leads to an altered structure and decreased affinity for DNA, whilst complete modification of this group results in total loss of activity.

Amino groups have been modified with β- naphthoquinone-4 - sulphonic acid and chlorofluoro - p - benzoquinone (Ishihama & Hurwitz, 1969), fluorescein isothiocyanate (Lowe, 1974), trinitrobenzenesulphonic acid (Krakow, 1973), and substrate analogues containing aldehyde moeities (Bull et al., 1975; Venegas et al., 1973; Wu & Wu, 1974; Spoor et al., 1970; Nixon et al., 1972; Armstrong et al., 1974). The results with affinity labelling amino group reagents suggest that lysines are present in the initiation and elongation sites. An essential lysine, with a pKa of 7·9, has been identified (Bull et al., 1975). With trinitrobenzenesulphonic acid 50 lysines are modified with complete loss of activity. DNA protection against inactivation has little effect on the number of lysines modified (Krakow, 1973).

Tryptophan and thiol modification with 2-hydroxy-5-nitrobenzyl bromide leads to a preferential inhibition of initiation (Novak et al., 1974), and imidazole modification with diazonium - 1 - H tetrazole or Rose Bengal + light lead to preferential loss of elongation (Ishihama & Hurwitz, 1969). Phosphorylation of one or more serine residues of σ leads to a stimulation of transcription of T4 DNA (Martelo et al., 1970; Martelo et al., 1974).

The subunits and subassemblies α , β, β′, αβ, αβ′, and ββ′ do not catalyse RNA chain formation, pyrophosphate exchange, nor covalent addition of UTP to a primer. However αβ′, ββ′ & β′ all bind to DNA, but with a lower affinity than core enzyme (Yarbrough, 1973).

In summary the β subunit has the initiation site, and binds inhibitors and σ. β′ is the DNA binding subunit. The elongation site may be on β or β′.

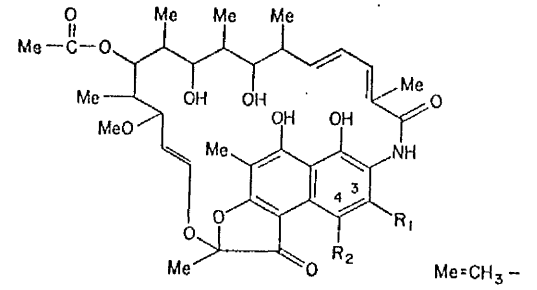
1.2.3.3 Inhibitors.

There are two classes of RNA polymerase inhibitors, those which interact with the enzyme, and those which interact with the DNA template.

A large number of DNA binding compounds are known which alter its ability to act as a template. These include actinomycin D (Goldberg & Reich, 1964), ethidium bromide, proflavine (Waring, 1965), and luteoskyrin (Ruet et al., 1973), which block elongation, but under certain conditions can also block initiation (Richardson, 1973).

Enzyme binding inhibitors can be divided into two groups, those which bind ionically and compete with the template for enzyme, and those which bind to a hydrophobic site. The first group includes RNA, heparin, and polyethane sulphonate (Chamberlin, 1974 b). The ansamycin antibiotics, such as rifamycin and streptolydigin (Riva & Silvestri, 1972) belong to the second group. Rifamycins (Fig 1.1) are potent inhibitors of RNA chain initiation which bind strongly to the enzyme, by a combination of hydrogen bonds and hydrophobic interactions, to form 1 : 1 complexes (Brufani et al., 1974). Streptolydigin (Fig 1.1) blocks both initiation and elongation, probably by binding to both sites on the enzyme (Siddhikil et al., 1969). Rifamycin derivatives with long hydrophobic side chains can also block template binding (Meilhac & Chambon, 1973). Other hydrophobic inhibitors include Rose Bengal (Wu & Wu, 1973 a, b) and phospholipids (Stevens, 1975). The polyanion poly (glu^1 tyr^1) is a potent inhibitor, whereas poly (glu), poly(asp), and poly(glu^9 leu^1) do not inhibit (Krakow, 1974). The high affinity of poly (glu^1 tyr^1) may be due to tyrosine interacting with hydrophobic groups in the active site of the enzyme. The "complementary" polypeptide poly (lys^1 tyr^1) binds strongly to DNA (section 1.3).

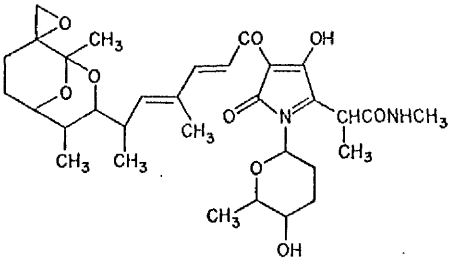
Fig 1.1 Structure of rifamycin and streptolydigin.



Rifamycin (I) R_1=-H R_2=-O-CH_2COOH

Rifampicin R_1=-CH=N-N⟨ ⟩N-CH_3 R_2=OH

Rifamycin SV R=-N⟨ ⟩O R_2=-OH



Streptolydigin

1.3 The role of aromatic and basic amino acids in DNA protein interactions.

1.3.1 Introduction.

Several mechanisms can be envisaged for the specific interaction between amino acids and nucleotides. One could involve amino acids being able to mimic all the normal interactions between nucleotides. Thus amino acids in specific three dimensional arrangements might hydrogen bond with the bases, while others might interact by amino acid-base stacking interactions. Many aromatic molecules, e.g. the phenanthridines such as ethidium bromide (T'sai et al., 1975), and actinomycin D (Sobel, 1973), can intercalate between the bases of single and double stranded DNA, and the aromatic amino acids may be capable of similar interactions. Evidence for these interactions, as well as eletrostatic bonding between basic amino acids and phosphates, comes from studies on synthetic peptide and protein nucleic acid interactions.

1.3.2 Polypeptide - nucleic acid interactions.

As early as 1955 Chargaff and his co-workers (Spitnik et al., 1955) began to use synthetic basic polyamines as models for studying the interactions of DNA with the basic proteins which occur in chromatin. Leng & Felsenfeld (1966) were able to show that polylysine interacts preferentially with A-T, whereas polyarginine favours G-C rich DNA. However these molecules had a low specificity, and this led to a study of polypeptides containing amino acids which could form hydrogen and hydrophobic bonds with nucleic acids.

Hydrogen bonding is difficult to demonstrate in aqueous solution, but in chloroform solution tyrosine and carboxylic amino acids (aspartic and glutamic acid) can form hydrogen bonds with adenine and uracil

(Sellini et al., 1973). Hydrogen bonding to guanine and cytosine have not been investigated, and no conclusion can be drawn about the specificity of the interaction.

The indole ring of tryptamine stacks with nucleotide bases at low temperatures (77°K), and this leads to characteristic changes in the fluorescence and NMR spectrum (Dimicoli & Hélène, 1971). Similar changes occur in complexes of basic oligopeptides containing aromatic amino acids and polynucleotides at room temperature (Hélène, 1971; Hélène et al., 1971; Dimicoli & Hélène, 1975 a, b; Gabbay et al., 1972). The observed changes, upfield shifts and line broadening of NMR signals from the aromatic rings of the amino acids and nucleotides, indole fluorescence quenching, and a decrease in the specific viscosity of the DNA, led the authors to conclude that aromatic amino acids were stacking between the nucleic acids bases. Hélène and his co-workers (Toulmé et al., 1974; Brun et al., 1975; Durand et al., 1975) have shown that an equilibrium exists between intercalating and purely ionic complexes. The equilibrium is shifted towards intercalation with single stranded DNA, showing that these peptides are able to discriminate between the helix and coiled forms of DNA. A large part of the binding energy for polypeptide - DNA complexes arises from electrostatic interactions since they are sensitive to ionic strength and pH.

The aromatic amino acids show some selectivity in their stacking interactions with nucleic acids. Tryptophan peptides bind to A-T rich sequences of DNA, whilst tyrosine peptides are less discriminating (Gabbay et al., 1973; Novak & Dohnal, 1973; Novak & Dohnal, 1974 a). Molecular complexes between tryptophan and nucleotides can be detected by difference spectroscopy, and they have the following order of stability IMP > CMP > AMP > TMP > UMP (Morita, 1974). The reasons for the observed specificities are not understood, and require further investigation.

Several reports contradict the intercalation model. Jacobsen & Wang (1973) found that aromatic amino acid amides had no effect on the sedimentation coefficient of supercoiled DNA, nor on supercoiling of DNA closed by a ligase. Since an unwinding of $0{\cdot}2^{\circ}$ per amino acid amide could have been detected, and the smallest unwinding angle for an intercalating base is 4° for daunomycin (Saucier et al., 1971 b), they suggested that intercalation did not occur. However a model has been built in which intercalation occurs without unwinding (Saucier et al., 1971 a), and CD measurements on the complexes favour a stacking mechanism in which the inter-base distance is not altered (Durand et al., 1974). Santella & Li (1974) showed that tyrosine in poly(lys^1 tyr^1) had little effect on the stability of the complex with DNA. However this may result from the nature of the polymer since complex stability in the series $tyr(gly)_{1-4}tyr$ is greatest for $tyr(gly)_2tyr$ (Novak & Dohnal, 1974 b).

Ternary complexes between ATP, Zn^{++}, and tryptophan occur in solution. The interaction is mainly due to stacking between the indole benzene and ATP imidazole rings (Naumann & Sigal, 1974). These complexes may be relevant to the mechanism of RNA polymerase action since it contains essential Zn(II) (Scrutton et al., 1971), and the ATP imidazole ring is essential for correct recognition at the initiation site (Chamberlin, 1974 b).

1.3.3 Protein - nucleic acid interactions.

Aromatic amino acids are found in the active sites of DNA, RNA, and nucleotide binding proteins, but in most cases their function is unknown. The best information comes from X-ray crystallographic studies on staphylococcus nuclease and flavodoxin, and additional information should soon be available for some nucleotide binding enzymes (Rossman et al., 1974) and an amino-acyl t-RNA synthetase. Chemical modification has been applied to a greater number of enzymes, but although it is possible

to decide that an amino-acid is essential, it is difficult to differentiate between a direct binding role and an indirect effect through the microenvironment of the active centre. For example, chemical modification suggested that tyrosine residues in flavodoxin were involved in substrate binding, whilst X-ray crystallography showed that they were too far away from the binding site for them to be involved (Burnett et al., 1974). Nucleotide quenching of protein fluorescence is occasionally observed, but this information is ambiguous because the effect may be direct, or indirect through the structure of the protein.

A number of DNA binding proteins, including histones, fd gene 5 protein, DNA ase I, lac repressor and staphylococcus nuclease have been shown to have basic and aromatic amino acids which interact with the DNA. Electrostatic bonding by calf thymus histones can be demonstrated with competitive labelling experiments, which show that the lysine ϵ-NH_2 groups in calf thymus DNA-histone complexes have abnormally high pKa values. (Malchy & Kaplan, 1974). Hydrophobic bonding is demonstrated by the different sensitivities of histones F1, and F3 and F2a1 to salt, propan-1-ol and urea dissociation. F1 has more positive and less hydrophobic groups than F3 or F2a1, and is more easily dissociated by salt, but not as easily by urea and propan-1-ol (Bartley & Chalkey, 1972). Phosphorescence and difference spectroscopy suggest that tyrosines are involved in the interactions (Matsuyama & Nagata, 1970; Palau & Padros, 1975). The affinity of fd gene 5 protein for DNA is lowered by salt, acetylation of amino groups, and nitration of tyrosine, but not by the introduction of a fluoro group on tyrosine (Anderson et al., 1975). The nitro group has a greater affect than the fluoro group on the "thickness" of the phenol side chain, and would be more disruptive to intercalation. Nitration also inhibits the activity of DNAase I (Hugli & Stein, 1971).

Proteolytic digestion (Platt et al., 1973) and genetic studies (Muller-Hill et al., 1968; Davies & Jacob, 1968; Adler et al., 1972)

suggest that the amino terminal 50 residues of lac repressor are responsible for complex formation with DNA. The region is unusual in that it contains 50 % of the tyrosines, but only 20 % of the molecule. Model building studies place these tyrosines in a position where they are able to intercalate with the DNA (Patel, 1975; Chou et al., 1975). Furthermore mutant replacement of tyr-17 with gly, ser or leu (Chou et al., 1975), and iodination of tyr -7, -12, -17 (Fanning, 1975), lead to loss of repressor activity.

Studies with staphylococcus nuclease illustrate the pitfalls of indirect evidence for stacking interactions. Fluorescence quenching (Pong, 1970), chemical modification (Cuatrecasas et al., 1968, 1969) and NMR (Markeley & Jardetsky, 1970) indicate that tyrosines are involved in substrate binding, but according to Arnone (1971) they do not form stacking interactions with the substrates. Modification studies with pancreatic RNA ase indicate that aromatic amino acids in proteins may provide selectivity for substrates. Replacement of phe-120 (which is in a position to interact with pyrimidine bases, Moore & Stein, 1973) with tyr-120 either synthetically, or naturally in giraffe RNA ase, leads to no loss of activity, but a marked change in selectivity for U as opposed to C residues (Hodges & Merrifield, 1974).

A number of nucleotide binding proteins have been studied by X-ray diffraction, chemical modification, and difference spectroscopy. Chemical modification shows that tyr and trp in clostridial flavodoxin are essential for FMN binding. X-ray crystallography shows the rings of trp-90 and FMN are imperfectly stacked, the angle between them is 27° (Burnett et al., 1974). Flavodoxin from Desulfovibris vulgaris has trp-80 inside and tyr-98 outside the FMN binding site. Tyr-98 is nearly coplanar, but trp-80 is at 45° to the plane of the FMN ring. Any rotation of trp-80 would probably lead to a conformational change

(Watenpaugh et al., 1973). Nitration of an active site tyrosine in arginine kinase inhibits nucleotide binding, possibly due to a conformational change (Kassab et al., 1970). Spectroscopic studies with heavy meromycin, an active fragment of myosin, suggests that adenine stacks with trp during the Mg^{++} ATP ase reaction (Morita et al., 1973; Yoshino et al., 1972).

1.4 Chemical modification in the study of macromolecular structure and funtion.

1.4.1 Chemical modification of proteins.

Chemical modification has been used to identify the amino acids involved in catalysis and substrate binding, to study protein structure, to prepare insoluble enzymes, to introduce haptens into proteins to enhance their immunogenicity, and to introduce heavy metals for X-ray crystallography (Means & Feeney, 1971). However the technique has many limitations, and they need to be realised so that results can be correctly interpreted.

Few reagents are specific for a functional group. Their behaviour can only be partially predicted from their specificity with model peptides and proteins because the local environment of each group will affect its reactivity, and this will vary from protein to protein. Hence, in almost every case, the specificity of a reagent must be carefully checked. The behaviour of a reagent also depends upon the environmental conditions, and these have to be carefully chosen in most cases. Finally it is often difficult to decide how the modification is affecting the activity. The modified group may be involved in catalysis, binding, or in maintenance of structure. In general the larger the protein, the greater will be the difficulties in resolving the effects of modifications.

The specificity of a reagent towards a protein is determined by the environment of the functional groups. Non-polar, charged, hydrogen bonding, and bulky neighbours can affect the reactivity of an amino acid, and they can also alter the reactivity of the reagent by selective absorption, electrostatic attraction or repulsion, and by catalysis. These properties can often be exploited to increase the specificity of a reagent. For example pyridoxal phosphate is more specific than trinitrobenzenesulphonic acid in the modification of the lysine groups

of RNA polymerase, probably because it mimics the hydrophobic and basic properties of the substrates and binds to a limited number of sites (section 1.2.3.2 ; Bull et al., 1975).

The specificity of a reagent can sometimes be increased by choosing the appropriate experimental conditions. With reagents that react with the unprotonated form of a nucleophile altering the pH will alter the groups which will react. At low pH tryptophan and methionine are most sensitive to photosensitised oxidation (Sluyterman, 1962), and alkylation (Gurd, 1967). When the pH is raised histidine, tyrosine, and lysine become more reactive. Electrophilic reagents, such as N-bromosuccinimide, are often less sensitive to pH control (Witkop, 1961). The specificity can occasionally be increased by reversibly protecting a more reactive group. Kassab et al. (1970) found that tetranitromethane preferentially oxidised the thiols of arginine kinase. By reversibly protecting the thiols with tetrathionate they were able to restrict modification to the nitration of tyrosines.

A variety of analytic techniques have been used to check the selectivity of modification. Continuous monitoring of a reaction by spectrophotometry is a simple and useful technique, but few reagents are known which produce derivative spectra sufficiently different from the reagent to be much use. An example of such a reagent is 5,5'-dithiobis (2-nitrobenzoic acid) (see chapter 4). Other, less direct, methods are amino acid analysis, controlled proteolysis followed by isolation and detection of the modified peptides, and incorporation of a radioactive label (see chapters 4 and 5). A good criterion of specific modification is complete reversibility of the modification with total recovery of activity. 5,5'-dithiobis (2-nitrobenzoic acid) is a readily reversible reagent (chapter 4).

Once it has been established that a reagent is capable of inactivating an enzyme, the next goal is to correlate the inactivation

with the modification of one or a group of amino acids. Often the first group to react causes inactivation, but in some cases the most reactive groups are not involved in activity. With fructose diphosphatase 6 out of the 10 tyrosines are acetylated by N-acetylimidazole without loss of activity. Further modification leads to inactivation (Pontremoli et al., 1966). Ideally both the rate and stoichiometry of modification should be correlated with the loss of activity. An example of unusual behaviour is the modification of E. coli tryptophan synthetase with [^{14}C] - N - ethylmaleimide. Incorporation of one mole per mole of reagent resulted in complete loss of activity, yet fragmentation showed that three thiols had partially reacted (Hardman & Yanofsky, 1965).

Once modification has been correlated with loss of activity, it is necessary to establish the cause of inactivation. Inactivation may have resulted from a conformational change, loss of substrate binding, subunit dissociation, or modification of a group involved in activity. It is often difficult to separate these effects unambiguously. A variety of techniques can be used to study structural changes, including gel filtration, ultracentrifugation, optical rotary dispersion, circular dichroism, fluorescence, nuclear magnetic resonance, hydrogen exchange, viscosity, and X-ray diffraction. The limitations of each technique must be considered in interpreting the effects of modification. Substrate binding can be measured by a number of techniques (chapter 4). Reagents vary in their ability to cause structural changes. Amidination of lysines with methyl acetimidate leads to no change in charge and hence presumably minimum perturbation of structure (Hunter & Ludwig, 1972), whilst succinylation produces a net change of two charge units and can result in disaggregation (Freisheim et al., 1967). The properties of methyl acetimidate can be exploited in differential labelling, a technique used to identify amino acids in the active site of an enzyme.

Dworschack et al., (1975) modified the lysines outside the active site or horse liver alcohol dehydrogenase by reacting the substrate protected enzyme with ethyl acetimidate. After the removal of substrates a single lysine remained available to modification with methyl -[^{14}C]acetimidate.

Chemical modification can also be used to study the structure of a protein. The relative reactivities of, for example, the thiols of a protein gives some information about their relative distribution between the surface and interior of a protein (section 1.2.3.2). Changes in the number of exposed and buried groups can be used to study conformational changes (chapter 4). Bifunctional reagents can be used to establish the proximity of functional groups, or subunits of a multimeric protein. Nitration, reduction, and crosslinking of tyr-85 and tyr-115 of staphycoccus nuclease was used to establish their proximity (Cuatrecasas et al., 1968). Crosslinking of aldolase with dimethyl suberimidate resulted in 4 species, corresponding to monomer, dimer, trimer, and tetramer, confirming other evidence for this enzyme being a tetramer (Davies & Stark, 1970).

1.4.2 Formaldehyde as a probe of DNA-protein interactions.

Since formaldehyde was originally shown to react with nucleic acids (Fraenkel-Conrat, 1954) it has been used for many purposes. Examples of these are to inactivate viruses to produce vaccines; to label non hydrogen-bonded regions in RNA; to study the dynamic structure of DNA; to quantitate the extent of DNA modification with nucleases, X-rays and ultraviolet light; and to study protein-nucleic acid interactions in viruses, ribosomes, and chromatin (Feldman, 1973).

Formaldehyde is one of the simplest known DNA denaturants. The chemistry of the reaction has been studied by several groups (Feldman, 1973) and most recently by McGee & von Hippel (1975a, b). Formaldehyde reacts with the exocyclic amino groups of A, G, & C residues, and with the endocyclic imino groups of T and G, by substitution of a hydrogen atom with the methylol group (- CH_2OH). Reaction with the exocyclic group can lead to mono- and di-substitution, but the equilibrium constants at 25°C favour the monosubstitution. Since the mono adduct is coplanar with the heterocyclic ring it can exist in two isomeric forms, only one of which interfers with the formation of normal Watson-Crick base pairs. Steric hindrance appears to favour the isomer which interferes with normal hydrogen-bonding. The proposed mechanism for the reaction is that formaldehyde approaches the base perpendicular to its plane, an intermediate zwitterion is formed, which then reverts to the planar adduct. In the case of imino groups the formaldehyde molecule reacts with the negatively charged form, and the charged intermediate is rapidly protonated to form the product. Since, in one case, the intermediate is tetrahedral, and in the other, ionisation of a group normally involved in hydrogen bonding is required, the reaction is inhibited by normal base stacking.

The reaction with DNA only occurs in denatured regions, is non-cooperative, and has little specificity. Characteristic absorbance changes occur due to adduct formation and DNA unwinding, which can be used to follow the kinetics of the reaction. These properties have been useful in studying the structure and stability of the DNA helix (Lazurkin et al., 1970; Dean & Lebowitz, 1971; von Hippel & Wong, 1971; Utiyama & Doty, 1971). These studies show that native DNA has transient regions in which about nine base pairs open cooperatively, and that supercoiling forms areas which are permanently open. Defects also act as permanently open regions, and can be introduced by DNA ase digestion (Bannikov & Trifonov, 1969), uv irradiation (Jonker & Blok, 1975), and the binding of proteins such as RNA ase (Kosaganov et al., 1969), and RNA polymerase (Kosaganov et al., 1971).

Since formaldehyde melting can be readily followed by spectrophotometry, and a kinetic analysis has been reported which could be used to obtain quantitative information (Trifonov et al., 1968), formaldehyde melting may be useful as an assay for RNA polymerase.

1.5 Scope of the Thesis.

RNA polymerase is a complex, highly controlled enzyme, which interacts with nucleic acids with varying degrees of specificity. To gain an insight into the properties of the enzyme it was predicted from work on model systems (synthetic peptides) and proteins, from the sensitivity of RNA polymerase to hydrophobic inhibitors, from fluorescence quenching, and from its ionic properties, that the aromatic and basic amino acids of RNA polymerase would be involved in its activity. Chemical modification, with N-bromosuccinimide (chapter 4), was used to investigate the aromatic amino acids tryptophan and tyrosine, and, with methyl acetimidate (chapter 5), the basic amino acid lysine. The enzyme's ability to destabilise the DNA helix was exploited in an investigation of the kinetics of formaldehyde melting as a probe of RNA polymerase-DNA interactions (chapter 6).

CHAPTER 2.

MATERIALS AND METHODS.

2.1. Materials.

Materials were obtained from the following commercial suppliers:

Amicon Ltd., High Wycombe, Bucks., U.K.	Ultrafiltration membranes PM10 & PM30.
Armour Pharmaceutical Co. Ltd., Eastbourne, Essex, U.K.	Bovine Serum Albumin.
B D H Chemicals Ltd., Poole, Dorset, U.K.	Ammonium sulphate (enzyme grade), "Cyanogum" 41, 4 - Dimethylaminobenzaldehyde (AnalaR), 5, 5′- Dithiobis(2 - nitrobenzoic acid), ε - DNP lysine, Folin & Ciocalteu's Phenol Reagent, Formaldehyde solution, Guanidine hydrochloride (specially pure), Hydrochloric acid (AVS 5N, CVS 1N, & Aristar), Hydroxylamine hydrochloride (reagent grade), Lysozyme, NN′- Methylenebisacrylamide, Polyethylene glycol 20M, Sodium hydroxide (CVS 1N), NNN′N′- Tetramethylenediamine, Urea.

Bio. Rad., Richmond, California, U.S.A.	Bio-Gel A-1.5m & A-5m, Mixed Bed Resin AG 501 - X8 (D).
The Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex, U.K.	Adenosine - 5′ - triphosphate, Aldolase (rabbit muscle), Guanosine - 5′ - triphosphate.
Camlab Ltd., Nuffield Rd., Cambridge, U.K.	Silica-gel G thin layer plates.
Eppendorf Gerätebau Netheler + Hiz GmbH, 2 Hamburg 63, W. Germany.	Conical plastic tubes - Eppendorf Reactionsgefasse
George T. Gurr, Searle Scientific Services, High Wycombe, Bucks., U.K.	Amido - Black 10B, Coomassie Brilliant Blue (R).
International Enzymes Ltd., Windsor, Berks., U.K.	Cytidine - 5′ - triphosphate, Uridine - 5′ - triphosphate.
Johnsons of Hendon, Hendon, London, U.K.	pH paper, narrow range (6.8 - 8.3, & 8.0 - 9.1).

Koch-Light Laboratories Ltd., Colnbrook, U.K.	Deoxyribonucleic acid from calf-thymus, 2, 5 - Diphenyloxazole, Mercaptoethanol (pure), Methanesulphonic acid (pure), Toluene (puriss), Trichloroacetic acid, Tryptamine hydrochloride (pure).
Nuclear Enterprises (GB) Ltd., Sighthill, Edinburgh, U.K.	Hyamine hydroxide.
Pharmacia Fine Chemicals AB, Uppsala, Sweden.	DEAE - Sephadex A25, Dextran-blue, Sephadex G25, G50, G200, Sepharose 6B.
Rohm and Haas (UK) Ltd., Lennig House, 2, Mason Av., Croydon, Surrey, U.K.	Triton X-100.
Spectrum Medical Industries Ltd., 60916 Terminal Annex, Los Angeles 90024, U.S.A.	Spectrapor 2 membrane.
The Radiochemical Centre, Amersham, U.K.	[8-^{3}H] Adenosine - 5'-triphosphate, [5-^{3}H] Uridine - 5'-triphosphate.

Sartorius Membranfilter GmbH, Gottinger, W. Germany.	Membrane filters (0.45μ pore size).
The Scientific Instrument Centre Ltd., 1, Leeke Street, London, U.K.	Dialysis tubing (Visking).
Sigma London Chemical Company Ltd., Kingston-upon-Thames, Surrey, U.K.	Agarose (for use in electrophoresis), Albumin, egg (grade V), Deoxyribonucleic acid from calf-thymus (type I), Dithiothreitol, Subtilisin, Thermolysin, TRIZMA base.
Whatman Biochemicals Ltd., Maidstone, Kent, U.K.	DEAE - cellulose (DE 52), Filter paper (No.1), Filter paper (acid hardened, No.52), Glass-fibre filters (GF/C & GF/F, 25 mm), Phosphocellulose P 11.
Worthington Biochemicals Corp., Freehold, New Jersey, U.S.A.	Chymotrypsin, Deoxyribonucleic acid from calf-thymus, Trypsin.

All unlisted chemicals were "AnalaR" grade from B D H.

2.2. Methods.

2.2.01 Amino-acid analysis.

The amino-acid content of RNA polymerase was determined using the methanesulphonic acid + tryptamine hydrochloride method (Liu et al., 1972), and the hydrochloric acid + 2-mercaptoethanol method. Hydrolysis was performed in heavy walled tubes (0·9 x 10 cm) which had been washed with H_2SO_4 : HNO_3 (3:1), rinsed with distilled water, and oven dried. RNA polymerase was extensively dialysed into 0·5 % ammonium bicarbonate, and 0·5 mg samples were freeze-dried in the hydrolysis tubes.

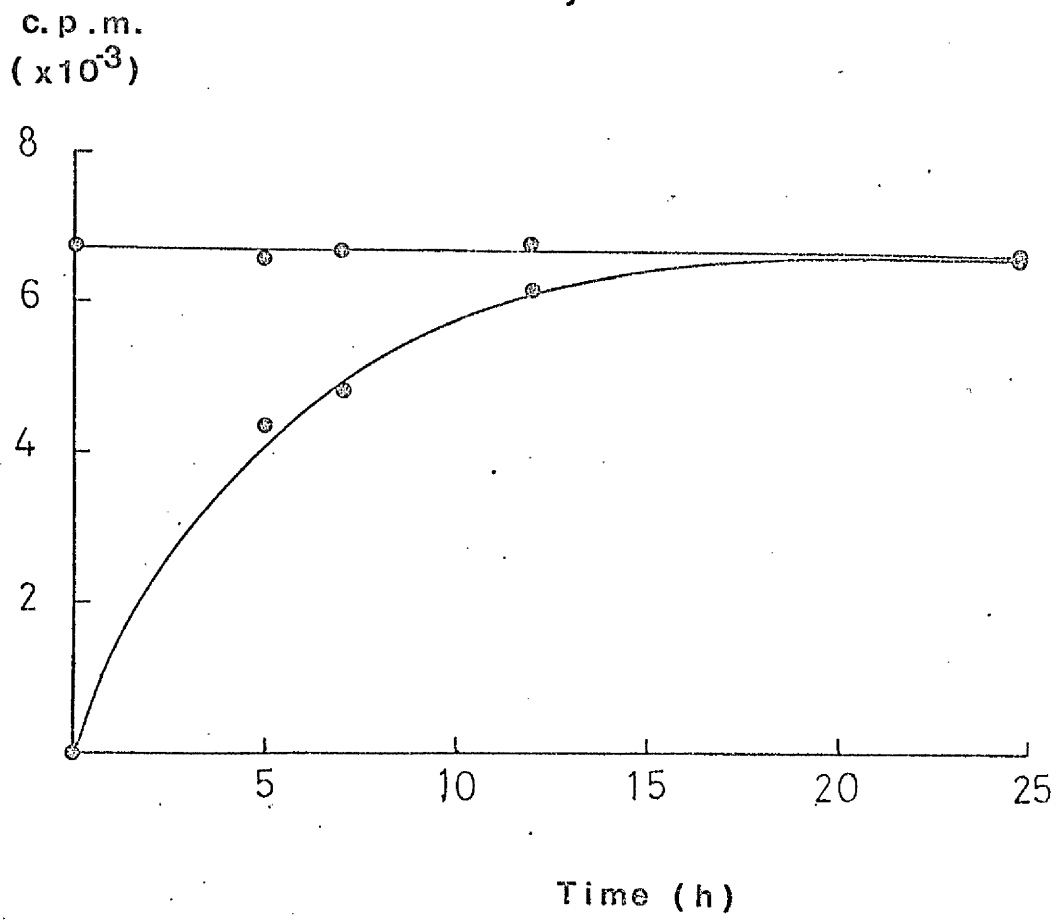
0·5 mg samples were hydrolysed in 0·5 ml of 4M methanesulphonic acid containing 0·5 mg of tryptamine hydrochloride. The samples were flushed with oxygen-free nitrogen and evacuated to 120 μmHg twice. Duplicate samples were hydrolysed for 48, 72, and 96 h at 110°C. The samples were cooled to 0°C, and the pH was adjusted to 2·0 - 2·2 with cooling. The samples were analysed on a Jeol JLC S AH amino-acid analyser (Japan Electron Optics Laboratory Co. Ltd., Chiyado-du, Tokyo, Japan). Tryptophan was eluted manually, from the short column, at 60°C. 0·5 mg samples were also hydrolysed in 0·5 ml hydrochloric acid (1 volume Aristar HCl : 1 volume distilled water) containing 1 % 2-mercaptoethanol. Duplicate samples were hydrolysed for 24, 48, and 72 h at 110°C, the HCl was removed under vacuum, and the samples were analysed as above.

2.2.02 Binding-constant determination.

The binding-constants for nucleoside triphosphates and RNA polymerase (core) were determined by the method of Hummel and Dreyer (1962) (see 2.2.04), and by equilibrium dialysis using two methods.

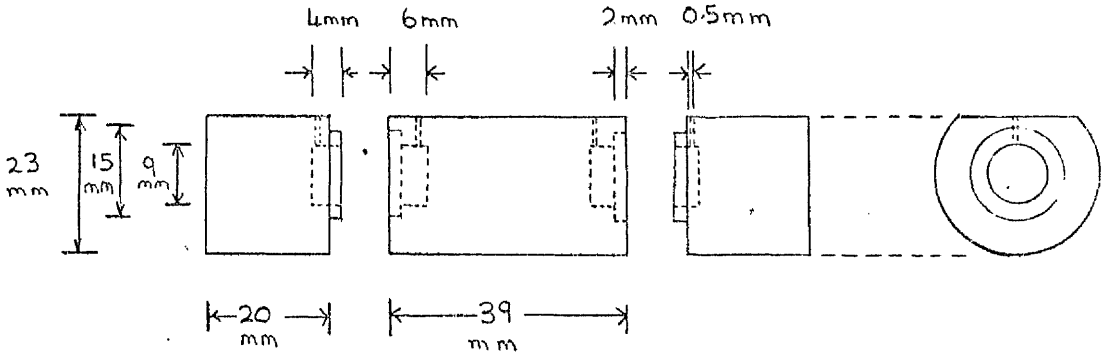
In the first method a small volume, in a specially constructed cell, was dialysed against an excess of buffer. The cell was made from a 1 dram vial (Johnsen & Jergensen Ltd., London, U.K.), whose bottom had been removed. The top was sealed with Visking tubing and an "O" ring. The vial was inverted and supported over the dialysis solution, with the dialysis membrane just below the surface. Only the dialysis buffer was stirred, but the geometry of the apparatus was such that equilibrium was attained in an acceptable time interval. The rate of equilibration was determined by dialysing 100 µl of buffer A + 0·2 M KCl against 50 ml of the same buffer + 1 µM [8-^{3}H] ATP (10 µCi/µM). 5 µl samples of buffer from each compartment were taken at intervals, and counted for radioactivity in 2 ml of triton-toluene scintillant. Equilibration took about 24 h (Fig 2.1).

Fig 2.1 Rate of equilibration of dialysis cell.



In the second method a microdialysis cell, similar to that described by Englund et al. (1969), was used. The apparatus was constructed by Mr. N.L. Harvey, and it consisted of perspex cells containing a twin set of dialysis chambers (Fig 2.2), clamped together with a perspex holder. The whole apparatus was clamped to a revolving drum in such a manner that the axis of rotation was parallel to the long axis of the cell. Mixing was achieved by rotation at 2 rpm, and with 1 mm glass beads placed in the dialysis chambers. Samples were introduced and withdrawn with Hamilton syringes (25 µl, 702N; 5 µl, 7005 NCH; Hamilton Micromeasure bv, The Hague, Holland).

Fig 2.2 Diagram of micro-dialysis cell.



Three membranes were tested for their permeability to nucleoside triphosphates:

i. Visking membrane.

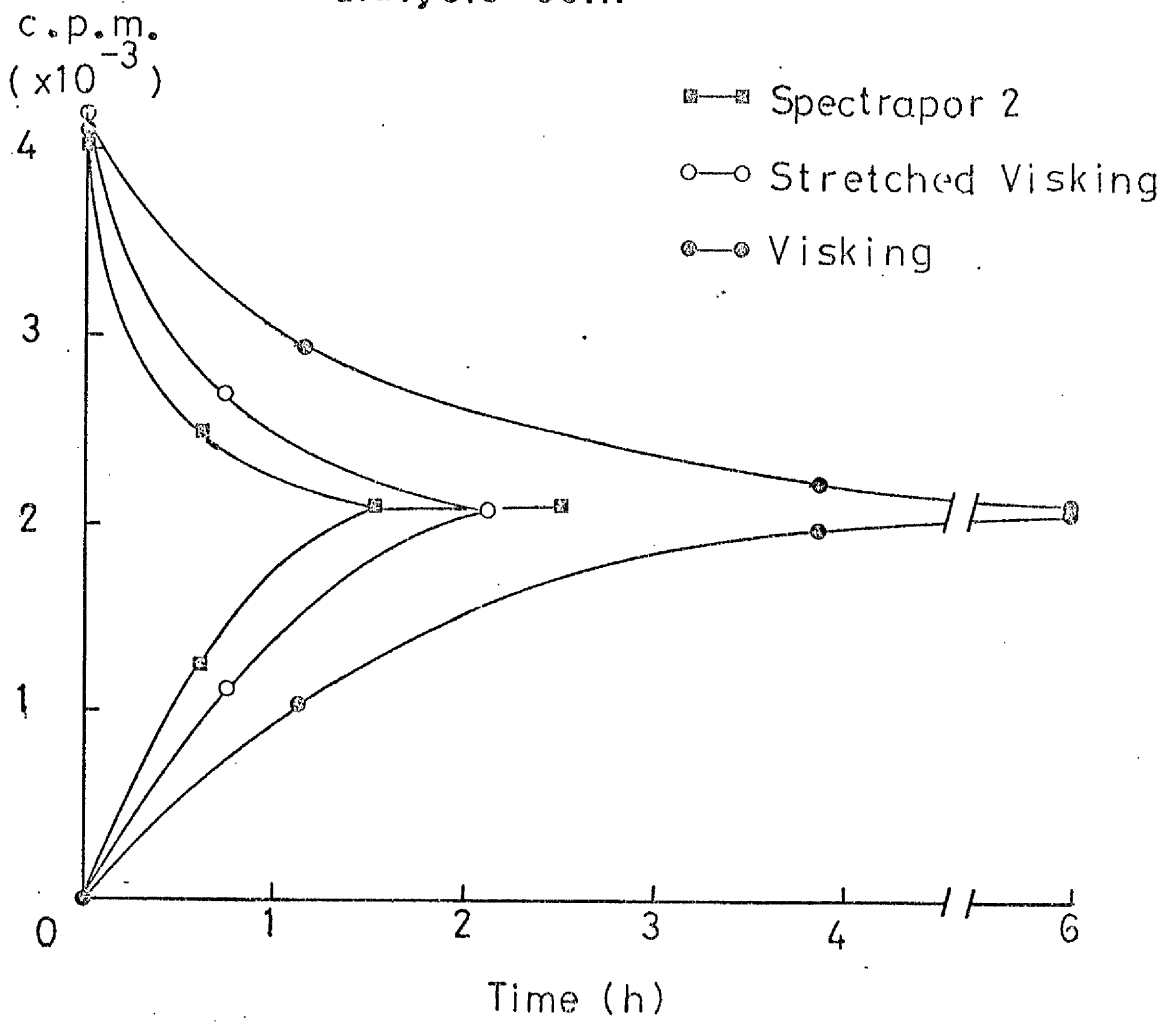
ii. Visking membrane stretched to increase its porosity (Craig and King, 1962).

iii. Spectrapor 2 membrane.

Visking tubing and Spectrapor 2 membrane were washed as described in 2.2.05. Stretched tubing was stored in 50 % ethanol, 4°C, and rehydrated before use in distilled water.

The rate of equilibration was determined by dialysing 20 µl buffer A + 0·2M KCl against 20 µl of A + 0·2M KCl + 50 µM [8-^{3}H] ATP (25 µCi/µM), at room temperature (17 ± 1°C). 1 µl samples were counted in 2 ml of triton-toluene (Fig 2.3). From the recovery of radioactivity it was clear that the membranes did not bind ATP. By dialysing a 10 mg/ml RNA polymerase solution against buffer it was shown that all three membranes did not bind, and were impermeable to, protein. In subsequent experiments Spectrapor 2 membrane was used.

Fig 2.3 Rate of equilibration of micro-dialysis cell.



2.2.03 Centrifugation.

i. Rate separation in a glycerol gradient.

Gradients of 10 - 30 % glycerol were poured at about 50 ml/h, and 4°C. The linear gradient (denser part first) was allowed to run down the side of a vertically mounted centrifuge tube, the tubing outlet being kept 2 - 3 mm from the surface of the forming gradient. A 3 ml sample and 55 ml gradient, or a 0·5 ml sample and 12 ml gradient, were used for the SW 25·2 or SW 40 rotors. The gradients were harvested at about 50 ml/h, through a stainless steel tube which had been lowered to the bottom of the centrifuge tube. The absorbance at 280 nm was monitored continuously with the 0·3 cm path length cell of the Gilford spectrophotometer, and 2·0 or 0·5 ml samples were collected.

ii. Centrifugal desalting.

The centrifugal method of Neal & Florini (1973) was sometimes used to desalt solutions of RNA polymerase. A 15 ml conical tube and 12 x 50 mm polyallomer tube, or a 50 ml conical tube and a 24 x 90 mm polyallomer tube were used to desalt volumes of about 1 or 8 ml respectively. A small vent-hole was made in the conical tube, at the point where it begins to narrow. Small holes were made in the bottom of the polyallomer tube, and they were overlayed with a GF/C filter. The plastic tubes were inserted into the conical tubes, and they were packed with sufficient Sephadex G25 (medium), at 1500 rpm (600 g), 2 min, to three-quarters fill them. The gel was washed about 7 times, with 1 or 8 ml of buffer, at 1000 rpm for 2 min, before it was used to desalt a protein solution. The sample to gel volume ratio was kept between 0·17 and 0·27. The efficiency of desalting was checked using a solution of 2 mg/ml dextran blue + 1 mM DTT. This method was faster than dialysis, and there was no dilution as occurs on column chromatography.

Disadvantages were the critical dependence on sample to gel volume ratio, and repeated washings required to regenerate the gel.

2.2.04 Column chromatography.

i. Equipment.

An Ultrorac fraction collector (MK 104-1), a Uvichord II, a chopper-bar recorder (6520-8) and a Varioperpex pump (LKB-producter AB, S-161 25 Bromma 1, Sweden) were used. Small volumes (0·1 - 1·0 ml) were collected by resting small conical tubes (Eppendorf) on the rim of the normal test-tubes.

Chromatography columns were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and polythene capillary tubing from Portex Ltd., Hythe, Kent. A number of glass columns were made to complement the commercial range. Push in adaptors (Pharmacia) were used as ends for the column tops. The column bottom consisted of a glass sinter and a constriction, the dead space between them being filled with glass beads.

ii. Gel-filtration.

Gel-filtration media were prepared, used, and stored according to standard procedures (manufacturers handbooks; Reiland, 1971).

Columns of Sephadex G25(F) were used to change the buffer components of RNA-polymerase solutions, and to stop reactions (eg. amidination and thionitrobenzoylation). Gel-filtration was found to be preferable to dialysis, because dialysis led to the inactivation of the enzyme (sometimes 50 % in 18 h). 0·7 x 12, 0·9 x 8, 0·9 x 14, 1·0 x 30 and 1·3 x 30 (internal diameter x length, cm) columns were used, with flow rates of 30 ml/h for the narrower columns, and 60 ml/h for the wider columns. Fractions of 0·1 - 1·0 ml were collected. The column size was chosen so that the bed volume was at least ten times greater than the sample volume. The columns were calibrated with a solution containing 2 mg/ml dextran blue, 1mM DTT or 0·1 M NaCl, and 0·5 mg/ml

ε DNP lysine. Chloride ions were detected with ammoniacal silver nitrate, and DTT with DTNB.

A column of Sephadex G50(F) was used to determine the binding-constant of Mg-ATP to RNA polymerase (core), by the method of Hummel & Dreyer (1962). A 0·1 ml sample of RNA polymerase (core), 10 mg/ml in elution buffer, was applied to a 0·5 x 28 cm column, which had been equilibrated with buffer A + 0·2 M KCl + 1 μM [8-^{3}H] ATP (10 μCi/μM). The column was eluted at 4 ml/h, 120 μl (2 drop) fractions were collected. 10 μl samples were counted in 2 ml of 5g/l PPO in toluene: Triton X-100 : absolute ethanol (7 : 3 : 1). Protein was determined by the method of Lowry et al. (1951).

iii. Ion-exchange chromatorgraphy.

Ion-exchange chromatography was used to purify RNA polymerase (DEAE-cellulose and phosphocellulose), to purify ATP (DEAE-Sephadex A 25) and to concentrate σ factor (DEAE-cellulose).

DEAE-Sephadex A 25 was preswollen in 1M triethylammonium bicarbonate (pH 7·5) for 24 h at room temperature, and then resuspended in several changes of 0·1 M triethylammonium bicarbonate (pH 7·5). It was equilibrated with several column volumes of 0·1 M triethylammonium bicarbonate (pH 7·5), at 4°C, until the pH and conductivity of the effluent was equal to that of the ongoing buffer.

DEAE-cellulose (DE 52), although purchased in a preswollen form, was precycled to ensure that it had its full capacity for protein (see Himelhoch, 1971). The exchanger was suspended in 0·5 M HCl (15 volumes per gram) for 30 min, washed to pH 4, suspended in 0·5 M NaOH for 30 min, washed to pH 8, suspended in 50 mM Tris HCl, pH 7·9, and titrated to pH 7·9.

Phosphocellulose (PC 11) was precycled in the same manner as DEAE-cellulose, except that the exchanger was suspended in 0·5 M NaOH first. This first step resulted in the release of a brown-coloured substance,

and ammonia, from the exchanger. The precycled exchanger was suspended in 50 mM Tris HCl, pH 7·9 at 20°C, and was titrated back to pH 7·9 with 6 M NaOH. The exchanger was allowed to stand for about 6 h at room temperature, and was retitrated to pH 7·9.

The exchangers were de-fined, and the columns poured from a thick suspension at 45 ml/h/cm^2. Columns were equilibrated at 4°C, until the pH and conductivity of the effluent and ongoing buffer were equal. This usually required about 4 column volumes for DEAE-cellulose, and 10 column volumes for phosphocellulose.

The components of interest were eluted from the ion-exchangers with steps, or a linear gradient, of increasing ion-strength. Linear gradients of salt (and glycerol) were produced using gradient-makers constructed from two interconnected containers of the same shape, and open to the air. They were mounted vertically, and were filled to the same level with the buffers containing the salt (glycerol) concentrations corresponding to the beginning and end of the gradient. The low salt (or high glycerol) buffer was placed in the stirred side connected to the column(centrifuge tube).

iv. Affinity chromatography.

DNA-agarose affinity chromatography was used to study the DNA binding ability of NBS-modified RNA polymerase.

DNA-agarose was prepared as described by Schaller et al. (1972). One gram of calf-thymus DNA (Koch-Light) dissolved in 67 ml of 0·02 M NaOH was heated to 50°C. A 4 % solution of agarose (Sigma) was autoclaved for 10 min at 10 psi, and it was rapidly cooled to 50°C. The two solutions were mixed, and rapidly solidified by pouring onto an ice-cooled glass dish. The gel was cut into small pieces, and was passed twice through a 60 mesh stainless-steel net placed in a syringe barrel. The fragments were suspended in a solution containing 10 mM Tris HCl pH 7·9, 1 mM EDTA, and 0·1 M NaCl, and were poured into a

column. The column was washed with the same solution until no further DNA eluted. The gel was stored in a solution containing 10 mM Tris HCl pH 7·9, 1 mM EDTA and 1·0 M NaCl, at 4°C. Little DNA was found to have eluted after 6 months storage (< 5 %). The DNA content was determined by dissolving 0·5 ml (bed volume) of DNA-agarose in 5 ml of 5 M $NaClO_4$ on a boiling water bath for 5 min. The absorbance at 260 nm was compared with a blank prepared from 2 % agarose. It was found that 70 % of the input DNA was retained on the agarose, or about 2·5 mg DNA per ml agarose.

Columns were equilibrated with at least 5 column volumes of buffer. Flow rates during packing and running did not exceed 12 $ml.h^{-1}.cm^{-2}$. Flow rates during loading were 0·5 - 1 column volumes per hour.

2.2.05 Cleaning laboratory apparatus.

i. Cuvettes.

Cuvettes were washed by soaking overnight in chromic acid (6·3 g potassium dichromate dissolved in ~30 ml of water, and then added with stirring to 1 l of concentrated sulphuric acid), followed by extensive rinsing in distilled water. The cuvettes were dried by rinsing with methanol (AnalaR). They were inverted to allow excess solvent to drain off, and they were blown-dry with filtered air.

ii. Dialysis tubing.

Dialysis tubing was washed just before use. Visking tubing was boiled for 15 min in 5 % sodium bicarbonate, rinsed in distilled water, boiled in 0·5 % EDTA, boiled in distilled water, and finally rinsed in distilled water. Spectrapor 2 membrane was washed in several changes of distilled water for 3 - 4 h.

iii. Glassware.

Laboratory glassware was rinsed after use, and soaked overnight in 0·4 % Haemo-Sol (Alfrex Cox (Surgical) Ltd., Coulston, Surrey, U.K.).

The glassware was washed, rinsed in tap water, followed by distilled water, and dried in an oven at 70°C.

Tubes for RNA polymerase assays were rinsed after use, soaked overnight in chromic acid, rinsed with distilled water, and oven dried.

Glassware contaminated with radioactivity was soaked for at least a week in 3 changes of 2 % Decon (Decon Ltd., Brighton, U.K.), and then rinsed in running water for several hours. It was then washed as normal laboratory glassware. Glassware which had been contaminated with more than 10 μCi of radioactivity was discarded.

Pipettes were soaked in 0.4 % Haemo-Sol. They were washed in a pipette-washer, rinsed in distilled water, and dried in a pipette-drier. Constriction pipettes were soaked in 2 % Decon after use. They were washed by sucking detergent, distilled water, and methanol through the pipettes with a water pump.

2.2.06 Computation.

A PDP/8L computer (Digital Equipment Co. Ltd., Reading, U.K.) was used to perform routine calculations. Programmes were written in Focal, 1969 to do the following:

i. analyse formaldehyde melting curves in terms of degree of unwinding, and fit the data to a straight line by a weighted least-squares method.

ii. to convert data from the amino-acid analyser to mole fraction, and number of amino-acids per molecule of protein.

iii. fit data to a straight line by the least-squares method, and calculate the standard error of the intercept and gradient.

iv. fit data to a quadratic equation using the least-squares method.

v. to correct the absorbance at 280 nm of a protein for light-scattering by linear extrapolation of log (absorbance) v log (wavelength) from the 320 - 450 nm region of the spectrum.

vi. to determine the mean, standard deviation, and standard error of a

set of data.

The computer programmes can be found in Appendices 1 - 6.

2.2.07 Concentration determination.

i. Formaldehyde.

Formaldehyde concentrations were determined by the hydroxylamine hydrochloride method. 0·3 g formaldehyde, 25 ml water, and 2 drops of bromophenol blue (0.5 % in ethanol) were carefully neutralised with 0·1 M NaOH. 10 ml of 10 % hydroxylamine hydrochloride was added, stirred for 10 min, and the liberated HCl was titrated with 0·1 M NaOH. A blank, lacking formaldehyde, was also titrated.

ii. Glycerol and polyethylene glycol.

Their concentrations were determined by comparing their refractive index with that of solutions of known concentration. The refractive indices were measured with an Abbé refractometer (Bellingham and Stanley Ltd., London, U.K.) and a sodium lamp.

iii. Isotopes.

Scintillation counting was used to quantitate compounds labelled with tritium and carbon-14. Homogeneous solutions were counted in a Phillips PW 4510/01 (Phillips Co. Ltd., Holland). This instrument corrects for quenching by the external-standard, channels-ratio method, and the output is in the form of disintegrations per minute. Occasionally a Nuclear-Chicago Isocap 300 scintillation counter (Nuclear-Chicago Corporation, Illinois 60018, U.S.A.) was used. The output was in the form of total counts per channel. The channel-ratios were checked to ensure that quenching was similar in all the samples counted.

Since tritium emits a low-energy β particle, the possibility of self-absorption arises when it is counted in a heterogeneous solution. Heterogeneous samples (in the form of $[^3H]$ RNA and $[^3H]$ ATP dried onto GF/C filters) were solubilised with 0·5 ml hyamine hydroxide

(1 M in methanol) at 60°C for 40 min. The solution was counted in 10 ml of 0·5 % PPO in toluene.

Polyacrylamide gel slices were solubilised by drying the gels at 60°C for 4 h, and then incubating them with 0·1 ml hydrogen peroxide (30 % w/v) for 4 h at 60°C. The isotope was counted in 3 ml of scintillant (0·5 % PPO in 2 volumes toluene + 1 volume triton X-100).

Aqueous samples were counted in 0·5 % PPO in 2 volumes toluene + 1 volume Triton X-100. The miscibility range was 0 - 50 µl and 250 µl - 1 ml in 3 ml of scintillant.

iv. Nucleoside triphosphates.

The concentration of nucleoside triphosphates was determined using the extinction coefficients given below:

	λ max (nm)	ε (l. mol^{-1}. cm^{-1}) x 10^{-3}
ATP	259	15·4
GTP	252	13·7
CTP	271	9·1
UTP	262	10·0

Each nucleoside triphosphate was dissolved in water to a concentration greater than that required. Aliquots were diluted with 10 mM KPi pH 7·0, and the absorbance was determined.

v. Proteins.

Protein concentrations were determined by a number of methods, depending upon the nature of the sample.

The concentration of pure, native RNA polymerase was determined using the extinction coefficient $E^{1\%}_{280} = 6{\cdot}5$ (Richardson, 1966). Dust was removed by filtration through a GF/F glass fibre filter. RNA-polymerase (core) aggregates in solutions of low ionic strength (< 0·27; Berg & Chamberlin, 1970), and this leads to light-scattering. The light-

scattering contribution to the absorbance at 280 nm was estimated by extrapolating a plot of log (apparent absorbance) versus log (wavelength) from the region 320 - 450 nm (Wetlaufer 1962). The gradient of this line was usually 4 ± 1. No light-scattering correction was necessary for filtered, high ionic strength solutions.

The concentration of protein in crude fractions of RNA polymerase was determined by the method of Waaburg & Christian (described by Layne, 1957), or by the method of Lowry et al. (1951) using a BSA standard ($E^{1\%}_{278} = 6.6$, Tanford & Roberts, 1952).

With dilute solutions of RNA polymerase, the normal buffer constituents Tris, triethanolamine, EDTA, and DTT interfere with the Lowry method. In this case the method of Schaffner & Weissmann (1973) was used. However, since this method depends partially on an ionic interaction between a dye and protein, the method of Murty & Leroux (1975) was used for dilute amidinated RNA polymerase solutions. Calibration curves were prepared using RNA-polymerase solutions of known concentration.

Occasionally the protein concentration of chemically modified RNA polymerase was determined by amino-acid analysis. 0.1 mg of protein, an equal volume of Aristar HCl, and 2-mercaptoethanol (1 %) were evacuated for 10 min with a vacuum pump, and then incubated at 110°C for 24 h. The HCl was removed under vacuum, and the resulting amino-acids determined on a Jeol S AH amino-acid analyser. The protein concentration was determined from the total recovery of an amino-acid relative to the recovery from a sample of known concentration. The results with leu, ile, val, thr, asp, pro, gly, and ala agreed to within 10 %. Occasionally norleucine or L-α-amino-β-guanidinopropionic acid internal standards were used. In this case the concentration was determined from the relative yields of amino acid and standard in the samples of unknown and known concentration. The results agreed within 10 % with

those determined from total recovery. This method was not used extensively because of the uncertainty arising from a decreased yield of amino-acids such as tyrosine and methionine, and since it was tedious in comparison with other methods.

vi. Salts.

Salt concentrations were determined by comparing the conductivity with solutions of known concentration. The conductivity was determined with a conductivity cell and meter (type CDM 2e, Radiometer, Copenhagen, Denmark).

2.2.08 Concentration of dilute RNA polymerase solutions.

Dilute solutions of RNA polymerase (< 0.2 mg/ml) could not be concentrated with ammonium sulphate because the enzyme lost activity, and the fine precipitate led to considerable loss of yield. No activity or yield was lost using perosmosis against polyethylene glycol, or ultrafiltration under N_2 (see table below).

RNA polymerase (0·2 mg/ml) was dialysed into buffer A + 20 % polyethylene glycol at $4^{\circ}C$ until its volume was reduced sufficiently to give a protein concentration of 3 - 5 mg/ml (about 5 h). Its volume was reduced further, and low molecular weight polyethylene glycol removed, by dialysis into storage buffer. An identical sample was dialysed into buffer C + 50 % saturated ammonium sulphate, centrifuged at 30,000 rpm, 1 h, in a Beckman '30' rotor, and the pellet was taken up in storage buffer. Another sample was concentrated using an Amicon ultrafiltration cell (Model No.52), and a PM 30 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.), followed by dialysis into storage buffer. Aliquots were used to determine the activity and absorbance at 280 nm. Ultrafiltration was used in subsequent experiments.

Table : Specific activity and yield of stored enzyme.

Method	Specific activity (%)	Yield %
Original enzyme	100	100
Ultrafiltration	100	98
Polyethylene glycol 20M	91	90
Ammonium sulphate	63	55

2.2.09 Peptide mapping.

Peptide mapping was used to identify the tryptophan peptides of RNA polymerase. RNA polymerase was carboxymethylated, digested with a variety of proteases, and mapped by the method of Bates et al. (1975).

RNA polymerase was carboxymethylated by dialysing a 1 mg/ml solution into 0·1 M Tris HCl, pH 8·2. Urea was added to 8M, DTT to 2 mM, and the solution was flushed with oxygen-free nitrogen. The solution was capped and stored in the dark, at room temperature, for 1 h. It was made 10 - 15 mM in iodoacetate (recrystallised from light petroleum, boiling range 40 - 60°C), flushed with N_2, and left in the dark for 1 h at room temperature. The sample was desalted on a G 25 (F) column into 0·5 % ammonium bicarbonate (pH 8·0).

The sample was concentrated by freeze-drying, and was redissolved in 0.5 % ammonium bicarbonate to 1 mg/ml. Samples were digested with the following combinations of proteases:

1) trypsin for 4 h at 37°C
2) trypsin for 4 h at 37°C, followed by chymotrypsin for 4 h at 37°C
3) trypsin for 4 h at 37°C, followed by thermolysin for 2 h at 60°C
4) thermolysin for 2 h at 60°C
5) subtilisin for 4 h at 37°C.

The solutions were boiled for 2 min after each proteolysis step. The

protease concentration was 2 % w/w, added from a 1 mg/ml solutions in 1 mM HCl.

The peptides were recovered by freeze-drying, dissolving the peptides in a small volume of 0·02 M NH_3, and transferring them to a small test tube. The tubes were centrifuged to bring the sample to the bottom, freeze-dried, and the resulting peptides taken up in sufficient 0·02 M NH_3 to make a solution >100 mg/ml.

Silica coated thin-layer plates (Polygram Sil G, 0·25 mm silica gel layer on a plastic sheet, 20 x 20 cm) were cut to the required size (10 x 10 cm). Samples of the RNA polymerase digest were applied to the silica sheets using a 1 µl microcap (Drummond Scientific Co., U.S.A.), to give a spot 1 - 2 mm in diameter. The sheets were dried between individual applications with a stream of cool air. 100 - 400 µg of RNA polymerase was applied to each sheet. The buffer was evenly applied to the sheets using wetted Whatman No.1 paper, placed 2 mm on either side of the origin. Further buffer was applied to the paper. The sample became wet by capillary action, and this helped concentrate the spot. The buffers used were:

1) 10 % pyridine, 0·5 % acetic acid, pH 6·5.

2) 0·5 % pyridine, 5 % acetic acid, pH 3·5.

3) 8 % acetic acid, 2 % formic acid, pH 2·0.

Electrophoresis was carried out at 500 V, 20 min on a home-made thin-layer electrophoresis apparatus (described later), with a BTL 500 power pack (Baird and Tatlock (London) Ltd., Chadwell Heath, Essex, U.K.). The sheets were thoroughly dried (20 - 30 min in a stream of cool air), and chromatographed upwards in the second dimension in butan-1-ol: acetic acid: pyridine: water (15: 3: 12: 10 by vol.) as described by Waley & Watson (1953). A set of marker amino-acids was occasionally electrophoresed under the same conditions as the RNA polymerase digest. The marker contained DNP asp, ε DNP lys, lys, his, gly, asp, trp, all

at 1 mg/ml.

Ninhydrin was used to detect peptides. The sheets were dipped in the ninhydrin-cadmium solution made up as follows:

15 ml of solution B was mixed with 85 ml solution A just before use.

solution A : 1 % ninhydrin in acetone

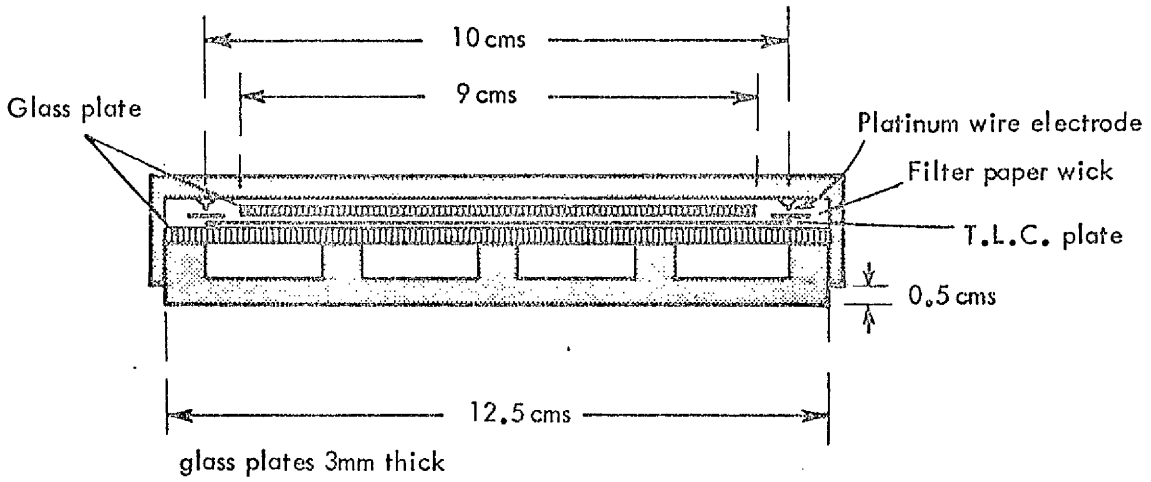
solution B : 1 g cadmium acetate, 50 ml acetic acid, 100 ml water. The sheets were allowed to dry at room temperature, and the colours formed were observed. The sheets were then heated in an oven at 100°C for a few minutes.

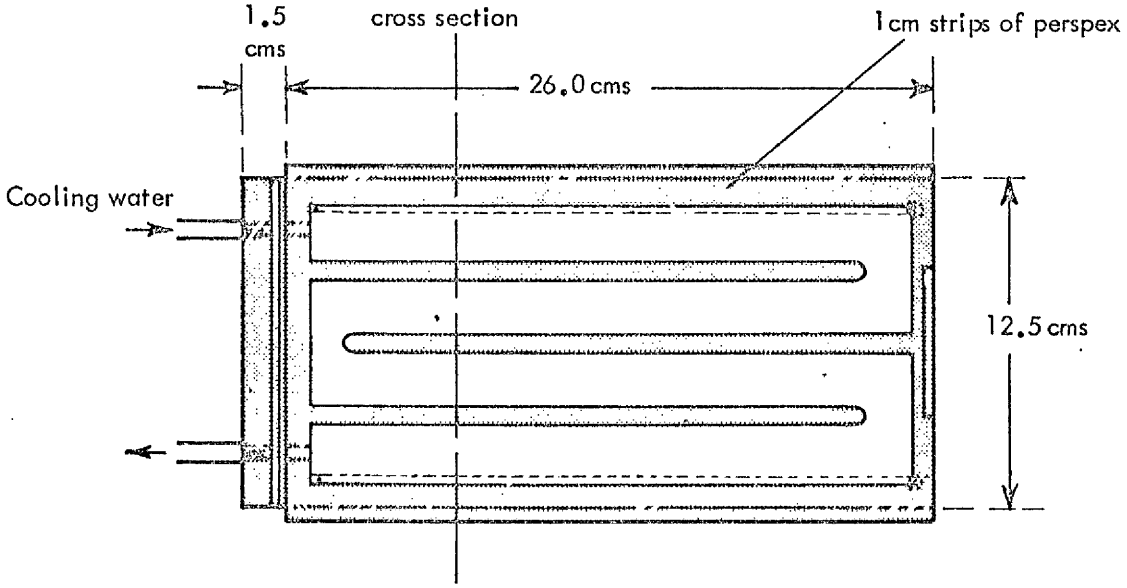
Tryptophan peptides were detected by dipping the sheets in 1 % p-dimethylaminobenzaldehyde in acetone, containing 10 % concentrated HCl. On standing at room temperature the tryptophan peptides gave a purple colour which gradually faded.

Care was taken to prevent contamination of silica-gel sheets with extraneous peptides and amino-acids. Plastic gloves were used for all manipulations. All glassware was acid washed, and a specially clean area was used to load the silica-gel sheets.

An apparatus, designed and built by Mr. N.L. Harvey, was used for electrophoresis. The cooling plate was constructed from a 26 x 12·5 x 0·3 cm glass sheet, and pieces of perspex sheeting (0·5 cm thick) glued together as in Fig.2.4. The electrodes were made of platinum wire (0·046 cm diameter, fixed 10 cm apart on a perspex lid). The apparatus was cooled by running cold tap water through the cooling plate. A glass plate was placed on top of the silica sheet to ensure good thermal contact with the cooling plate, and to minimise solvent evaporation. Thin pads of filter paper provided electrical contact between the silica sheets and the electrodes. The apparatus was large enough to run two 10 x 10 cm sheets.

Fig 2.4 Electrophoresis apparatus.





2.2.10 Polyacrylamide gel electrophoresis.

i. SDS gels.

The method is based on that described by Weber et al. (1972). The sample for electrophoresis was prepared by adding sufficient incubation buffer (0·01 M sodium phosphate pH 7·2, 1 % SDS, 1 % 2-mercaptoethanol) to the protein to give a concentration of 0·05 - 1 mg/ml, and an SDS to protein ratio of 3 to 1. The sample was incubated at 100°C for 2 min, and then dialysed overnight, at 37°C, against 0·01 M sodium phosphate pH 7·2, 0·1 % SDS, 0·1 % 2-mercaptoethanol. For dilute solutions, a final dialysis into the above buffer containing 50 % glycerol was used to increase the concentration (upto 5 fold). 1 volume of bromophenol blue marker (10 ml saturated bromophenol blue + 90 ml glycerol) was added to 4 volumes of sample. The final sample should have 1 - 20 µg of each polypeptide to be visualised in 50 - 200 µl.

7 cm gels were prepared in 0·6 x 10 cm tubes made from constant-bore glass tubing. The following solutions were mixed to give 5 % gels:

6·75 ml solution A (22·2 g acrylamide, 0·6 g methylenebisacrylamide, water to 100 ml)

6·75 ml water

15·0 ml gel buffer (0·2 M sodium phosphate pH 7·2, 0·2 % SDS)

The solution was evacuated for 1 min, and 1·5 ml of ammonium persulphate (15 mg/ml) and 0·045 ml of N N N′N′ tetramethylenediamine were added. The gel tubes were filled to a height of 7 cm, and the gel solution overlayed with a small volume of water. The gels were allowed to polymerise for at least 1 h at room temperature. The samples were loaded onto the gels, and electrophoresis was allowed to proceed at a constant current of 8 mA/tube, using the buffer 0·1 M sodium phosphate pH 7·2, 0·1 % SDS, until the dye reached the bottom of the tube (4 - 5 h at room temperature). The position of the dye was marked with a

stainless-steel wire. Gels were stained with 0.1 % Coomassie Brilliant Blue R 250 in 50 % methanol, 10 % acetic acid for 2 - 12 h at room temperature, and destained in 10 % methanol, 10 % acetic acid. The rate of destaining was faster at 37°C, and in the presence of ion-exchanger (AG 501 - X8 (D)). Protein bands could be seen after about 1 h and complete destaining took 1 - 2 days.

Molecular weights were estimated from the relative mobilities of the dye and protein bands on gel scans. Molecular weight standards were prepared from ovalbumin, aldolase, glutamate dehydrogenase and lysozyme by the method of Carpenter & Harrington (1972).

ii. Urea gels.

Samples were prepared by dialysing the protein against 0.005 M Tris, 0.04 M glycine, pH 8.3, and adding solid urea and 0.1 M DTT to 8 M and 10 mM respectively. The gel solution contained, in 25 ml, 0.75 g of "cyanogum" 41, 1.14 g Tris, 20 ml 10 M urea, 0.05 ml NN$\acute{N}\acute{N}$ tetramethylenediamine, 1.5 ml 1 M HCl, and 1 ml 7 % ammonium persulphate. The final pH was 8.9. Electrophoresis was performed using a buffer of 0.005 M Tris, 0.04 M glycine, pH 8.3, at a constant potential of 100 V, until the bromophenol blue marker had run off the bottom of the gel. Gels were stained and destained as for SDS gels. However urea gels took longer to destain and sometimes excess dye was removed electrophoretically. Gels were soaked in destaining solution (10 % methanol, 10 % acetic acid), and placed in 0.8 x 10 cm gel tubes sealed at the lower end with a short length of gel. Electrophoresis was carried out, with the solution 10 % methanol, 10 % acetic acid, at a constant voltage of 50 V, and with the original polarity reversed.

2.2.11 Purification of chemicals.

i. ATP.

The procedure described by Roach (1972) was used. The volatile buffer, triethylammonium bicarbonate, was prepared by bubbling CO_2 through a solution of triethylamine. Triethylamine (BDH) was redistilled before use, the fraction distilling between 88 and 89°C was collected, and stored in the dark at -10°C. A 1 M triethylammonium bicarbonate solution was prepared by bubbling precooled CO_2 into the solution (140 ml triethylamine in 1 l) cooled in an ice-bath, until the pH of a 1 : 10 dilution was 7·5 at 20°C.

A 2·5 x 30 cm column of DEAE Sephadex A 25 was equilibrated with 0·1 M triethylammonium bicarbonate (pH 7·5) at 4°C. 10 ml of 1·5 % [8-^{3}H] ATP (2 mCi/mM) was applied to the column. The ion exchanger was washed with two volumes of 0·1 M triethylammonium bicarbonate buffer, and eluted with a 450 ml 0·1 - 1·0 M linear gradient of buffer, at 36 ml/h. The large peak was pooled, and evaporated to dryness under vacuum at 25°C. The residue was twice redissolved in 100 ml of water, and evaporated to dryness.

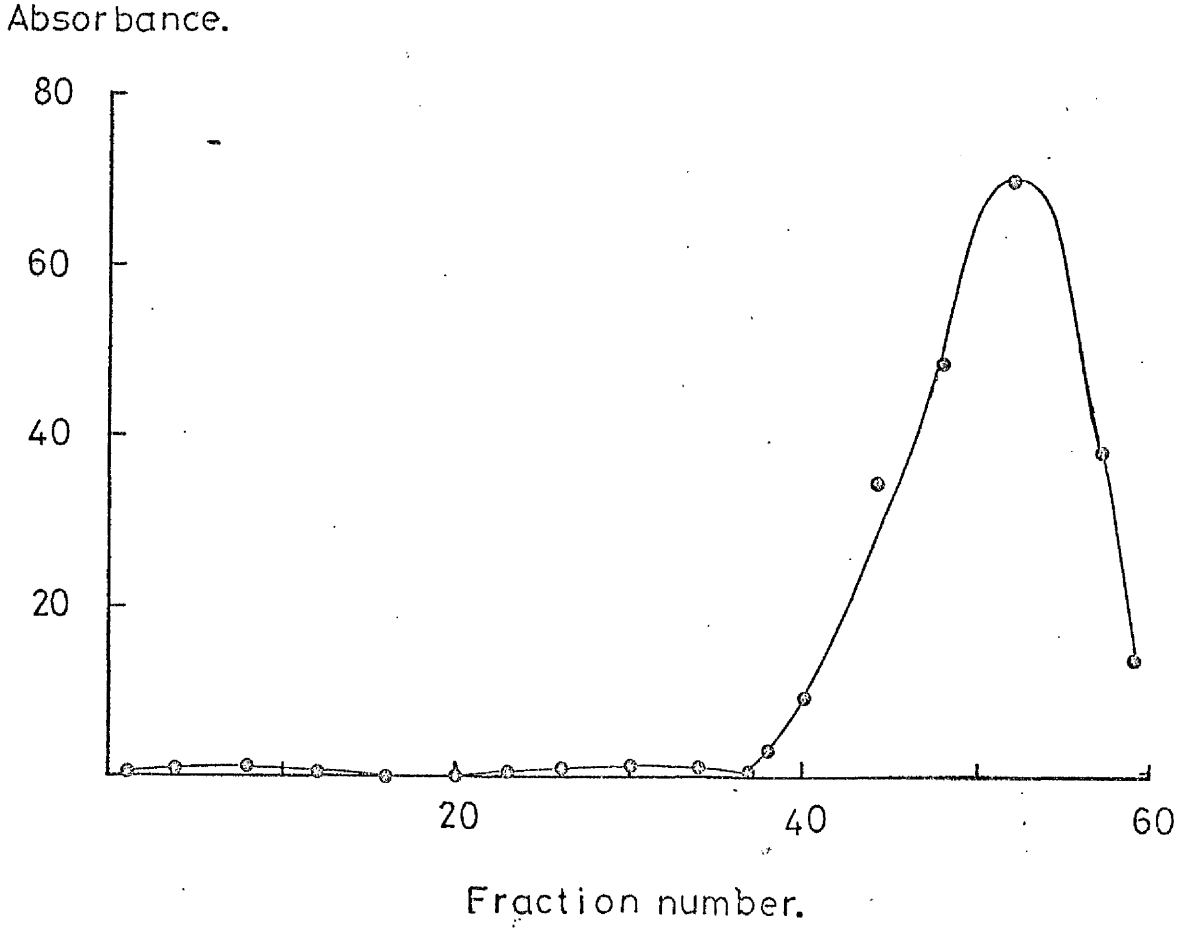
The three peaks, which eluted in positions corresponding to AMP, ADP and ATP, had the following relative sizes:

	Total absorbance (%)	Total radioactivity (%)
ATP	100	100
ADP	2	0·4
AMP	0·2	0·02

Purified [8-^{3}H] ATP, and unpurified [8-^{3}H] ATP in the presence of the polynucleotide phosphorylase inhibitor potassium phosphate, were found to give the same results in the assays of crude fractions of RNA polymerase. In general, unpurified [8-^{3}H] ATP, and 0·4 mM potassium

phosphate, were used in the assay of RNA polymerase.

Fig 2.5 Peaks eluted from DEAE-Sephadex A-25 with a linear gradient of 0·1–1 M TEAC, pH 7·5.



ii. Formaldehyde.

Formaldehyde was purified as described by Walker (1964). Paraformaldehyde was prepared by distilling formalin, collecting the fraction distilling at about 97°C. On cooling the distillate polymerises to give a white solid. Alternatively paraformaldehyde was prepared by storing formalin at -10°C. The white solid was washed with water, dried over phosphorus pentoxide, and stored. Solutions of formaldehyde

were prepared by decomposing the polymer in water, at pH 7·0, 100°C. Undissolved polymer was removed by filtration, and the formaldehyde concentration determined as described in section 2.2.07.

iii. Polyethylene glycol.

Polyethylene glycol 20 M is a polydispersed polymer, ranging in molecular weight from 6000 - 38,000. The small molecular weight species, which can diffuse through dialysis tubing, were removed as described by Polson (1973). A 5 % solution of polyethylene glycol was made 28 % in ammonium sulphate, and centrifuged at 5,200 rpm, ½ h, in the 6 x 750 head of the MSE 6L. The bottom layer, containing the high molecular weight species (20,000 - 38,000), was concentrated by extraction into chloroform. The chloroform was removed under vacuum.

iv. T7 DNA.

T7 DNA, purified by the method of Thomas & Abelson (1966), was a gift from Dr. A.D.B. Malcolm. Its 260 to 280 nm absorbance ratio was 1·63, and its sedimentation coefficient, in neutral sucrose, gave a molecular weight of 25 x 10^6, and in alkaline sucrose, 8 x 10^6.

v. Urea.

Urea (AnalaR) was recrystallised from 95 % ethanol. The hot saturated solution was filtered and cooled to 0°C. The crystals were filtered, washed with a little cold ethanol, and dried, first between sheets of Whatman 3 mm paper, and then under vacuum. Before use a 10 M solution was prepared, and deionised by passing it down a column of mixed bed resin (AG 501 - X8(D)).

2.2.12 RNA polymerase assays.

RNA polymerase was assayed for its ability to incorporate a radioactive precursor nucleoside triphosphate into acid insoluble RNA. The basic incubation conditions were:

Solution	Stock (mM)	Volume added (µl)	Final concentration (mM)
Calf-thymus DNA (Sigma I or Worthington)	4·5		0·9
Tris HCl, pH 7·9	200	50	40
EDTA	0·5		0·1
$MgCl_2$	150		12
$MnCl_2$	12·5	20	1
KCl	2,500		200
DTT	25	10	1
Potassium phosphate, pH 7·9	10	10	0·4
[8-^{3}H] ATP (~ 2 mCi/mM)	4		0·8
GTP	4	50	0·8
CTP	4		0·8
UTP	4		0·8

Water and enzyme were added to 250 µl. The DNA concentration was estimated using $\varepsilon(P) = 6{\cdot}6 \times 10^3$ (Mahler & Cordes, 1966). The stock solutions were stored at -20°C, in aliquots large enough for 50 assays.

The solutions were mixed at 0°C, incubated at 37°C for 10 min, and then rapidly cooled to 0°C. The reaction was stopped with 500 µl cold BSA (0·2 mg/ml) and 500 µl cold trichloroacetic acid (12·5 %), sodium pyrophosphate (1 %). The precipitate was allowed to stand for 15 min at 0°C, and it was filtered through a 2·5 cm GF/C glass fibre filter. The test-tube was washed with 4 x 3 ml aliquots, and the filter was then washed with 4 x 10 ml aliquots, of cold trichloroacetic acid (5 %), sodium pyrophosphate (1 %). The filter was then washed with 2 x 5 ml of

cold methanol, and dried under an infra-red lamp. The precipitate was solubilised with 0·5 ml hyamine hydroxide (1 M in methanol) for 40 min at 60°C, and counted in 10 ml of 0·5 % PPO in toluene. Blanks lacking either enzyme or DNA were used as controls.

A unit of enzyme activity was defined as the amount of activity which incorporated 1 nmole ATP into acid precipitable RNA under the above conditions. The counts in 10 µl of the nucleoside triphosphate stock solution was equivalent to 40 units.

Holoenzyme activity was measured with T7 DNA (60 µg/assay) instead of calf-thymus DNA. σ factor activity was measured with T7 DNA (60 µg/assay) and RNA polymerase (core) (20 µg/assay) replacing calf-thymus DNA. In this case the blank contained all the components except σ factor.

2.2.13 Spectrophotometry.

i. Absorption spectrophotometry.

Absorption spectrophotometry was used to measure the absorption spectra of solutions, and to scan polyacrylamide gels. An SP 8000 (Pye-Unicam Ltd., Crawley, Sussex, U.K.) was routinely used to measure the absorption spectra, in the range 210 - 750 nm, of solutions with volumes $\geq$ 1 ml. With solutions of low absorbance, a Cary 15 (Varian, Walton-upon-Thames, Surrey, U.K.) equipped with a 0 - 0·1 absorbance slide-wire was used. A Gilford 240 spectrophotometer, and a Servoscribe recorder, RE 511.20 (Gilford Instruments Ltd., Teddington, Middlesex, U.K.) were used to measure the absorbance of small volumes of solution (0·3 ml), to scan stained polyacrylamide gels, and to monitor density gradients from the ultracentrifuge.

The wavelength calibration of the SP 8000 was occasionally checked with the holmium and didymium filters supplied with the instrument. A solution of potassium chromate was used to check the absorbance

calibration (Haupt, 1952). The temperature was controlled using a thermostated cell holder, or a jacketed cell and a thermostated circulating water-bath (Haake, type F4391, Berlin, Germany). The temperature inside the thermostated cuvette (± 0·5°C) was determined from the absorbance of a solution of cresol-red in 3·3 % ammonium chloride, pH 8·5. The absorbance of this solution was found to have a useful temperature dependance in the range of 25 - 95°C, and to decrease, on average, ~ 0·020 D/°C. The cresol-red solution was calibrated with an SP 800 spectrophotometer equipped with a thermostated heating block. The temperature of the cresol-red solution was assumed to be equal to that in a similar cuvette equipped with a thermocouple and a calibrated galvanometer (Pye-Unicam).

Stained polyacrylamide gels (0·6 x 7 cm) were scanned in a 0·6 x 1·0 x 10 cm cuvette (type 2414, Gilford), at 1 cm/min and with a slit of 0·05 x 2·36 mm. The relative amounts of protein in the bands was determined by weighing tracings of the scan peaks.

ii. Fluorescence.

Fluorescence measurements were made with a Hitachi, model HPF-2A, fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan), equipped with a thermostated cell holder. Fluorescence measurements were made at 25 ± 0·1°C, with 1 cm pathlength fluorimetric cells requiring a 2 ml sample volume.

Emission and excitation spectra were determined as described in the instruction manual supplied with the instrument. A number of precautions were necessary to ensure that reliable fluorescence data were obtained. The concentration of RNA-polymerase was kept below that at which fluorescence became a non-linear function of concentration. The maximum concentration was determined from the equation:

$$C_{max} = \frac{0.05}{a_\lambda \times b}$$

C_{max} = concentration above which fluorescence becomes a non-linear function of concentration.

a_λ = molar absorptivity at the wavelength of excitation.

b = sample path length along axis of excitation.

To ensure that good resolution of the emission peak was obtained, the band-pass was chosen so that it was less than $1/5$ of the half-band width (the band width at half the intensity maximum). Since solvent components can interfere with fluorescence measurements, it was shown that solvent alone, and solvent and NBS (a varying solvent component in some experiments), gave no emission bands under the conditions used. It was shown that the fluorescence emission spectrum of RNA polymerase did not vary in the length of time it took for an experiment to be completed. Fluorescence spectra are temperature sensitive, and ordinarily one would expect a 1 to 2 % decrease/^{o}C increase in temperature. The sample was allowed to warm up for several minutes in a special cell-holder outside the instrument, thermostated at the same temperature as the fluorimeter, before its spectrum was recorded.

CHAPTER 3.

PURIFICATION OF RNA POLYMERASE.

3.1. Introduction.

Three general methods have been used to purify RNA-polymerase from large quantities of *E. coli*. They differ in the way the enzyme is separated from the tightly bound RNA polymerase-DNA-RNA complex present in the cell (Bremer & Konrad, 1964). Berg, Chamberlin, and their co-workers (Berg *et al.*, 1971; Mangel, 1974) have used a combination of DEAE-cellulose and protamine-sulphate, whilst Zillig *et al.* (1970a) employed polymin P extraction. A third method utilises DNA ase digestion, ultracentrifugation and ammonium sulphate fractionation (Burgess & Travers, 1971). After the enzyme has been separated from the nucleic acid, it may be purified by standard procedures. Two useful properties of the enzyme are its ability to bind to both anionic and cationic ion-exchangers at pH 8, and its salt dependent monomer dimer equilibrium (Burgess, 1969a).

Since chemical and structural studies generally require large quantities of enzyme, an attempt was made to purify RNA polymerase from large quantities of *E. coli*. Using a modification of the Burgess & Travers (1971) procedure, the enzyme has been successfully purified from up to 1 kg (wet weight) of *E. coli*.

3.2. Materials.

Stock solutions:

1 M Tris.HCl, pH 7·9, at 20°C

1 M Tris HCl, pH 7·5, at 4°C

1 M $MgCl_2$

0·1 M Na EDTA, pH 7·0

0·1 M DTT.

Stock solutions were stored frozen.

Buffers:

G : 50 mM Tris HCl, pH 7·5

10 mM $MgCl_2$

200 mM KCl

0·1 mM Na_2EDTA

0·1 mM DTT

5 % (v/v) glycerol.

A : 10 mM Tris HCl, pH 7·9

10 mM $MgCl_2$

0·1 mM Na_2EDTA

0·1 mM DTT

5 % glycerol.

C : 50 mM Tris HCl, pH 7·9

0·1 mM Na_2EDTA

0·1 mM DTT

5 % glycerol.

Storage buffer:

10 mM Tris HCl, pH 7·9

10 mM $MgCl_2$

100 mM KCl

0·1 mM Na_2EDTA

0·1 mM DTT

50 % glycerol.

Buffer A + 1·0 M KCl meant the solution contained, in addition, 1·0 M KCl. Buffers were freshly prepared before use.

Cells: E. coli MRE 600 were obtained from The Microbiological Research Establishment, Porton, Salisbury, England. The cells were grown in continuous culture, at 37°C, under conditions of carbon limitation, and processed as described by Elsworth et al. (1968). The cells were washed with a buffer containing 10 mM Tris HCl, pH 7·4, 10 mM $MgAc_2$ and stored at -70°C.

3.3. Methods.

The procedure is summarised in Fig 3.01.

Cell disruption.

The frozen cells (400-1000 g) were broken into small pieces with a mallet, and suspended in buffer G (1 ml per g cells) in an Atomix blender (M.S.E.). 90 ml portions were homogenised, in the presence of cold glass beads (0.1 mm), by shaking at 80 Hz for 2 min, at 4°C, in a Zillig homogeniser (Zillig & Holzel, 1958). The extract was poured into a beaker, and DNA ase I (0·5 mg per 100 g cells) was added. The pH of a 1 : 10 dilution was adjusted to 7·5 (at 4°C) and the extract was allowed to stand for 30 min at 4°C. Separation of the extract from the glass beads was achieved by decanting the supernatant from the settled beads, washing them several times with buffer G, and extracting them with gentle suction. Alternatively the cells were homogenised by stirring in an Atomix blender, at medium speed, until the cells had completely thawed, and then for a further 20 min. Occasional cooling in a solid CO_2 - glycol mixture was used to keep the temperature between 0 and 5°C. The extract was blended for a further 30 s with DNA ase I, the pH adjusted to 7·5, and the extract allowed to stand for 30 min at 4°C. Sufficient buffer G was added to adjust the volume to 200 ml per 100 g cells (Fraction I).

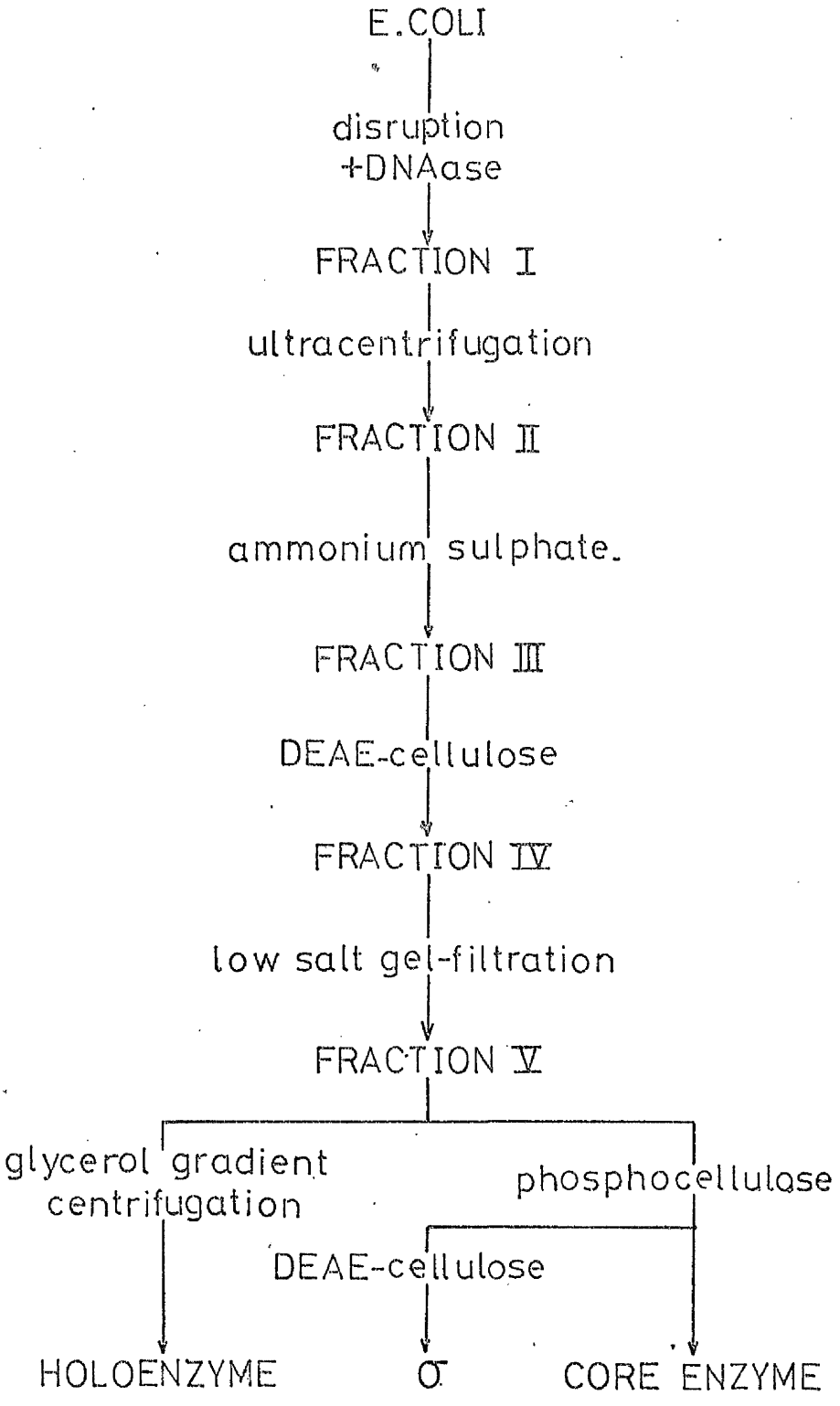
High-speed centrifugation.

Fraction I was centrifuged at 30,000 rpm for 2½ h, at 4°C, in a Beckman 30 rotor, or at 25,000 rpm for 3½ h, at 4°C, in an MSE 10 x 100 ml rotor. About 140 ml per 100 g cells of clear amber supernate was obtained (Fraction II).

Ammonium sulphate fractionation.

An ammonium sulphate cut, from 30 to 47 % saturation, was taken,

Fig 3·01 Summary of purification procedure



and backwashed twice with 42 % ammonium sulphate (Burgess & Travers, 1971). The pellet was dissolved in buffer A (about 100 ml per 100 g cells) to a conductivity less than or equal to buffer A + 0·13 M KCl (Fraction III).

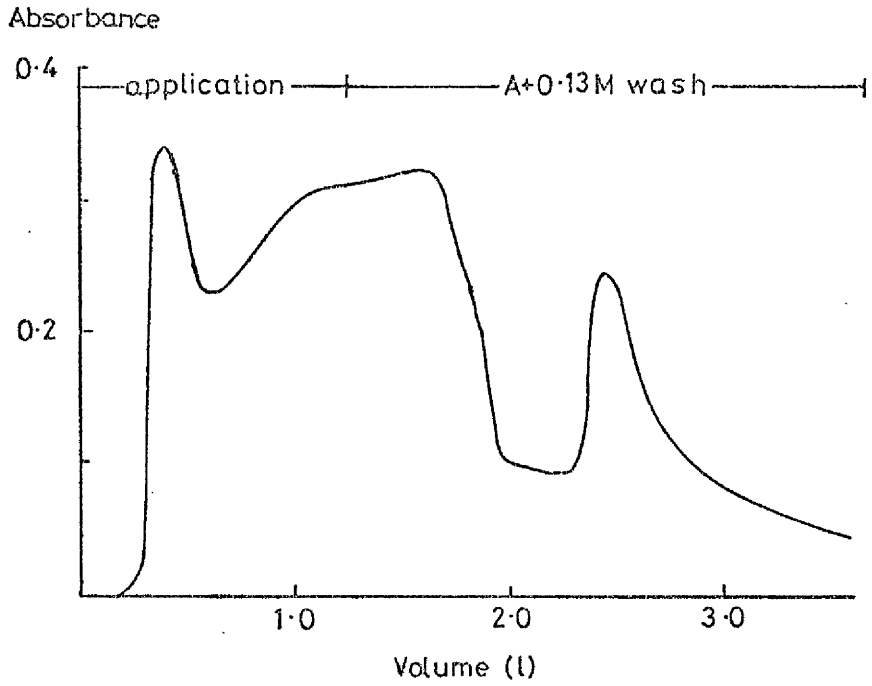
DEAE - cellulose.

DEAE-cellulose was prepared as described in section 2.2.04. The ion-exchanger (50 ml bed volume per 100 g cells) was equilibrated with buffer A, at 4°C. Fraction III was applied at 0·5 - 1·0 column volumes per h, the column was washed with 3 - 4 volumes of buffer A + 0·13 M KCl, and the enzyme eluted with a linear salt gradient from A + 0·13 M to A + 0·30 M KCl. (300 ml per 100 ml bed volume). RNA polymerase eluted at 0·17 M KCl (Fig 3.02), just before a peak of nucleic acid (Fraction IV). The 280 : 260 nm absorbance ratio was checked, to ensure it was greater than 1·1, before low salt gel filtration.

Low salt gel filtration.

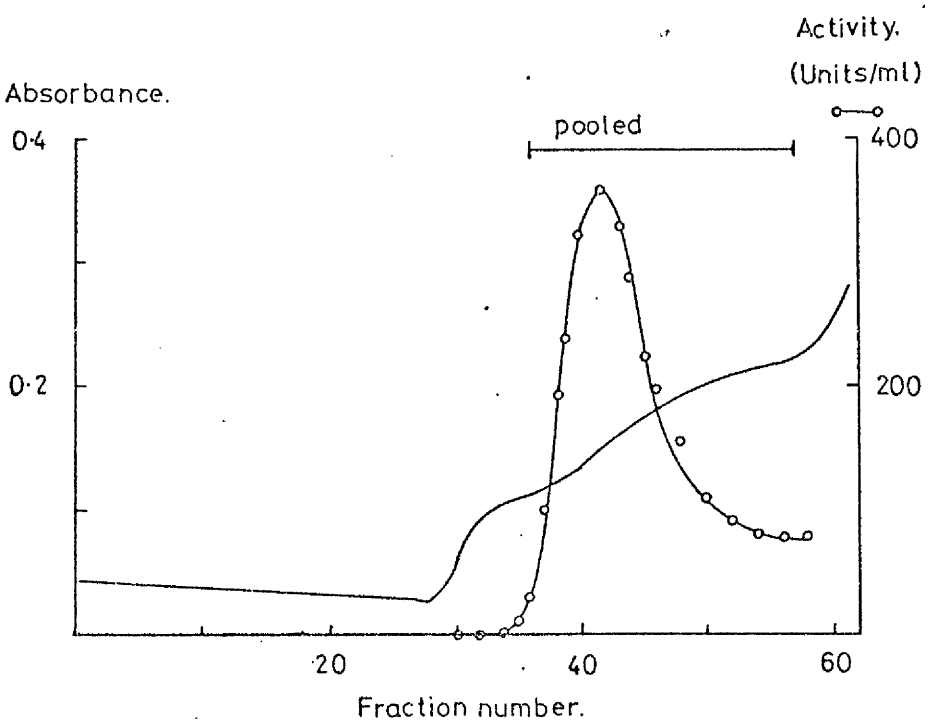
Fraction IV was concentrated by adding 35 g of ammonium sulphate per 100 ml. The suspension was stirred for 30 min at 4°C, centrifuged for 30 min at 10,000 rpm, and the pellet dissolved in 10 ml buffer C. A 5 x 60 cm column of Bio-Gel A 5m was equilibrated with buffer C at 4°C. The enzyme was applied and eluted at 60 ml per h (Fig 3.03). It eluted at 1·45 times the void volume (Fraction V).

Fig 3·02a DEAE-cellulose chromatography, application and wash.



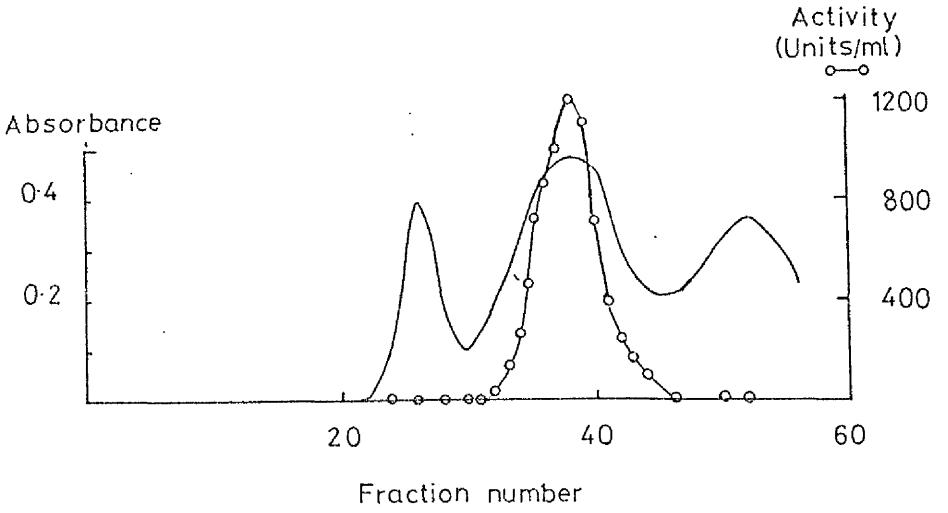
Fraction III, from 1000 g cells, was applied to a 500 ml (5 x 25 cm) DEAE-cellulose column. The ion-exchanger was washed with 2·5 l of buffer A + 0·13 M KCl. (Flow rate = 300 ml/h).

Fig 3·02b DEAE-Cellulose, gradient elution.



The enzyme was eluted with a linear, 1·5 l, A + 0·13 - A 0·30 M KCl gradient (Fraction size = 15 ml).

Fig 3·03 Agarose chromatography in low salt.

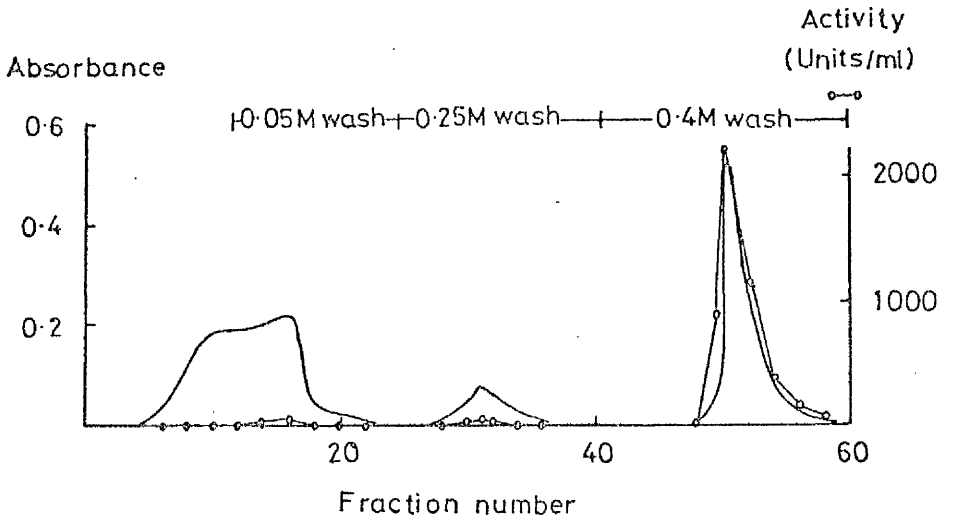


Fraction IV, dissolved in 10 ml of buffer C, was applied to a 5 x 60 cm Bio-Gel A 5m column. The column was eluted at 60 ml/h, 12 ml fractions were collected.

Phosphocellulose chromatography.

Phosphocellulose was prepared as described in section 2.2.04. The ion-exchanger (20 ml bed volume per 100 g cells) was equilibrated with buffer C + 0·05 M KCl at 4°C. Fraction V was adjusted to 0·05 M KCl, and applied at 0·5 - 1·0 column volumes per h. After washing the ion-exchanger with 2 - 4 column volumes of buffer C + 0·05 M KCl, followed by buffer C + 0·25 M KCl, the enzyme was eluted with buffer C + 0·4 M KCl (Fig 3.04). It was concentrated by ultrafiltration under N_2 (see section 2.2.08) to about 5 mg per ml, and dialysed against storage buffer. The enzyme was stored at -20°C, at a concentration of 5 mg per ml or greater.

Fig 3·04 Phosphocellulose chromatography

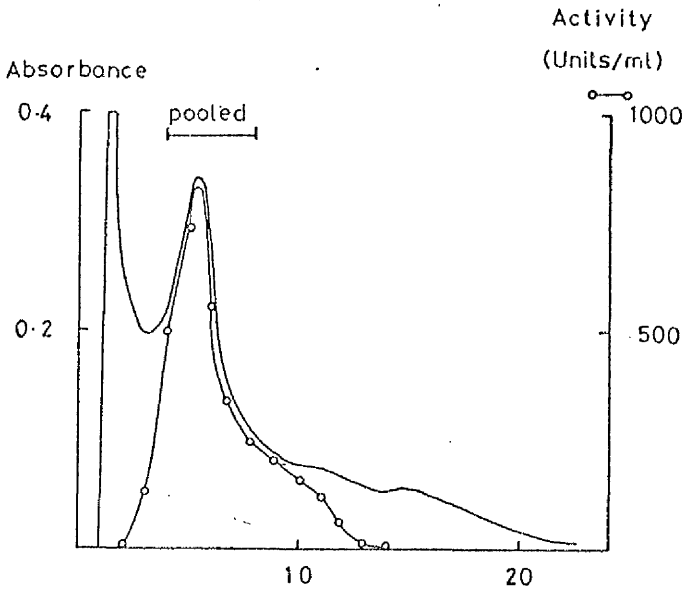


Fraction V, (1000 g cells), in buffer C + 0·05 M KCl, was applied to a 150 ml (2·6 x 28 cm) phosphocellulose column. The column was washed with 300 ml of buffer C + 0·05 M KCl, 300 ml buffer C + 0·25 M KCl, and buffer C + 0·4 M KCl. (Flow rate = 150 ml/h, fraction size = 12 ml).

Glycerol - gradient centrifugation.

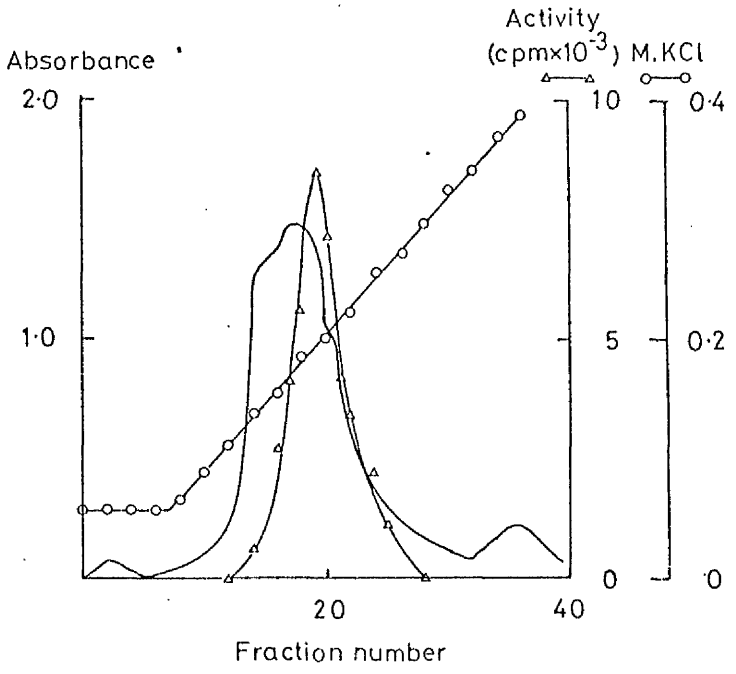
Fraction V was concentrated by adding 35 g ammonium sulphate to 100 ml. After stirring for 30 min at 4°C, and centrifugation for 30 min at 10,000 rpm, the pellet was dissolved in buffer A (0·5 ml per 100 g cells) and dialysed into buffer A + 1·0 M KCl (- glycerol). It was layered onto a 10 - 30 % glycerol gradient, in buffer A + 1.0 M KCl, and centrifuged for 22 h at 40,000 rpm, 4°C, in a Beckman SW 40 rotor (Fig 3.05), or for 36 h at 25,000 rpm, in an SW 25·2 rotor. The enzyme was dialysed into storage buffer, and stored at -20°C.

Fig 3·05 High-salt glycerol gradient centrifugation.



0·5 ml samples of Fraction V (10 mg/ml) were layered onto a 12 ml, 10 - 30 % glycerol gradients in buffer A + 1·0 M KCl, and centrifuged at 40,000 rpm, for 22 h, at 4°C, in a Beckman SW 40 rotor. 0·5 ml fractions were collected.

Fig 3·06 DEAE-cellulose chromatography of σ factor.



The phosphocellulose flow-through (400 g cells) was applied to a 0·9 x 12 cm DEAE-cellulose column at 14 ml/h. The ion-exchanger was washed with 20 ml buffer C + 0·05 M KCl, and eluted with a 80 ml gradient from C + 0·05 - C + 0·65 M KCl, 1·5 ml fractions were collected. 25 µl samples were assayed for σ activity.

Second DEAE - cellulose chromatography.

The flow-through from the phosphocellulose column was applied to a DEAE-cellulose column (2 ml per 100 g cells) equilibrated with buffer C + 0.05 M KCl, at 4°C. The ion-exchanger was washed with 3 - 4 column volumes of buffer C + 0.05 M KCl, followed by a linear salt gradient (10 column volume) from buffer C + 0.05 M KCl to buffer C + 0.65 M KCl. The fractions were assayed for σ activity (Fig 3.06).

Purity of RNA polymerase.

Purity of RNA polymerase from each preparation was estimated by SDS-polyacrylamide gel electrophoresis, specific activity, and the ratio of absorbance at 280 and 260 nm.

3.4. Results and Discussion.

3.4.1 Purification of core enzyme.

Procedure.

Complete purification of core enzyme took three days from cell disruption to the storage of the enzyme. This time was kept to a minimum because the enzyme was found to be labile in crude extracts.

The cells were disrupted by shaking with glass beads, or stirring in a blender. Both methods were equally efficient, as judged by the amount of protein in the early fractions, and the final yield of enzyme. Stirring probably led to cell disruption by thawing (Lowe, 1974), and was more convenient because there were no glass beads to remove. DNA ase I was dissolved in buffer G and added immediately, since it was labile in this buffer (Melling & Atkinson, 1972).

Ultracentrifugation was the most difficult step to scale up, and the largest amount which could be handled conveniently was 1000 g cells. This step removed cell debris and ribosomes, and most of the remaining nucleic acid was removed by ammonium sulphate fractionation and DEAE-

cellulose chromatography. If too much nucleic acid was applied to the column the activity trailed into the nucleic acid peak. This could be overcome by improving the A + 42 % ammonium sulphate washing procedure, or by increasing the size of the column. If the 280 : 260 nm absorbance ratio of Fraction IV was less than 1·0, the enzyme bound to the nucleic acid during gel-filtration and eluted partially in the void volume. If the absorbance ratio was too low excess nucleic acid was removed on a 2·6 x 100 cm Bio-Gel A 1·5m column equilibrated with buffer A + 1·0 M KCl. Fraction V was approximately 50 % pure on SDS polyacrylamide gels, and in the majority of preparations phosphocellulose chromatography was used to give pure core enzyme.

Fig 3.07 Summary of E. coli MRE 600 RNA polymerase purification[1].

Fraction	Description	Total protein[2] (mg)	Total activity[3] (units)	Specific activity (units/mg)	Yield[4] (%)
I	Crude extract	7,000	9,000	1·3	100
II	Ultracentrifuge supernate	3,800	9,000	2·5	100
III	Ammonium sulphate fraction	1,000	9,000	9·0	100
IV	DEAE-cellulose peak	170	11,000	65	120
V	Agarose A 5 m peak	37	13,000	350	145
	Phosphocellulose peak, final core enzyme.	10	7,000	700	80
	Glycerol gradient peak, Final holoenzyme.	22	12,500	570	140

1. The results are given per 100 g of cells.
2. Determined as described in section 2.2.07.
3. Assayed with a calf-thymus DNA template.
4. The yield is not meaningful for Fractions I - III due to the presence of DNA ase I and nucleic acids.

Yield and purification.

A summary of the purification is given in Fig 3.07, and SDS-polyacrylamide gels of various fractions in Fig 3.08 & 3.09.

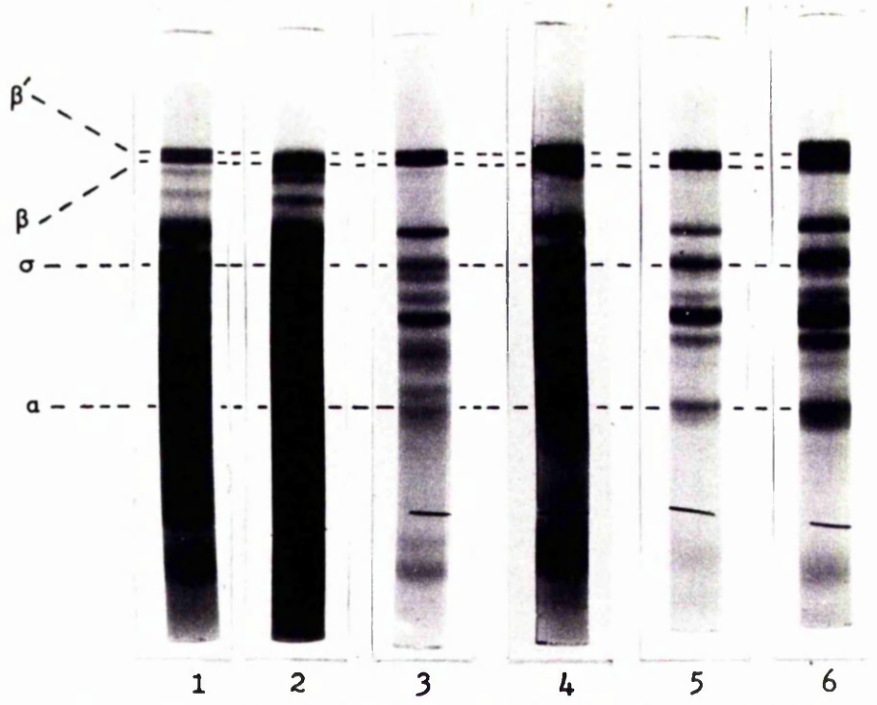
The yields of activity for Fractions I - III were not meaningful due to the presence of DNAase I and nucleic acid. A more realistic estimate for fraction III was 65 - 70 % since some enzyme was probably trapped in the ultracentrifugation and ammonium sulphate pellets. A further 30 % could be lost by overloading the DEAE-cellulose with nucleic acid. Fraction V contained 50 % RNA polymerase, and the yield was at best 60 - 70 %. Further purification on phosphocellulose led to a 45 - 50 % loss of both activity and enzyme subunits. This was probably an overestimate because σ , which was lost in the flow-through, stimulates transcription on calf-thymus DNA (Sagar-Sethi, 1971). RNA polymerase subunits could be detected in the flow through and column wash (Fig 3.09), but these fractions contained little activity (Fig 3.04). This suggested that the column removed, or created, inactive subassemblies of the enzyme. Some enzyme was probably also lost by non-specific adsorption, since a relatively large volume of ion-exchanger was required, and a fairly pure enzyme sample was applied. The final yield was 10 mg per 100 g cells.

Purity.

A pure preparation, with a constant subunit composition, and lacking interfering enzyme activities, was required for chemical studies. Purity was estimated from the specific activity, absorbance ratio at 280 and 260 nm, and by electrophoresis in the presence of SDS and urea.

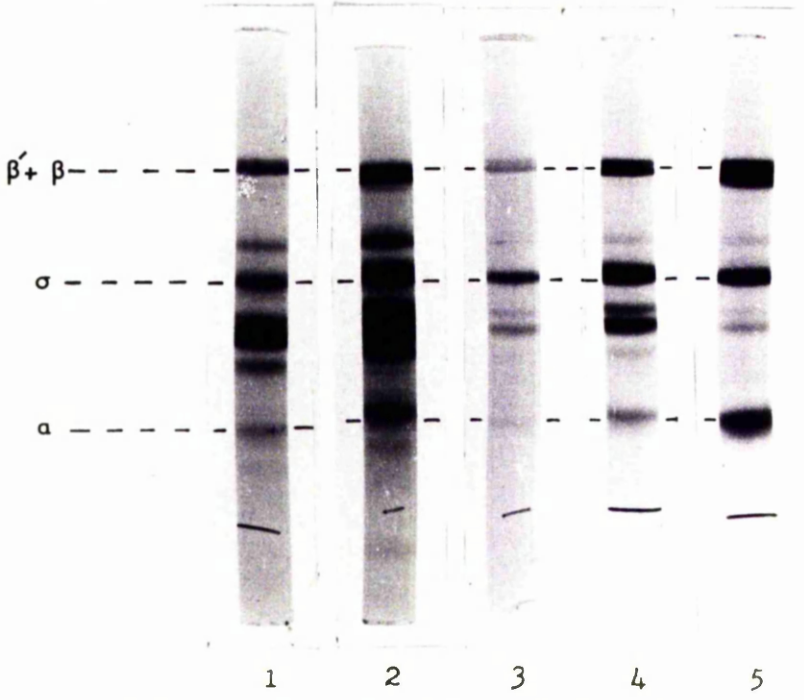
SDS gels (Fig 3.10) of 10 µg of core enzyme showed the characteristic subunits of the enzyme, with a ratio of 0·85 - 0·95 : 1 ($\beta + \beta' : \alpha$) of bound Coomassie Blue. Heavily loaded gels revealed

Fig 3.08 Electrophoretic analysis of Fractions III - V



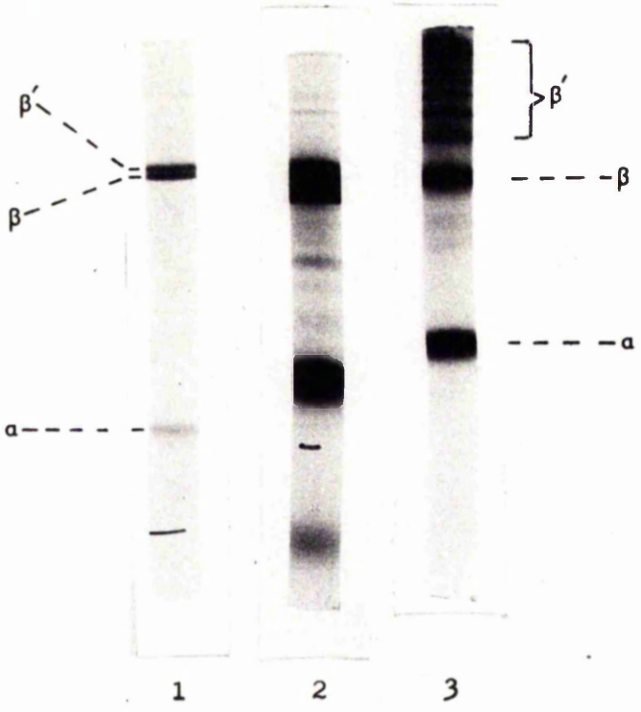
SDS polyacrylamide gels (5 %) were run with 1) 25 µg, 2) 100 µg, Fraction III; 3) 10 µg, 4) 100 µg, Fraction IV; 5) 10 µg, 6) 50 µg, Fraction V.

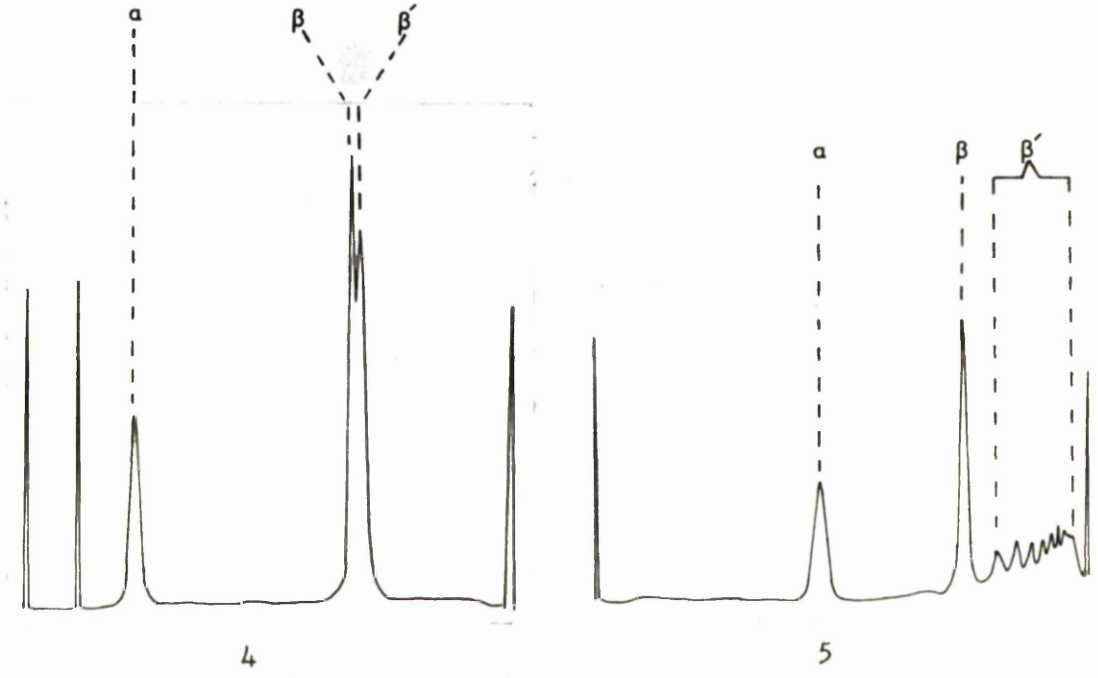
Fig 3.09 Electrophoretic analysis of phosphocellulose fractions.



SDS polyacrylamide gels were run with 1) 8 µg, 2) 40 µg, phosphocellulose flow-through; 3) 8 µg, 4) 40 µg, fraction 17 (Fig 3.04); 5) 14 µg C + 0·25M wash.

Fig 3.10 Electrophoretic analysis of core enzyme.





SDS polyacrylamide gels were run with 1) 10 µg, 2) 100 µg, and a urea gel with 3) 50 µg, of core enzyme. 4) and 5) are scans of the SDS and urea gels respectively.

small amounts of contaminating proteins. Gel scans showed that these contaminants were less than 5 % of the total protein (Fig 3.10). Occasionally contaminants were seen which ran in the position of σ and ω (Burgess, 1971, Chamberlin; 1974b), and less frequently a protein immediately ahead of α. Urea gels showed two discrete bands, α and β, and a series of high molecular species which appeared to arise from crosslinking of β' (Lowe, 1974). No other bands could be detected. Specific activities of 600 - 800 units per mg, and 280 : 260 nm absorbance ratios of 1·6 - 1·9 were found. No RNA forming activity could be detected when 25 µg was incubated in the normal assay solutions lacking DNA and potassium phosphate (a polynucleotide phosphorylase inhibitor). The properties of the pure enzyme are summarised in Fig 3.11.

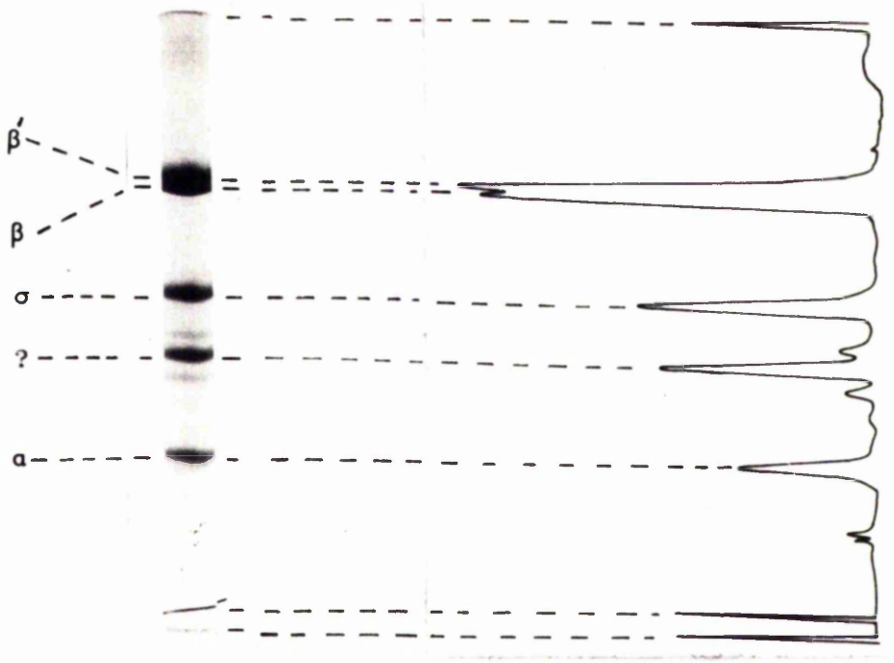
Fig 3.11 Properties of pure core-enzyme.

Specific activity	600 - 800 units/mg
Absorbance ratio (280 nm/260 nm)	1·6 - 1·9
Gel-electrophoresis	95 % of detectable protein.
Polynucleotide phosphorylase	undetectable.

3.4.2 Purification of holoenzyme.

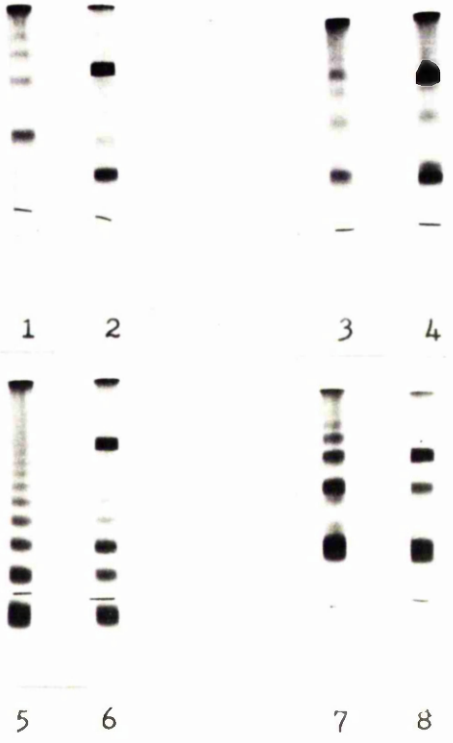
Fraction V was purified further by high salt glycerol gradient centrifugation. The resulting enzyme was about 75 % pure holoenzyme (Fig 3.12). The major remaining contaminant had a molecular weight of about 60,000. This contaminant eluted from DEAE-cellulose and Bio Gel A 5m in the same position as holoenzyme, and ran ahead of it on high salt glycerol gradient centrifugation (results not shown). The properties of the enzyme are summarised in Fig 3.13.

Fig 3.12 Electrophoretic analysis of holoenzyme.



An SDS polyacrylamide gel was run with 36 µg holoenzyme, and it was scanned.

Fig 3.15 SDS polyacrylamide gel determination of RNA-polymerase subunit molecular weights.



Molecular weight standards were prepared from glutamate dehydrogenase, aldolase, lysozyme, and ovalbumin, by the method of Carpenter & Harrington (1972). The gels were loaded with 1) 125 µg of glutamate dehydrogenase, 2) 63 µg of glutamate dehydrogenase + 30 µg core, 3) 125 µg of aldolase, 4) 63 µg of aldolase + 30 µg core, 5) 125 µg lysozyme, 6) 63 µg of lysozyme + 30 µg core, 7) 125 µg of ovalbumin, 8) 63 µg ovalbumin + 30 µg core.

Fig 3.13 Properties of the holoenzyme preparation.

Specific activity	=	550 Units/mg
Absorbance ratio (280 nm:260 nm)	=	1·7
SDS polyacrylamide gels	=	75 % pure (main contaminant = 60,000 mol.wt, 15 % of total protein).
σ content	=	0·6 - 1·0
Polynucleotide phosphorylase	=	undectable

3.4.3 Purification of σ factor.

Impure σ factor was a by-product during the purification of core-enzyme, and it was expected that it could be easily separated from the remaining impurities because of its lower isoelectric point and smaller molecular weight. A partial purification was achieved by chromatography on DEAE-cellulose (Fig 3.06). The main impurities eluted at 0·16 - 0·17 M KCl, whilst σ eluted at 0·2 M KCl. SDS polyacrylamide gels (Fig 3.14) showed that the purest fraction contained 30 % σ , and the main impurity was the 60,000 mol. wt. component. Since this protein ran as a high molecular weight aggregate, ahead of holoenzyme on high salt glycerol gradients, this could be used as a further purification step. The latter fractions of the phosphocellulose flow through contained partially purified σ (Fig 3.09), possibly because of an affinity for the core enzyme-phosphocellulose complex, or a slow rate of dissociation of holoenzyme.

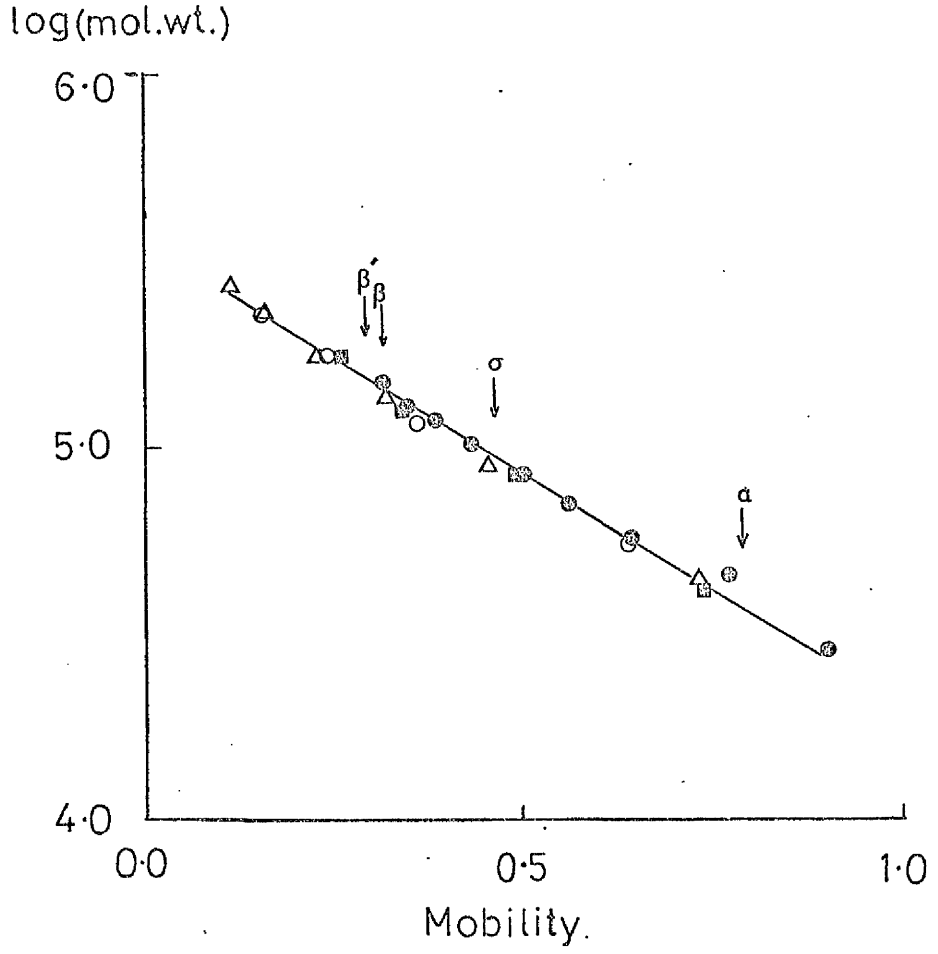
Fig 3.14 Purification of σ on DEAE-cellulose.

Fraction number (see Fig 3.06)	Weight fraction (%) of polypeptides on SDS gels.				
	39,000	54,000	60,000	σ	160,000
Original material	6	9	63	12	3
14	3	54	22	8	4
19	2	13	54	17	12
20 + 21	2	1	47	30	9
24	2	4	70	9	11

3.4.4 Molecular weights of RNA polymerase subunits.

It is difficult to estimate the molecular weights of the large subunits of RNA polymerase, and to unambiguously assign compositions to crosslinked species (King et al., 1974b) because of the lack of suitable high molecular weight markers. Preliminary results with amidinated RNA polymerase suggested that cross-linking occurred which was stable to 1% SDS, 1% 2-mercaptoethanol at 100°C, and markers were required to characterise them. Carpenter and Harrington (1972) described a method of generating markers by crosslinking protein-SDS micelles. When aldolase (4 x 42,000; Sine & Hass, 1969), lysozyme (14,300; Weber et al., 1971); ovalbumin (43,000; Weber et al., 1971) and glutamate dehydrogenase (6 - 8 x 56,000; Krause et al., 1974) were crosslinked, species upto 270,000 mol. wt. were generated (Fig 3.15). A plot of log (mol. wt.) against mobility gave a straight line which was used to calculate the mol. wt. of the RNA polymerase subunits (Fig 3.16). The results are summarised in Fig 3.17 together with other reported values.

Fig 3·16 Plot of log(mol.wt.) v mobility for the crosslinked standards.



The mobilities of the molecular weight standards and enzyme subunits were determined relative to the bromophenol blue marker (Fig 3.15). The curve fits the equation $y = -1{\cdot}22\ (\pm\ 0{\cdot}02)x + 5{\cdot}55\ (\pm\ 0{\cdot}01)$.
(●—● = lysozyme, ○—○ = glutamate dehydrogenase,
▣—▣ = aldolase, △—△ = ovalbumin).

Fig 3.17 Molecular weights of RNA polymerase subunits.

Strain of *E. coli*	Subunit				Reference
	σ	β′	β	α	
MRE 600	85,000 ± 5,000	155,000 ± 5,000	145,000 ± 5,000	37,500 ± 500	Fig 3.16
MRE 600	78,000	145,000		37,000	Lochhead(1971)
B	86,000	150,000	145,000	41,000	Berg *et al.* (1971)
B	95,000	170,000		42,000	Lowe (1974) King & Nicholson(1971)
K12	86,000	150,000	145,000	41,000	Berg *et al.* (1971)
K12	95,000	165,000	155,000	39,000	Burgess(1969 b)

The values agreed with other published results. Evidently crosslinking had not affected the structure of the SDS-protein complex (Reynolds & Tanford, 1970) and they could be used as markers.

3.5 Summary.

RNA polymerase core enzyme was prepared from upto 1 kg of *E. coli*, with a yield of 10 mg per 100 g cells, and a purity $\geq$95 %. Holoenzyme was purified to 75 %, and σ to 30 % purity. Core enzyme was considered to be suitable for chemical studies because of its purity and homogeneity. Holoenzyme was less suitable because of its larger size and more complex properties than core enzyme, and the variable yields of σ . It was used for the formaldehyde melting studies.

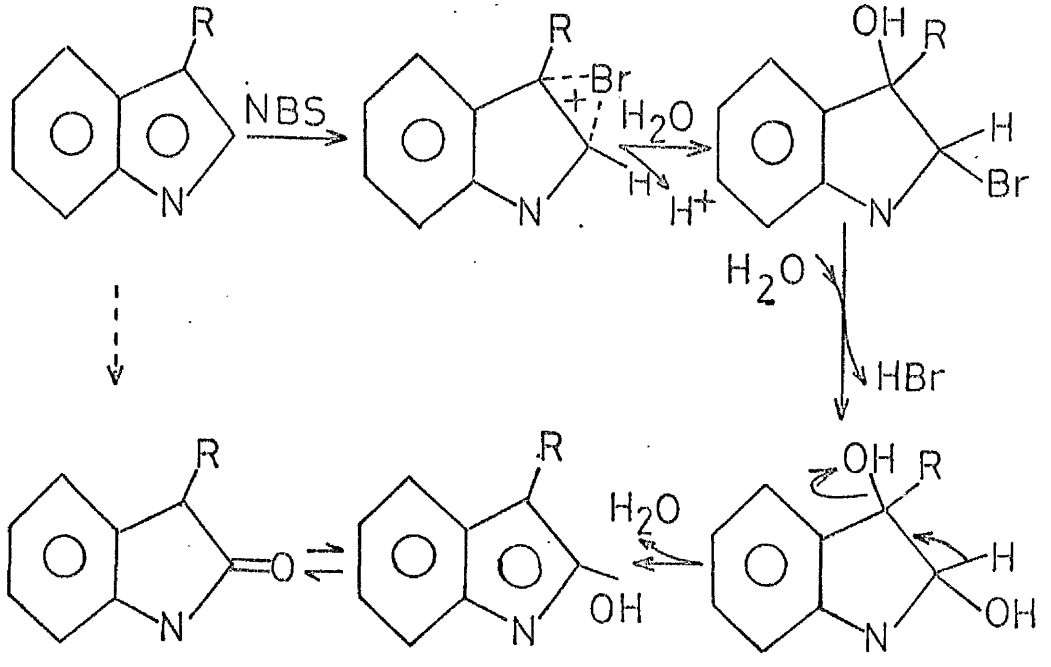
CHAPTER 4.

N-BROMOSUCCINIMIDE OXIDATION OF CORE ENZYME.

4.1. Introduction.

N-Bromosuccinimide has been used to study tryptophan residues in a number of proteins. This reagent was used to identify essential tryptophans in dihydrofolate reductase (Williams, 1975; Liu & Dunlap, 1974; Warwick et al., 1972; Freisheim & Huennekens, 1969), ribonuclease T 1 (Kawashima & Toshio, 1969), lysozyme (Hayashi et al., 1965), DNA ase (Poulos & Price, 1971), and αamylase (Fujimori et al., 1974).

At acid pH, and low molar ratios of reagent to protein, NBS selectively oxidises tryptophan residues (Spande & Witkop, 1967). The reagent is a source of bromonium ions, which react with the indole side chain to give an oxindole. According to Green & Witkop (1964) the reaction mechanism is:



Oxidation is accompanied by a loss of absorbance at 280 nm, which can be used to follow the reaction (Spande & Witkop, 1967).

The reagent will also react with tyrosine and histidine. For model peptides the reactivity is trp > tyr > his in 50 % aqueous acetic acid (Schmir & Cohen, 1961). As the pH is raised towards neutrality there is an increased reactivity of tyrosine and histidine, relative to tryptophan (Spande et al., 1970; Kronman et al., 1967). In some cases the selectivity at neutral pH can be completely reversed, and histidine is attacked preferentially (Brand & Shaltiel, 1963; Brand & Shaltiel, 1964; Williams, 1975), whilst in others it only attacks tryptophan (Liu & Dunlap, 1974). Cysteine, cystine, and methionine are also known to react with NBS (Witkop, 1961; Freisheim & Huennekens, 1969; Williams, 1975). Tryosine oxidation interferes with the use of absorbance changes to monitor loss of tryptophan (Kronman et al., 1967; Schmir & Cohen, 1961).

In model studies 1 - 2 moles of NBS are required to oxidise one tryptophan. In proteins, even when all side reactions with other amino acids are excluded, up to 9 moles per mole are required (Liu & Dunlap, 1974). If excess reagent is added, a variety of side reactions can occur, such as the formation of 5-bromo-oxindoles (Green & Witkop, 1964), and selective cleavage of tryptophan and tyrosine peptide bonds (Spande & Witkop, 1967; Spande et al., 1970).

In these studies RNA polymerase (core) has been modified, at pH 7.9, with low molar ratios of reagent. Studies at lower pH values were not possible, owing to the instability of the enzyme (Novak & Doty, 1970).

4.2. Results and Discussion.

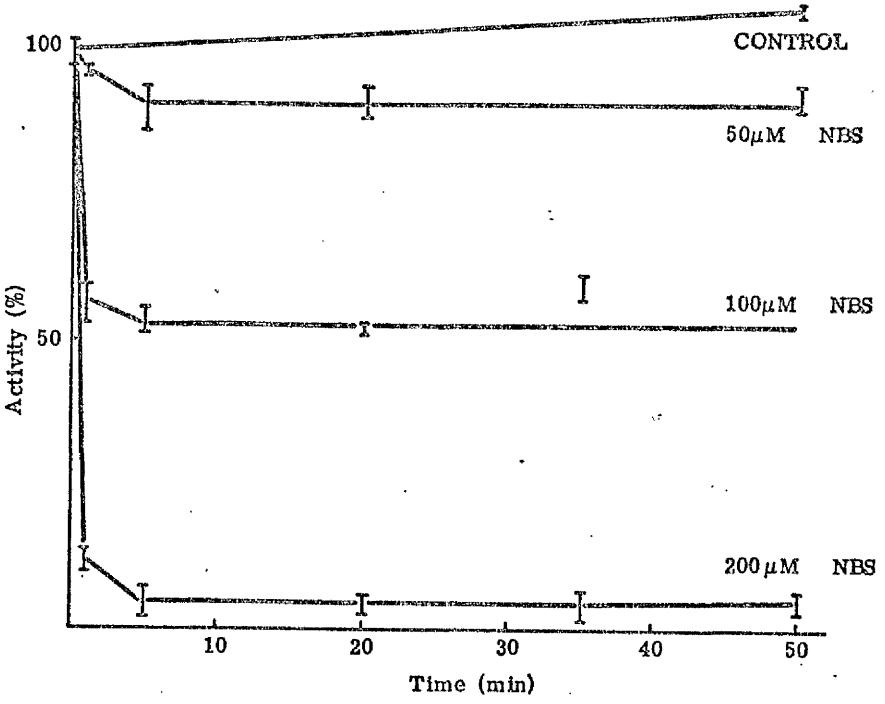
4.2.1 Oxidation of core enzyme.

NBS was found to inactivate RNA polymerase core enzyme, rapidly. The reaction was complete within 5 min at either 37°C (Fig 4.01), or 25°C (Fig 4.02), which was consistentwith the known reactivity of the

reagent (Fujimori et al., 1974; Spande & Witkop, 1967).

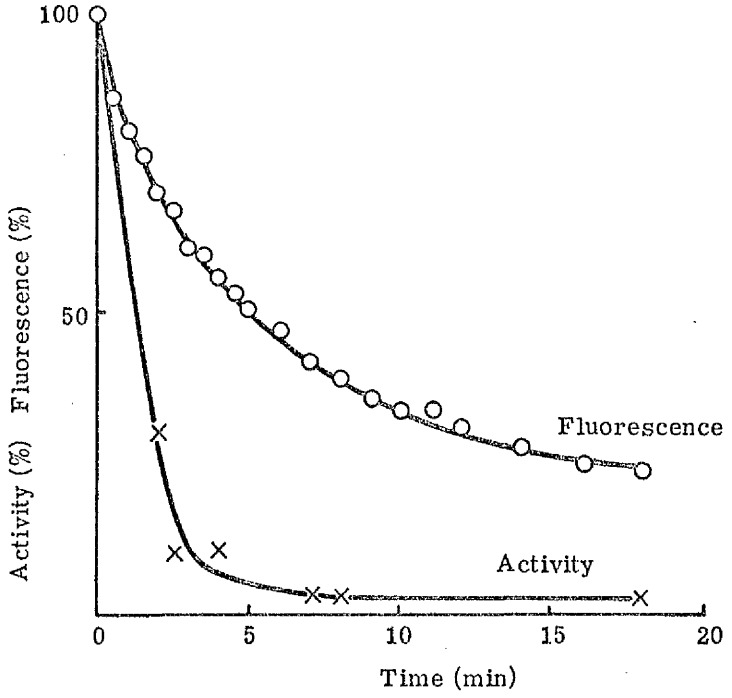
Fluorescence loss was a slower process, but since excess reagent was added, the rate of reaction in this case was probably dictated by the rate of exposure of previously unreactive groups (see later). The extent of inactivation in five minutes depended upon the molar ratio of reagent to enzyme (Fig 4.01). DTT was present in the buffer used for this experiment, and since the NBS could react with thiols, probably only a small proportion of the reagent was consumed in inactivating the enzyme.

Fig 4·01 LOSS OF ACTIVITY OF RNA POLYMERASE (CORE) ON OXIDATION WITH N-BROMOSUCCINIMIDE



RNA polymerase (0·3 μM), in buffer A, at 37°C, was reacted with NBS (freshly prepared before use). Samples were diluted tenfold into the normal assay mixture, and the residual activity was determined.

Fig4·02 LOSS OF ACTIVITY AND FLUORESCENCE WITH EXCESS N-BROMOSUCCINIMIDE

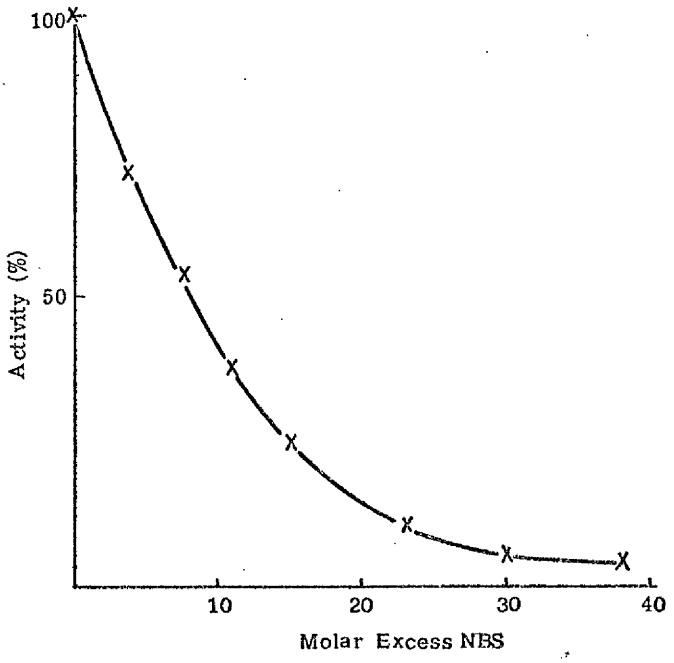


2 ml of RNA polymerase core enzyme (0·32 μM), in buffer M (10 mM Tris HCl pH 7·9, 10 mM Mg Cl_2, 0·1 mM EDTA, 0·2M KCl, 5 % glycerol), at 25°C, was reacted with 40 μl of 5 mM NBS (330 fold molar excess). Changes in fluorescence emission were followed, and, at intervals, 20 μl samples were diluted with 180 μl assay mixture (-NTP) at 0°C. The assay solutions contained 0·2 mM indole to stop the reaction with NBS. Residual activity was measured at 25°C, for 20 min. (Excitation wavelength = 285 nm (10 nm slit), emission wavelength = 335 nm (10 nm slit), sample sensitivity = 4, reference sensitivity = 2).

When core enzyme was reacted with NBS for 5 min, at 25°C, in a DTT free buffer, it was found that, with up to a 20 fold molar excess of reagent there was a progressive loss of activity, and that a 30 fold excess was required for complete inactivation (Fig 4.03). Since about 2 moles of reagent are required for the oxidation of an amino acid, and up to 9 moles can be required to modify just one tryptophan in a protein (Spande & Witkop, 1967; Kronman et al., 1967; Liu & Dunlap,

1974) then 2 - 15 residues in RNA polymerase were being oxidised during inactivation. This was a small proportion of the 250 NBS sensitive residues (cys + trp + tyr + his + met, see section 4.2.3).

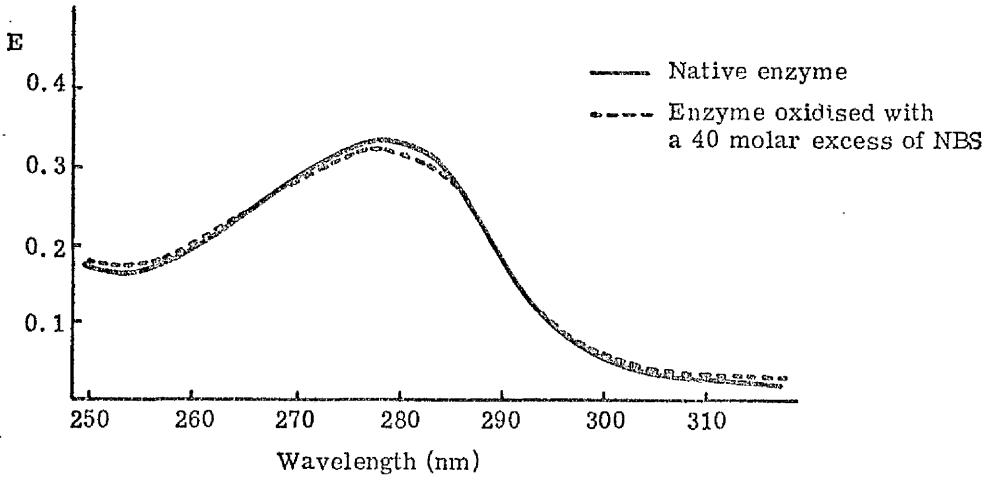
FIG 4.03 ACTIVITY OF RNA POLYMERASE (CORE) AS A FUNCTION OF N-BROMOSUCCINIMIDE ADDED



RNA polymerase (core), in buffer M, was reacted with NBS for 5 min, at 25°C. The reaction mixture was cooled to 0°C, indole added to 0.5 mM, and aliquots assayed for residual activity, in the absence of DTT, for 20 min, at 25°C.

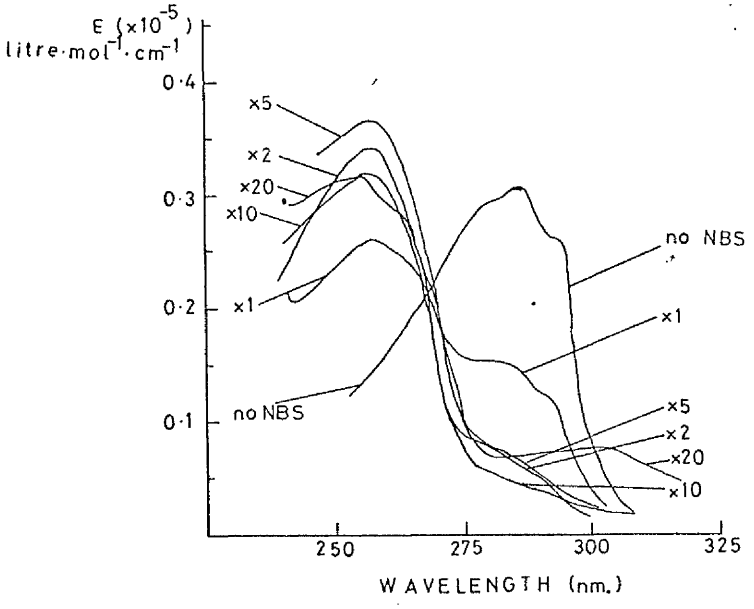
The absorption spectrum of RNA polymerase, oxidised with a 40 molar excess of NBS, was found to have a decreased absorbance between 290 and 265 nm, an increased absorbance between 265 and 245 nm, and an isosbestic point at 265 nm (Fig 4.04). These changes were qualitatively similar to those observed when N-acetyl L-tryptophan was oxidised with NBS (Fig 4.05), and were consistent with tryptophan oxidation.

Fig 4·04 CHANGE IN ABSORPTION SPECTRUM RESULTING FROM N-BROMOSUCCINIMIDE OXIDATION OF RNA-POLYMERASE (CORE)



The absorption spectrum of core enzyme (1·2 μM) in buffer M, before and after modification, was determined against buffer M ± NBS, on a Cary 15 spectrophotometer.

FIG 4·05 EXTINCTION CHANGES ON N-BROMOSUCCINIMIDE OXIDATION OF N-ACETYL L-TRYPTOPHAN.



0·1 mM N-acetyl L tryptophan, in 10 mM Tris HCl pH 7·9, 10 mM $MgCl_2$, was reacted with a 1 - 20 fold excess of NBS, and the absorbance was determined against buffer containing NBS.

The decrease in extinction at 280 nm was used to quantitate the number of tryptophans oxidised, using the equation (Spande & Witkop, 1967):

$$n = \frac{\%\text{ decrease in absorbance} \times 1{\cdot}26 \times \text{mol. wt.}}{100 \times 5500 \times \text{a.f.}}$$

where n = number of trp oxidised per mole of protein

a.f. = absorptivity factor, to convert absorbance at 280 nm to mg/ml

mol. wt. = 400,000 for core enzyme

5500 = molar extinction coefficient for trp at 280 nm

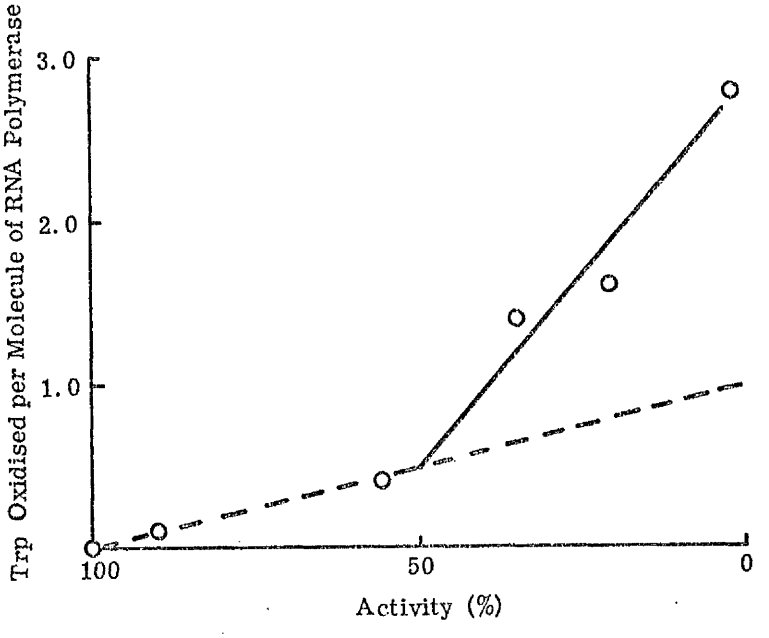
1·26 = factor to correct for residual absorbance of the tryptophan oxidation product.

The factor 1·26 ($\pm$ 0·03) was calculated from the data in Fig 4.05, and was similar to the value of 1·33 determined at pH 4·0 (Spande & Witkop, 1967).

When the extinction changes were followed in buffer A, at 37°C, it was found that 1 - 2 tryptophans were being oxidised during complete inactivation. The early values could be extrapolated to the loss of one tryptophan with 100 % loss of activity (Fig 4.06). However, the extinction changes were small (about 1·5 % per tryptophan) and hence there was the possibility of a comparatively large error in the determination. The sensitivity of the experiment was increased by following the extinction changes by difference spectrophotometry (Fig 4.07). When a 30 molar excess of reagent was added (sufficient to completely inactivate the enzyme), the change in extinction corresponded to the oxidation of one tryptophan (Fig 4.06). Adding more reagent gave further oxidation, and at about a 40 fold molar excess there was a break in the curve, followed by an increase in the susceptibility of tryptophan to oxidation. This may have resulted from decreased

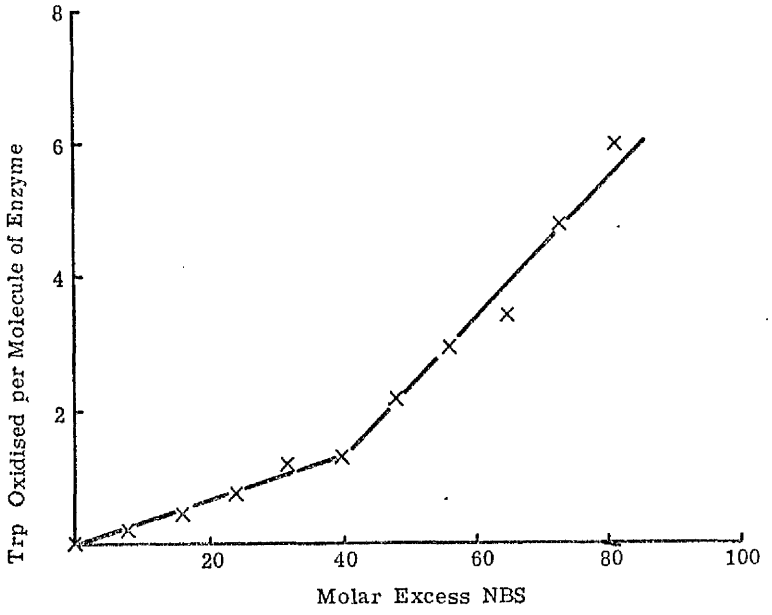
competition of side reactions for available reagent, or (and) from a conformational change leading to exposure of a further group of tryptophans.

Fig 4·06 TRYPTOPHAN OXIDATION FOLLOWED BY CHANGES IN EXTINCTION AT 280nm



The extinction changes of 1 ml of core enzyme (0·4 μM in buffer A) were followed with an SP 8000 spectrophotometer. 3 x 20 μl samples were taken for assay, and 30 μl enzyme (2 x concentration) and 30 μl NBS (0·48 mM in buffer A) added. The solutions were mixed by inversion, and after 5 min the absorbances were determined against references from which 60 μl had been withdrawn and 30 μl NBS and 30 μl buffer A added (temp. = 37°C).

Fig 4·07 TRYPTOPHAN OXIDATION FOLLOWED BY EXTINCTION CHANGES AT 280nm



10 µl aliquots of 1·5 mM NBS were added to 1 ml RNA polymerase (1·85 µM) in N_2 flushed buffer M. The solutions were mixed, and the reaction allowed to proceed for 5 min at 25°C. Extinction changes at 280 nm were recorded on a Cary 15 spectrophotometer against RNA polymerase to which 10 µl water was added. Extinction changes due to NBS alone were subtracted from these values.

Reference solutions containing just the Tris buffer and NBS gave absorbance changes similar in size to those resulting from tryptophan oxidation. The experiments were repeated in potassium phosphate in which the absorbance changes due to reagent alone were less than 5 % of the total. In this case, when sufficient reagent was added to inactivate the enzyme completely, the absorbance change corresponded to the oxidation of about 2 tryptophans (Fig 4.08).

Fig 4.08 Tryptophan oxidation followed by extinction changes at 280 nm.

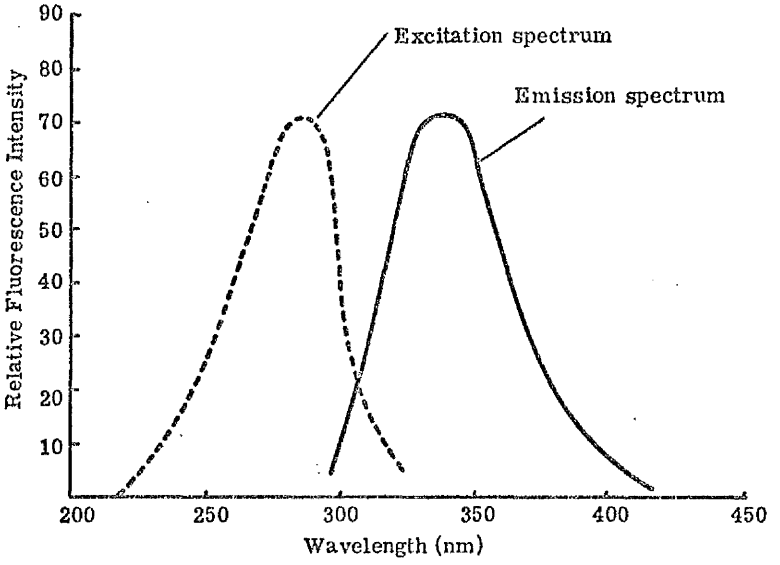
Molar excess NBS	Tryptophans oxidised per molecule of enzyme.
13	2.0
26	2.0
39	2.4
52	3.0
65	3.6

10 µl aliquots of 1.5 mM NBS were added to 1 ml RNA polymerase (1.85 µM) in N_2 flushed 0.14 M KPi, pH 7.5. After 5 min at 25°C the absorbance at 280 nm was recorded against a blank containing KPi and NBS.

These experiments, under three different conditions, gave similar results, and indicated that 1 or 2 tryptophans were being oxidised during complete inactivation.

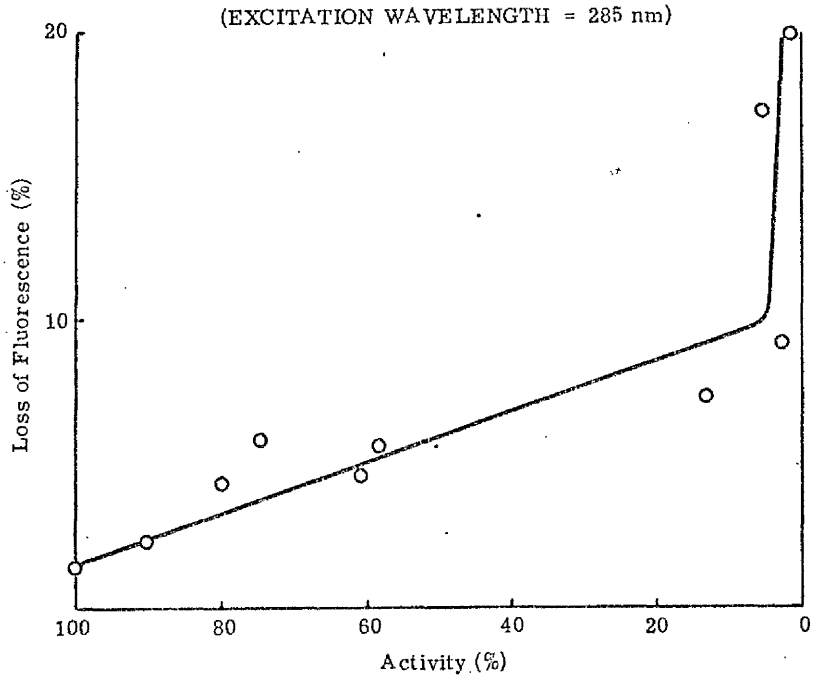
It was predicted that tryptophan oxidation would lead to a decrease in the enzyme fluorescence. (Imoto et al., 1972). The fluorescence spectrum of RNA polymerase had excitation and emission maxima at 285 and 335 nm respectively (Fig 4.09). These wavelengths are characteristic of tryptophan fluorescence, and the observed emission is mainly due to this amino acid. Most proteins containing both tryptophan and tyrosine do not show, or have a weak tyrosine emission band (Brand & Witholt, 1967). Adding NBS led to a progressive loss of fluorescence, and there was a 9 % decrease on complete inactivation (Fig 4.10). Some (or all) of this loss may have been due to a conformation change, since the flourescence spectrum of RNA polymerase has been found to be sensitive to such changes. Yarbrough & Hurwitz (1974) showed that an inactive conformation of RNA polymerase, on the renaturation pathway to active core enzyme, had an emission maximum at 338 nm. A conformational change to the active form gave a 2 - 3 % decrease in fluorescence.

Fig 4·09 FLUORESCENCE SPECTRA OF RNA-POLYMERASE (CORE)



The fluorescence spectrum of 0·2 μM RNA polymerase in N_2 flushed buffer M, at 25°C, was recorded on a Perkin-Elmer spectrofluorimeter. The excitation and emission wavelengths were 285 nm and 335 nm.

Fig 4·10 LOSS OF FLUORESCENCE WITH N-BROMOSUCCINIMIDE INACTIVATION OF RNA-POLYMERASE (CORE)
(EXCITATION WAVELENGTH = 285 nm)

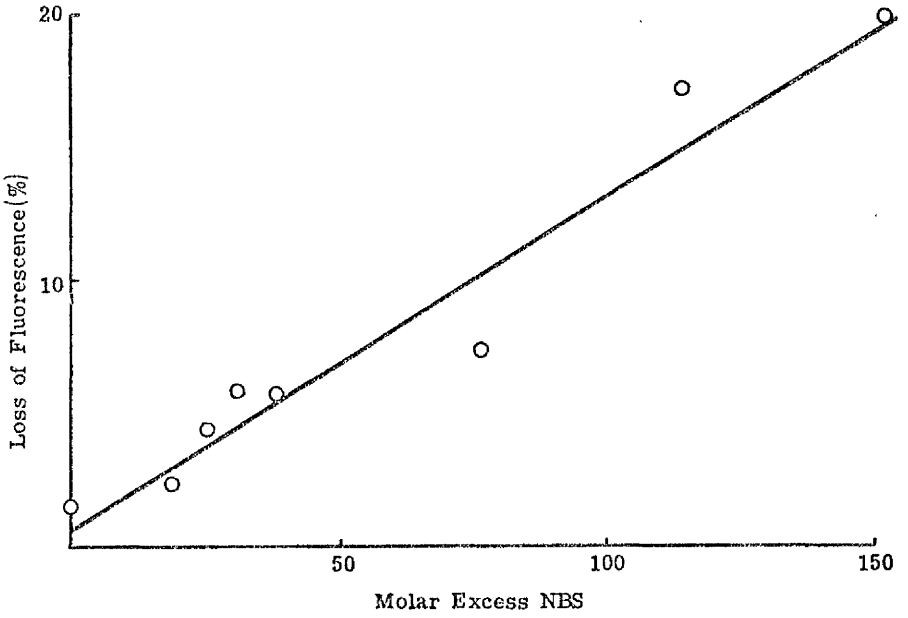


10 μl of NBS was added to 2 ml 0·2 μM RNA polymerase at 25°C. The solution was stirred, and the emission was recorded until a constant value was obtained (less than 5 min). 40 μl of 0·1 M DTT was added, and 3 x 50 μl aliquots used for assay. (Excitation wavelength = 285 nm, 10 nm slit; emission wavelength = 335 nm, 5 nm slit; sample sensitivity = 6, reference sensitivity = 3).

When more NBS was added than required for complete inactivation, there was further loss of fluorescence (Fig 4.11). Addition of up to a 150 fold excess led to a fluorescence loss which was essentially complete in 5 min. However, when a 330 fold excess was added, a slower reaction was observed, possibly due to a slow exposure of tryptophan to the reagent (Fig 4.02). A 330 fold excess led to the loss of 75 % of the fluorescence.

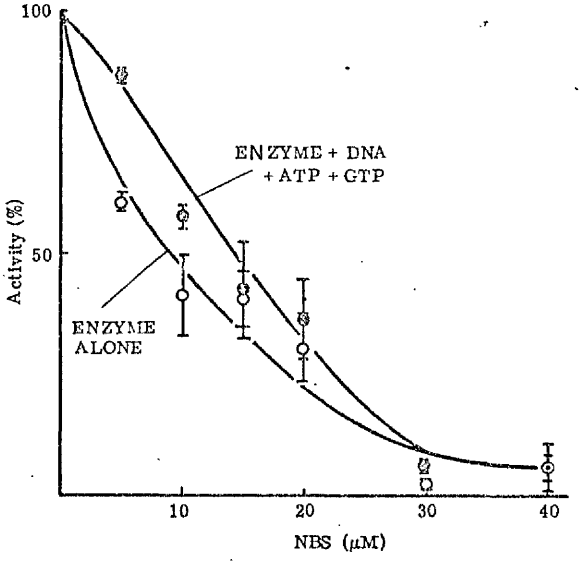
Substrate protection has often been used to show that an essential amino acid is in the active site of an enzyme (Means & Feeney, 1971). When RNA polymerase was incubated with DNA, ATP, and GTP little or no protection against NBS inactivation could be detected (Fig 4.12). Since a small change in the rate of activity loss would not have been detected in this experiment, the kinetics were followed by quenching excess reagent with indole. However no difference in the rate of inactivation could be detected (Fig.4.13). Therefore, either the substrate did not protect against NBS oxidation, or, although a difference in rate did exist, this reagent could completely inactivate the enzyme within the time of the experiment. Infact few cases of substrate protection have been observed with NBS. Biotin has been found to prevent NBS oxidation of avidin (Green, 1963), but the dissociation constant in this case was about 10^{-15}M. N-acetyl D-glucosamine does not protect against NBS oxidation of lysozyme, even though tryptophan is known to be involved in the binding (Imoto et al., 1972). The dissociation constant in this case was 10^{-2}M. The dissociation constants of ATP and GTP from RNA polymerase are about 10^{-5}M to their strong binding site, and about 10^{-4}M to the weak site (Fig 4.31), and for DNA it is about 10^{-10}M (Hinkle & Chamberlin, 1972 a).

Fig 4·11 LOSS OF FLUORESCENCE WITH N-BROMOSUCCINIMIDE OXIDATION OF RNA-POLYMERASE (CORE). (EXCITATION WAVELENGTH = 285nm)



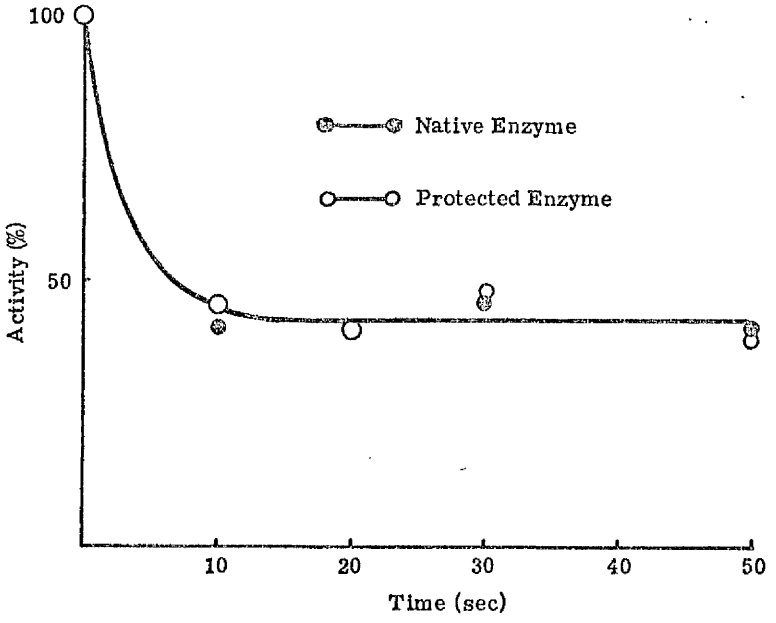
Conditions were as described in Fig 4.10.

FIG 4·12 PROTECTION OF RNA-POLYMERASE (CORE) BY DNA + ATP + GTP AGAINST N-BROMOSUCCINIMIDE INACTIVATION



0·6 μM RNA polymerase, in deoxygenated buffer M, was incubated with or without 0·3 mg/ml calf thymus DNA, 0·8 mM ATP, 0·8 mM GTP, at 37°C. NBS was added, and after 5 min 20 μl samples were assayed for residual activity (in the absence of DTT). Varying the length of preincubation from 1 - 10 min had no effect on protection.

Fig4·13 RATE OF N-BROMOSUCCINIMIDE INACTIVATION OF NATIVE AND DNA + ATP + GTP PROTECTED RNA-POLYMERASE (CORE)



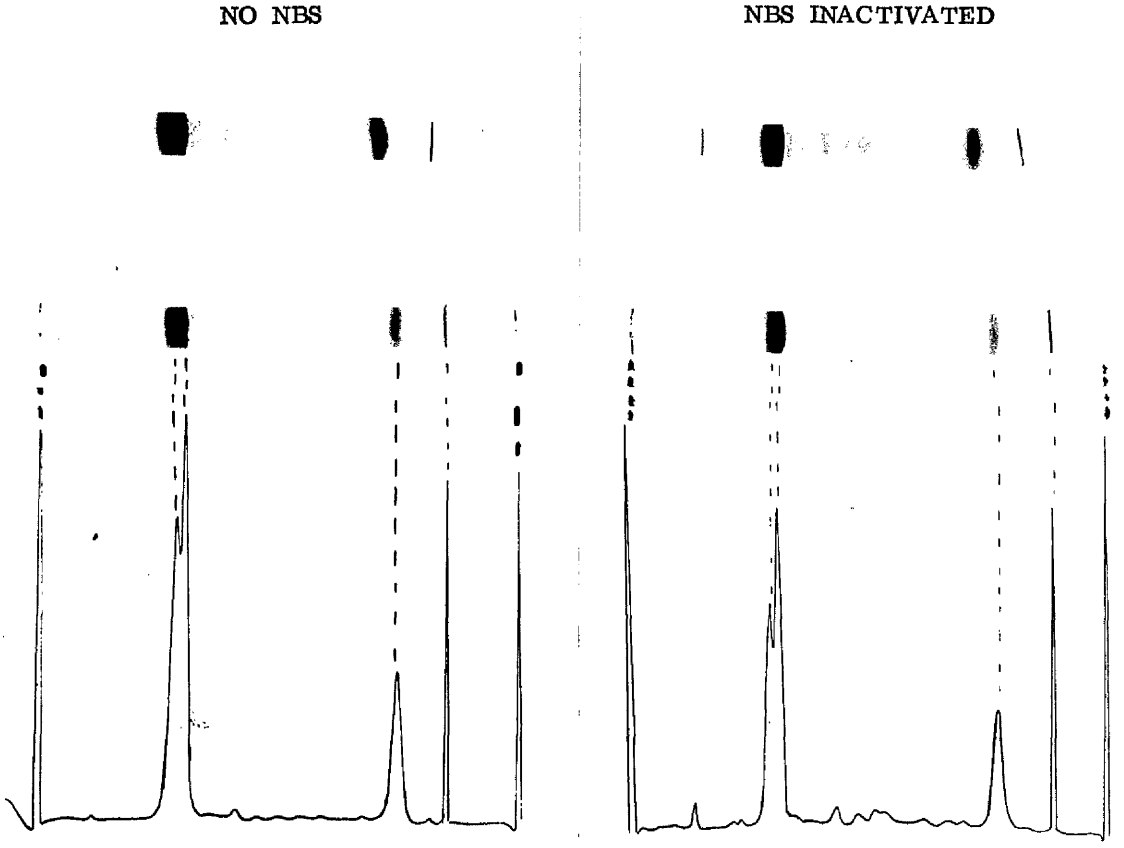
0·6 μM RNA polymerase, in deoxygenated buffer M, with or without substrates (as in Fig 4.12) was reacted with an 18 fold excess of NBS, at 25°C. The reaction was stopped with 0·5 mM indole. Adding indole before NBS led to no loss of activity compared to a sample lacking both indole and NBS.

To show that tryptophan was essential for RNA polymerase activity, it was necessary to exclude all possible side reactions which could lead to inactivation. RNA polymerase activity has been shown to be sensitive to proteolytic inactivation (King et al., 1974a; Lill & Hartmann, 1975), and NBS has been used, under certain conditions, to cleave polypeptide chains at tryptophan, tyrosine and histidine side chains (Spande et al., 1970). To exclude chain cleavage as the cause of inactivation, SDS-polyacrylamide gels of modified enzyme were used to show that no extra peptides were generated. It was found that modification led to no change in the ratio of α to $\beta + \beta'$, no change in their mobilities, and no extra major bands. (Fig 4.14). Cleavages at the N or C terminals giving polypeptides of $\leqslant$ 1000 mol. wt. for α, or $\leqslant$ 10,000 for β and β', would not have been detected. However, since mild conditions and limiting amounts of reagent were used, and the region susceptible to proteolysis is one third of the way along β or β', it was unlikely that chain cleavage accounted for inactivation.

A more likely side reaction was the oxidation of thiols. It was observed that incubating NBS inactivated RNA polymerase with 1 mM DTT led to recovery of activity (Fig 4.15). With low molar ratios of reagent the activity could be restored to the original level, but with more reagent an increasing proportion of the activity could not be recovered. No further reactivation was observed when the DTT concentration was increased to 10 mM, and the incubation to 8 h at 37°C.

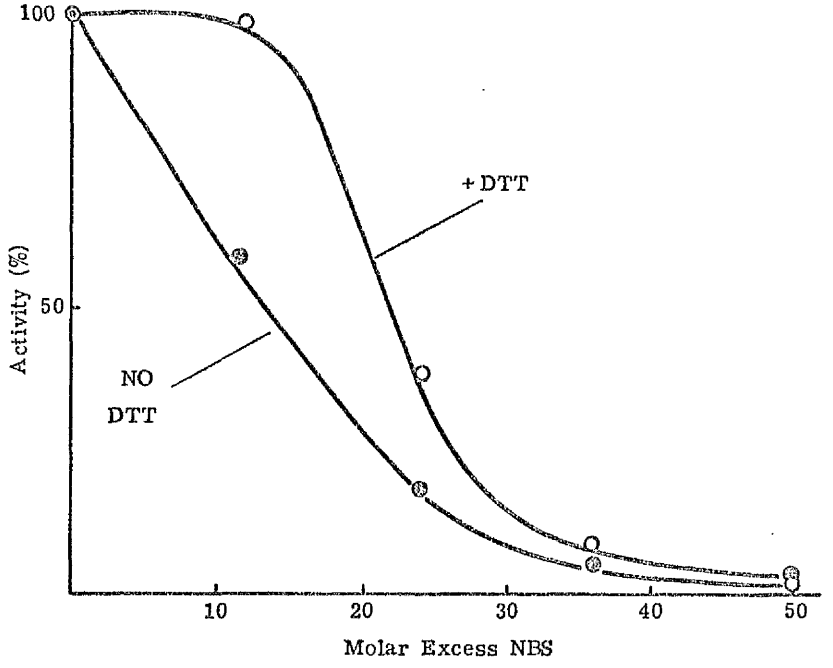
This suggested that some of the inactivation was due to thiol oxidation. Oxidation beyond the disulphide or sulphenic acid stage (Freisheim & Huennekens, 1969) would not have been reversed with DTT, but these are less sensitive to oxidation than thiols.

Fig 4.14 Electrophoretic analysis of NBS modified RNA polymerase.



5 % SDS-polyacrylamide gels were run of RNA polymerase before and after inactivation with a 40 molar excess of NBS, at 25°C, in buffer M. The molar ratio of α to $\beta + \beta'$ was 1 : 0·9 for both modified and unmodified enzyme.

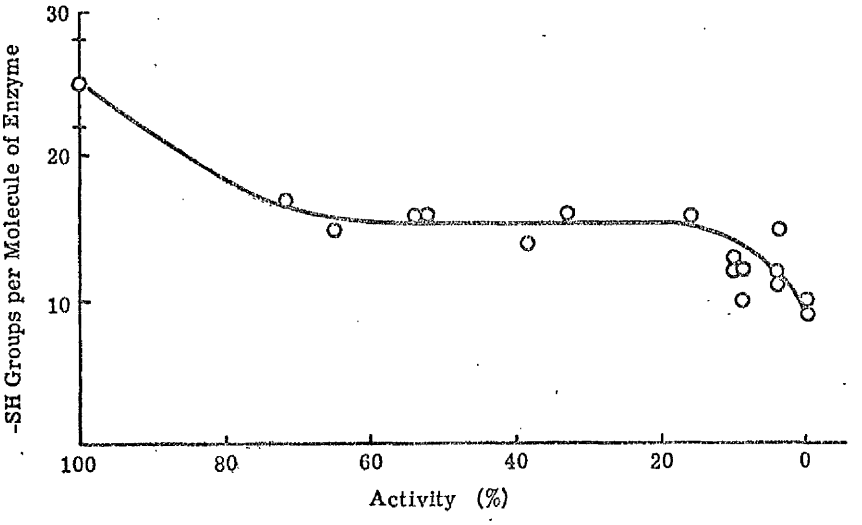
Fig4·15 REACTIVATION OF N-BROMOSUCCINIMIDE MODIFIED RNA-POLYMERASE (CORE) WITH DITHIOTHREITOL



NBS was added to 0·42 μM RNA polymerase in deoxygenerated buffer M, at 37°C. The reaction was stopped after 5 min with 0·5 mM indole, and the enzyme was incubated with 1 mM DTT, for 10 min, at 37°C. 20 μl aliquots were taken for assay in assay solutions lacking DTT. There was a loss of about 10 % of the activity in solutions to which no NBS was added. Indole itself had no effect on activity.

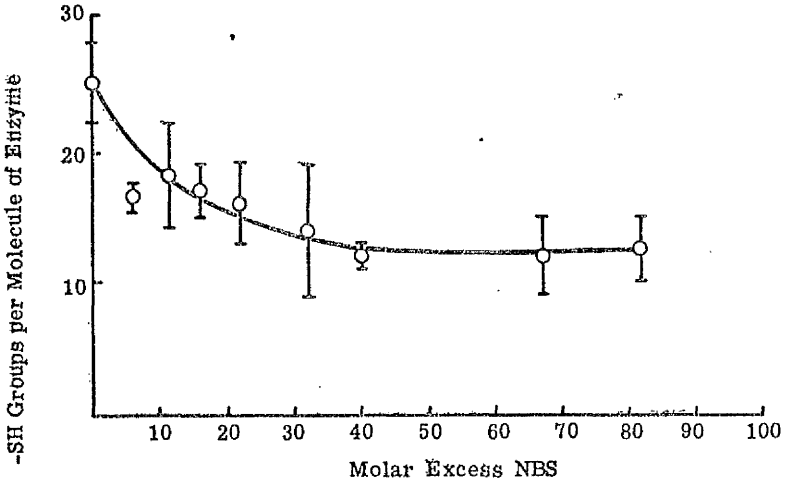
The extent of thiol oxidation by NBS was followed with 5, 5′-dithiobis(2-nitrobenzoic acid) (Habeeb, 1972). It was found that about 10 thiols were oxidised during the first 30 % of the inactivation, and that further loss of activity led to no further oxidation (Fig 4.16). When the number of thiols were followed as a function of NBS concentration, it was found that, beyond a 40 fold molar excess of reagent, no further thiol oxidation occurred (Fig 4.17). This may have accounted for the increased susceptibility to tryptophan oxidation observed with this amount of reagent (Fig 4.07).

Fig 4.16 SULPHYDRYL CONTENT AND ACTIVITY OF RNA POLYMERASE (CORE) AFTER N-BROMOSUCCINIMIDE OXIDATION



0·8 μM RNA polymerase, in deoxygenated buffer M, at 25°C, was reacted with NBS for 5 min, and the reaction stopped with 0·5 mM indole. Samples were taken for assay, and the remaining enzyme was gel filtered, at 4°C, on Sephadex G25 equilibrated with buffer M. 10 μl DTNB (80 mg/ml in 0·1M Tris HCl pH 7·9) was added to 1 ml of polymerase, at 25°C, and absorbance changes at 412 nm were followed. After 10 min 100 μl 20 % SDS was added.

Fig 4·17 OXIDATION OF THE SULPHYDRYL GROUPS OF RNA-POLYMERASE (CORE) WITH N-BROMOSUCCINIMIDE

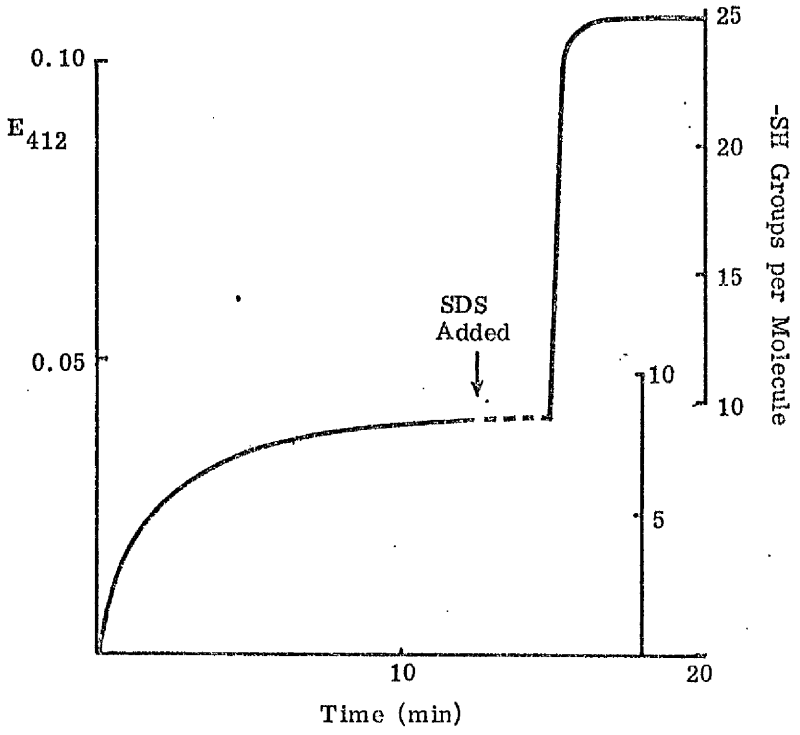


Conditions were as in Fig 4.16.

The rate of DTNB reaction was used to distinguish two groups of thiols on RNA polymerase, those exposed and buried to this reagent (Fig 4.18). It was found that, at 25°C, a small number of thiols reacted rapidly, and that the reaction slowed down considerably after 10 min. Addition of SDS exposed a further group of thiols. These were

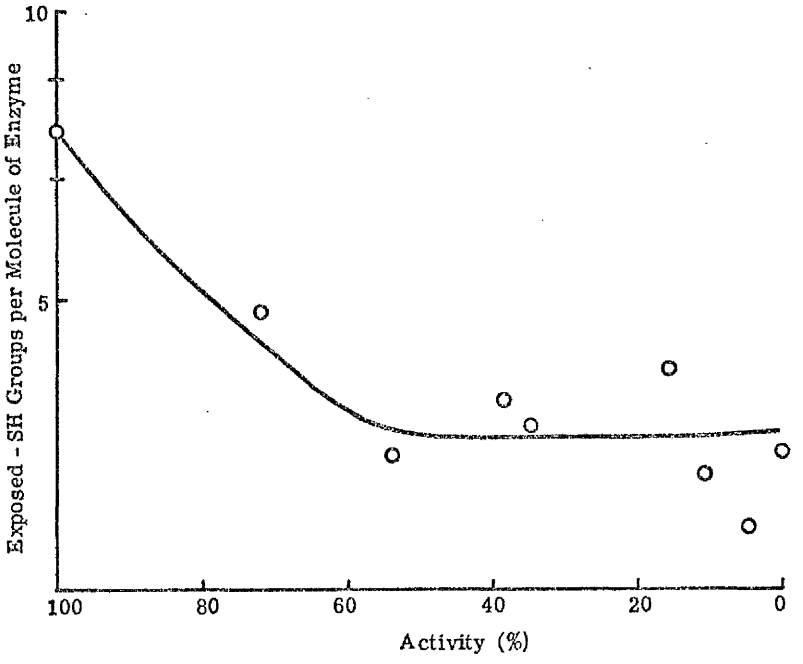
defined as the exposed and buried groups, respectively. NBS inactivation to 40 % led to the loss of about 6 of the 8 exposed thiols, and further inactivation led to little or no further loss (Fig 4.19). There was no loss of buried groups over 90 % of the inactivation (Fig 4.20). This was taken to mean that only surface thiols were oxidised, and that the structurally important buried groups were not attacked. Since the number of buried groups remained constant it seemed likely that no gross structural alterations had occurred.

Fig 4·18 REACTION OF DTNB WITH THE EXPOSED AND BURIED SULPHYDRYL GROUPS OF RNA-POLYMERASE (CORE)



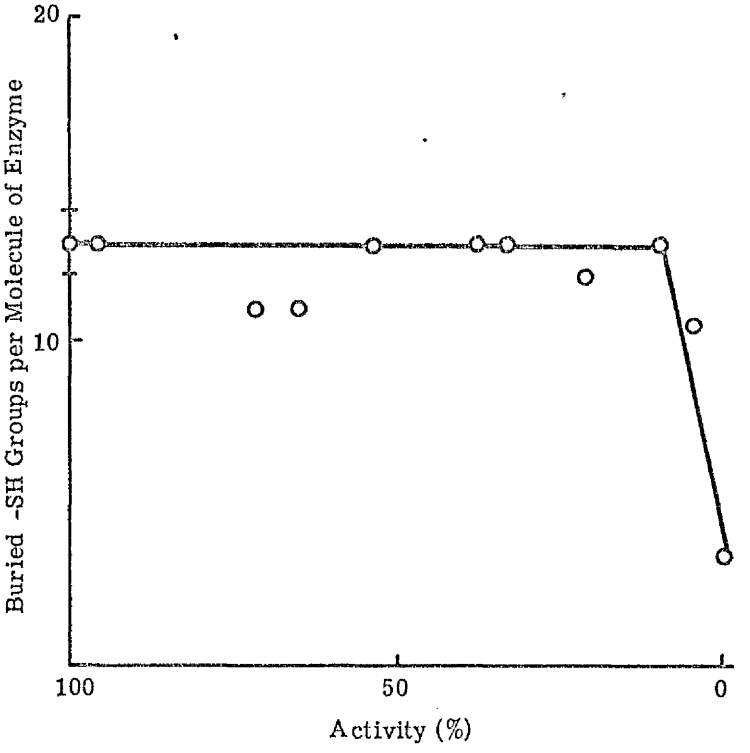
Conditions were as in Fig 4.16, except that no NBS was added.

Fig 4-19 LOSS OF EXPOSED SULPHYDRYL GROUPS WITH N-BROMOSUCCINIMIDE INACTIVATION OF RNA-POLYMERASE (CORE)



Conditions were as in Fig 4.16.

Fig 4-20 LOSS OF BURIED SULPHYDRYL GROUPS WITH N-BROMOSUCCINIMIDE INACTIVATION OF RNA POLYMERASE (CORE)



Conditions were as in Fig 4.16.

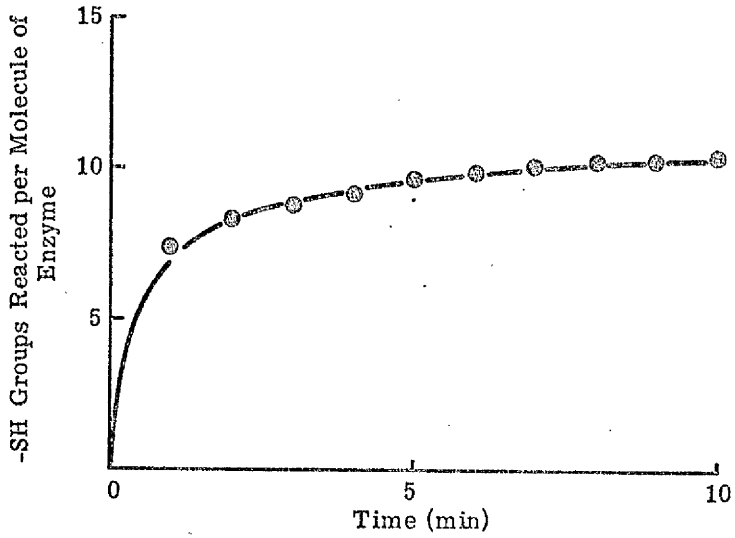
To exclude thiol oxidation, completely, as the cause of inactivation, it was necessary to reverse the oxidation, or prevent it occurring. Several thiol protecting agents have been described. Kassab et al. (1970) used tetrathionate to prevent tetranitromethane oxidation of arginine kinase thiols. Smith et al.(1975) described a series of simple blocking groups of increasing size, which may be introduced onto thiols from either alkyl alkanethiosulphonates or alkoxycarbonyl alkyl disulphides. It was felt that DTNB would be a suitable protecting group for RNA polymerase because the reaction could be restricted to a small number of thiols and these appeared to belong to the NBS sensitive group.

4.2.2 Oxidation of thionitrobenzoyl enzyme.

A good protecting group should have a number of characteristics. It should be capable of being introduced and removed under mild conditions. The protecting group should have no effect on the overall structure and activity of the enzyme, or if it does, these effects should be completely reversible. It should also be stable under the conditions of modification, and protect completely against any modification of the protected amino acid which lead to inactivation. DTNB was found to satisfy most of these criteria.

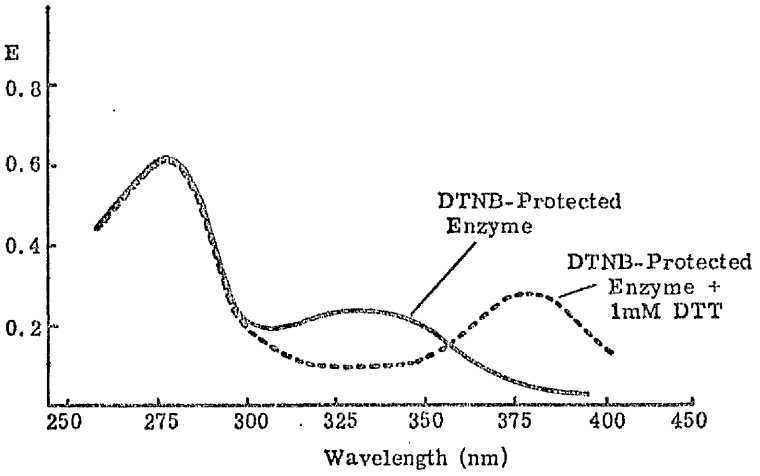
Fig 4.21 shows that, under the conditions used for protection, about 10 thiols reacted with DTNB. The absorption spectrum of protected enzyme had a peak at 330 nm, similar to the absorption maximum at 323 nm of the mixed 2-mercaptoethanol-nitrobenzoate disulphide (Means & Feeney, 1971). On treating the thionitrobenzoylated enzyme with 1 mM DTT the spectrum reverted to one indicative of a mixture of RNA polymerase and thionitrobenzoate anions (Fig 4.22). Residual absorbance at 330 nm was completely removed by gel-filtration on Sephadex G25.

FIG 4.21 REACTION OF DTNB WITH THE SURFACE SULPHYDRYL GROUPS OF RNA POLYMERASE



Core enzyme (5 mg/ml) was dialysed into 3 changes of 300 volumes of N_2 flushed buffer M, for 5 h, at 4°C. 190 µl of DTNB (80 mg/ml in 0.1 M Tris HCl pH 7.9) per ml of enzyme was added. After 10 min at 25°C the solution was cooled, and the reaction stopped by chromatography, at 4°C, on Sephadex G25 equilibrated with buffer M.

FIG 4·22 ABSORPTION SPECTRA OF DTNB-PROTECTED ENZYME WITH AND WITHOUT 1mM DTT



The spectrum of RNA polymerase, prepared as in Fig 4.21, and after reaction with 1 mM DTT at 25°C, was recorded on an SP8000 spectrophotometer.

The protected enzyme had about 30 % of its original activity (Fig 4.23). This activity could be completely and quickly restored with DTT. Protected and NBS modified enzyme regained no more activity than was expected from removal of thionitrobenzoyl groups. In other words, enzyme which was 90 % inactivated relative to a protected sample, was still 90 % inactivated relative to unprotected enzyme when it was treated with DTT. This was true irrespective of the degree of inactivation (see later).

Thionitrobenzoate anions could be lost from the protein by disulphide interchange with unreacted thiol groups in the partially protected enzyme (Fernandez-Diez et al., 1964). The protecting groups were found to be stable under the conditions used for modification, about 0·5 moles per mole were lost in 3 h at 25°C (Fig 4.24). This was similar to the results of Steinert et al. (1974) for DTNB reacted alcohol dehydrogenase. DTT removed about 9 protecting groups, in good agreement with the 10 which were originally introduced.

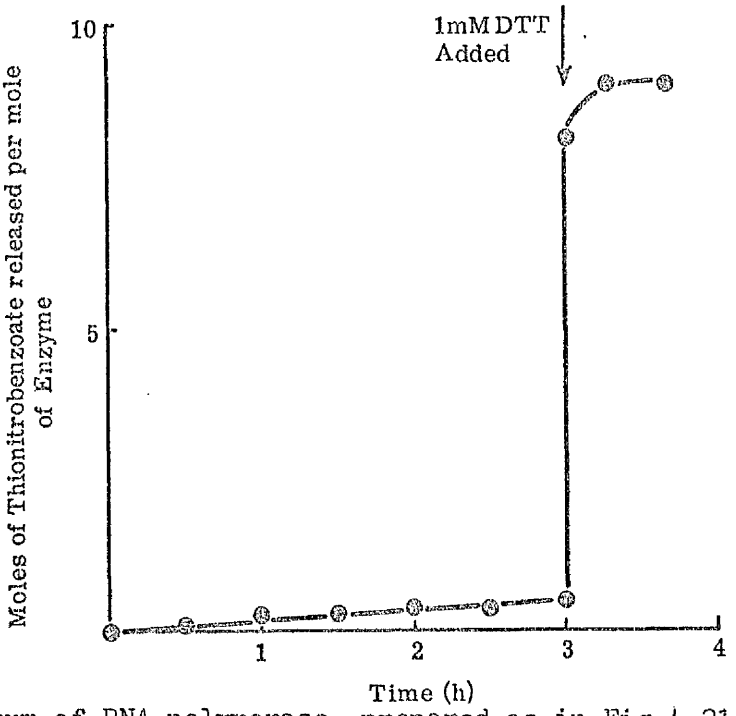
Fig 4.23 Recovery of activity after DTT reduction of the thionitrobenzoylated enzyme.

Time (min)	No NBS	Modified with NBS (25 fold excess)	Unreacted with DTNB
0	28	2.8	107 %
15	101	8.5	-
30	104	8.9	-
60	91	9.2	-
180	100	9.6	-

Thionitrobenzoylated enzyme, prepared as described in Fig 4.21, was allowed to warm up to 25°C for 1 min. NBS was added, the reaction allowed to proceed for 5 min at 25°C, and then DTT was added to 2 mM. Samples were taken for assay before and after the reversal of protection. The specific activity after reversal was 780 units/mg. Activities were expressed relative to the 180 min unmodified value.

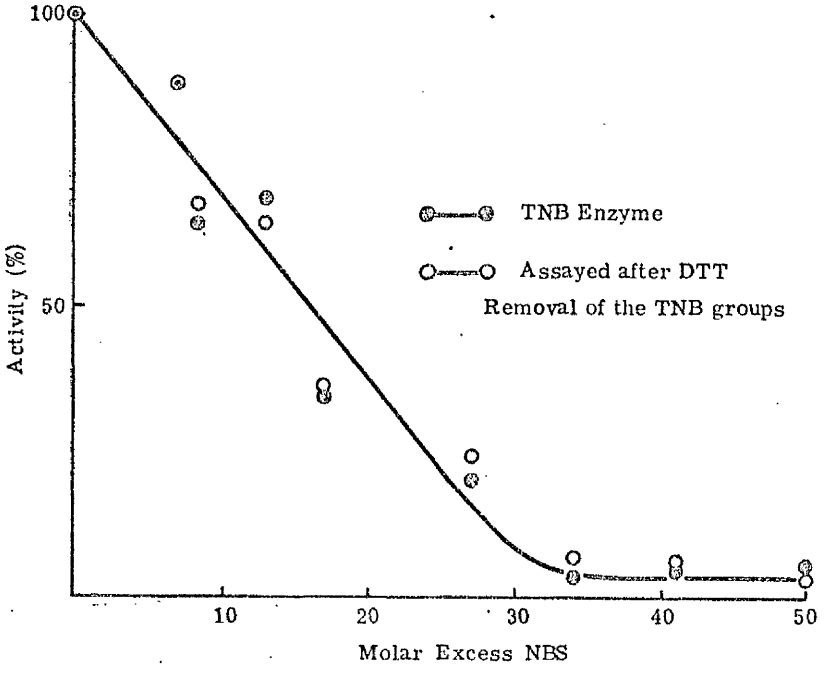
When TNB-enzyme was modified with NBS, it was found that there was a progressive loss of activity until, with a 30 molar excess of reagent, there was no remaining activity (Fig 4.25). No more DTT reactivation of the enzyme was observed, other than that expected from reversal of the protection. This could be compared with the NBS modified unprotected enzyme, which could be partially reactivated (Fig 4.14). In spite of protection against thiol oxidation, the molar excess of reagent required for complete inactivation was similar to that for native enzyme. This may have resulted from an increase in the number of other groups oxidised, or an increased non-specific loss of reagent.

FIG 4·24 STABILITY OF THIONITROBENZOYL GROUPS ON RNA POLYMERASE



The spectrum of RNA polymerase, prepared as in Fig 4.21, was recorded immediately. The temperature was raised to 25°C, and the absorbance at 412 nm was recorded for 3 h. DTT was added to 1 mM, and the final absorbance at 412 nm, attained after 15 min, remained constant for a further 12 h. The original absorbance at 412 nm, before the temperature was raised, was equivalent to about 2 TNB groups. This probably arose from the overlap of the thionitrobenzoyl-enzyme spectrum.

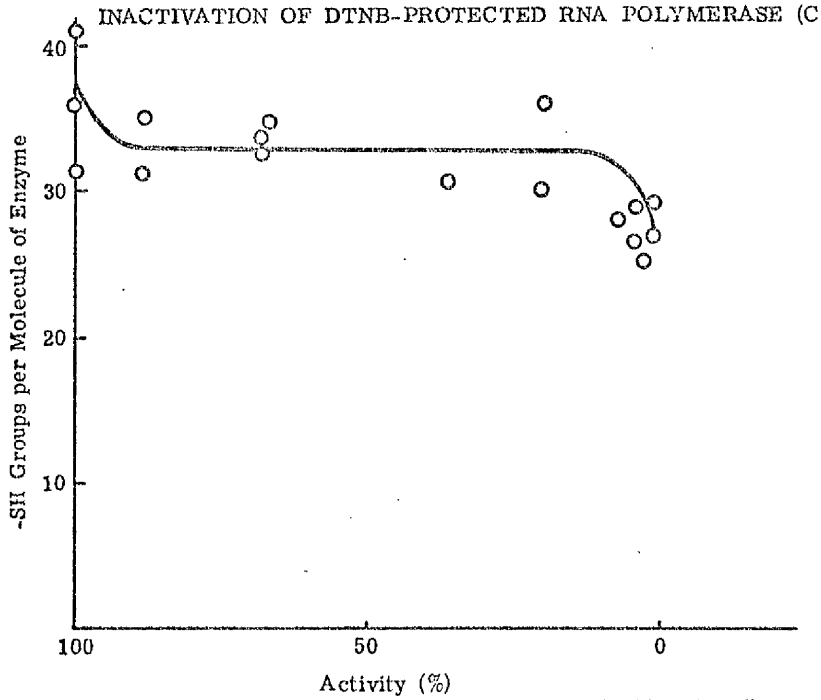
FIG 4·25 INACTIVATION OF DTNB-PROTECTED RNA POLYMERASE WITH N-BROMOSUCCINIMIDE



Protected core enzyme, prepared as described in Fig 4.21, was reacted with NBS at 25°C for 5 min. Aliquots were assayed. DTT was added to 2 mM and, after 3 h at 25°C, further aliquots were assayed.

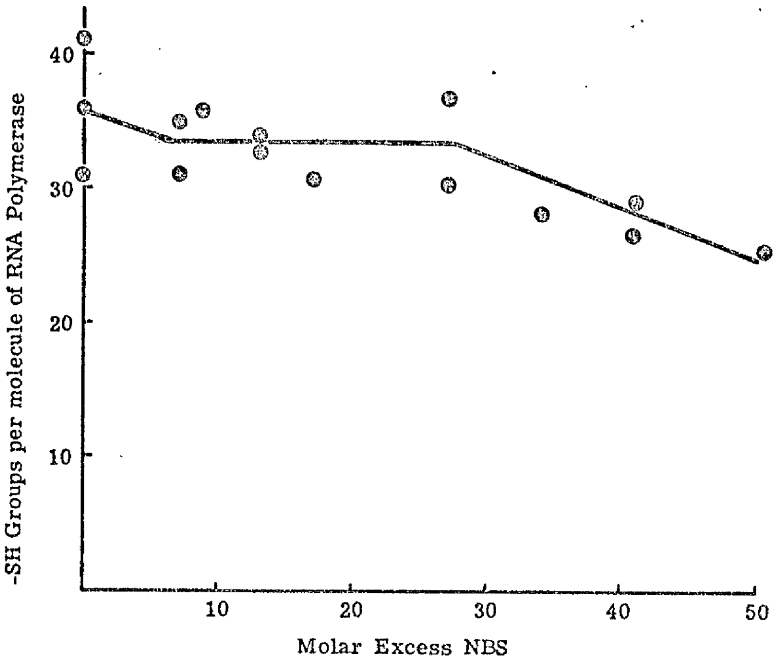
The thiol groups remaining after NBS modification were determined with DTNB. With small amounts of reagent there was a loss of about 3 thiols, and less than 10 % of the activity (Fig 4.26). A straight line fitted, by least squares analysis, to the number of thiols remaining between 90 and 10 % activity indicated that 0·7 (± 2·8) SH groups were being oxidised. Since it is not known that there is a unique essential thiol in core enzyme (section 1.2.3.2) it seems likely that the major cause of inactivation was some other oxidation. Thus it seemed that over the course of 90 % of the inactivation and less than 4 thiols were being oxidised, and they accounted for no more than 10 % of the activity loss. Similarly when the data were plotted as a function of NBS concentration there was little destruction of thiols up to a 30 molar excess of reagent, but further addition led to oxidation (Fig 4.27). 36(±4) thiols were detectable in unmodified RNA polymerase, which was in good agreement with values of 32 (Burgess, 1969b), 33 (Fujiki & Zurek, 1975), and 34 (Yarbrough & Wu, 1974). 25(±3) thiols were detected when sodium dodecyl sulphate was used as a denaturant at a higher ionic strength (235 compared to 15 mM). Since denaturation depends upon the free monomer concentration of detergent (Reynolds & Tanford, 1970), and increasing ionic strength lowers the critical micellar concentration, the lower number of detectable thiols might have resulted from incomplete denaturation of RNA polymerase.

FIG 4·26 LOSS OF SULPHYDRYL GROUPS WITH N-BROMOSUCCINIMIDE INACTIVATION OF DTNB-PROTECTED RNA POLYMERASE (CORE)



Samples, prepared as described in Fig 4.21 and Fig 4.25, were chromatographed on a 1 x 30 cm Sephadex G25 column equilibrated with 10 mM Tris HCl pH 7·9, 1 mM EDTA. 1 ml samples were treated with 0·1 ml 20 % SDS and 10 µl DTNB (20 mM in 0·1 Tris HCl pH 7·9), and the absorbance at 412 nm determined.

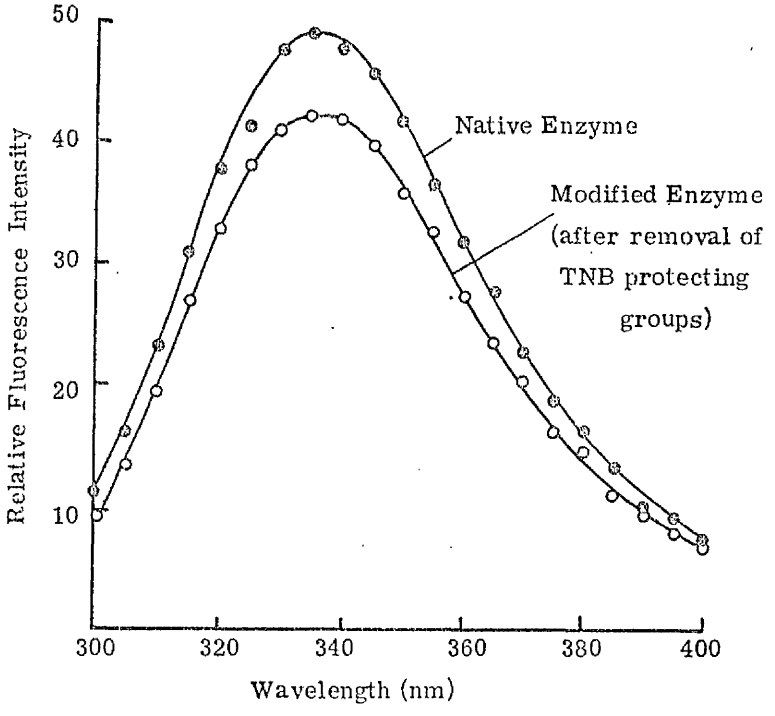
FIG 4·27 SULPHYDRYL CONTENT OF N-BROMOSUCCINIMIDE OXIDISED RNA-POLYMERASE



Conditions were as described in Fig 4.26.

NBS inactivated core enzyme, after removal of the protecting groups, had a decreased fluorescence emission compared with native enzyme (Fig 4.28). The 11($\pm$2) % decrease was similar to 9 % loss found with unprotected enzyme (Fig 4.10). Since the fluorescence decrease could have resulted from a conformational change, an independent method was used to detect structural changes. Far ultraviolet circular dichroism can be used to monitor the conformation of the polypeptide backbone of a protein (Sears & Beychok, 1973). The CD spectrum of core enzyme had negative peaks at 220 and 207 nm, the latter being the more intense (Fig 4.29). The overall spectrum was similar to the spectra reported by Harding & Beychok (1974) and Novak & Doty (1970), whereas the molar

FIG 4.28 FLUORESCENCE EMISSION SPECTRUM OF RNA-POLYMERASE (CORE). (EXCITATION AT 285 nm)



TNB-RNA polymerase was prepared and modified as described above. Thionitrobenzoate ions were removed with 2 mM DTT, followed by dialysis into buffer A + 0·5 M KCl at 4°C. The fluorescence spectra of 0·12 μM solution of modified (5 % original activity), and unmodified enzyme were compared. (Excitation wavelength = 285 nm, 10 nm slit; emission = 8 nm slit; sample sensitivity = 5; reference sensitivity = 5).

ellipticity at 220 nm, -8,700 (deg. cm^2. $dmol^{-1}$), agreed with -8,900 obtained by the former, but not with the value of -13,000 obtained by the latter authors. Inactivation of RNA polymerase with NBS had little effect on the spectrum (<2 %). Other modifications lead to larger changes in the CD spectrum. Novak et al. (1974) found an 8 % decrease in the molar ellipticity when core enzyme was 50 % inactivated with 2-hydroxy-5-nitrobenzyl bromide, and Harding & Beychok (1974) found a 14 % difference between native core enzyme and an inactive intermediate on the renaturation pathway. Williams (1975) found that NBS modification of dihydrofolate reductase led to a shift in the negative peak from 220 to 208 nm, and an apparent change in the β structure of 10 %. The effect was not due to tryptophan oxidation alone because, although the tryptophan CD band shifts from 225 to 210 nm, it accounts for a small fraction of the total ultraviolet circular dichroism.

Using the method of Greenfield & Fasman (1969) it was calculated that RNA polymerase core enzyme had 23 % α helix, 16 % β structure, and 60 % was "unstructured", or 24 %, 20 %, and 56 % if a correction was made for the aromatic contribution (Sears & Beychok, 1973). These figures are comparable to α helical contents of 13 % (Nicholson, 1971) and 30 % (Lochhead, 1971) obtained from optical rotary dispersion measurements. The deduced structure is only approximate because the model compounds used do not represent all the structures present in a protein, although the predicted structure is better for some proteins than others (Barela & Darnall, 1974). In spite of the difficulties in predicting a structure for RNA polymerase, it seemed likely that the structure changed by less than 2 % on modification. Small local conformational changes would not have been detected by this method.

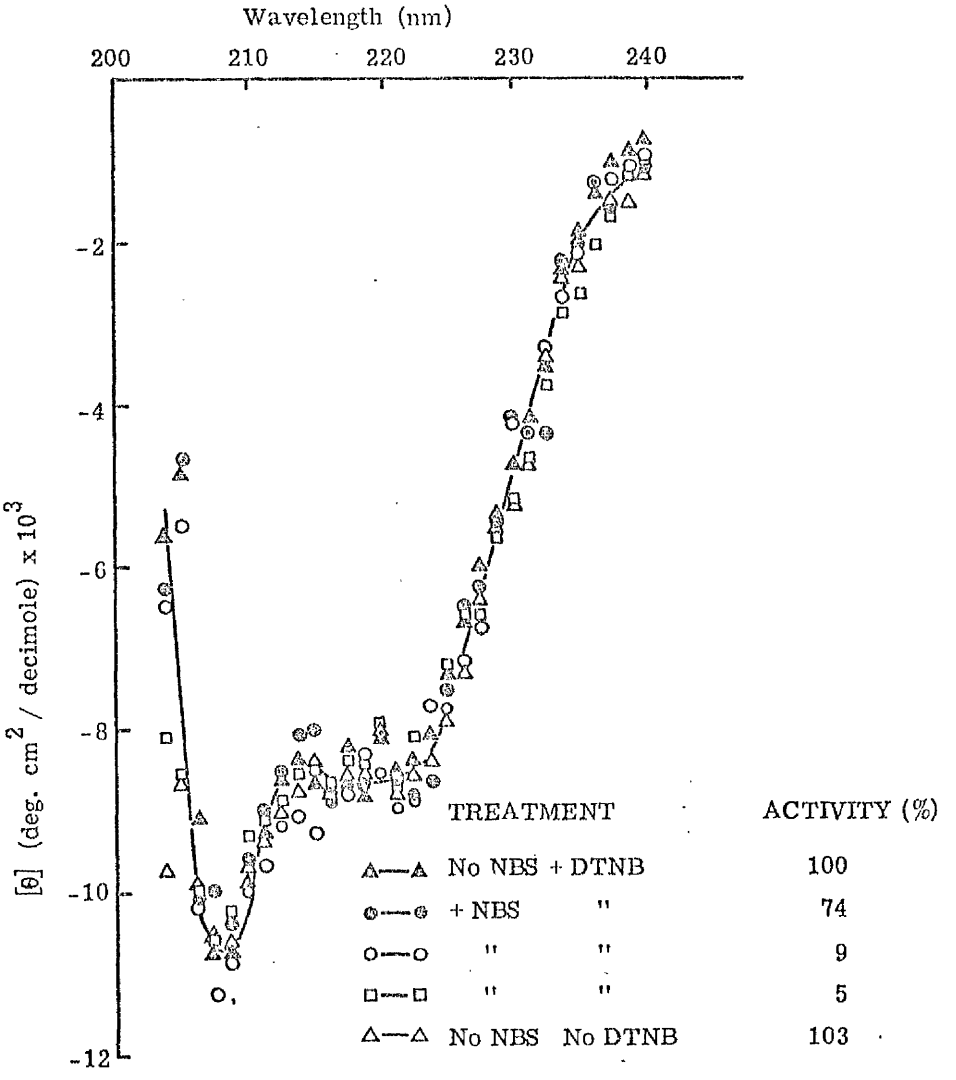


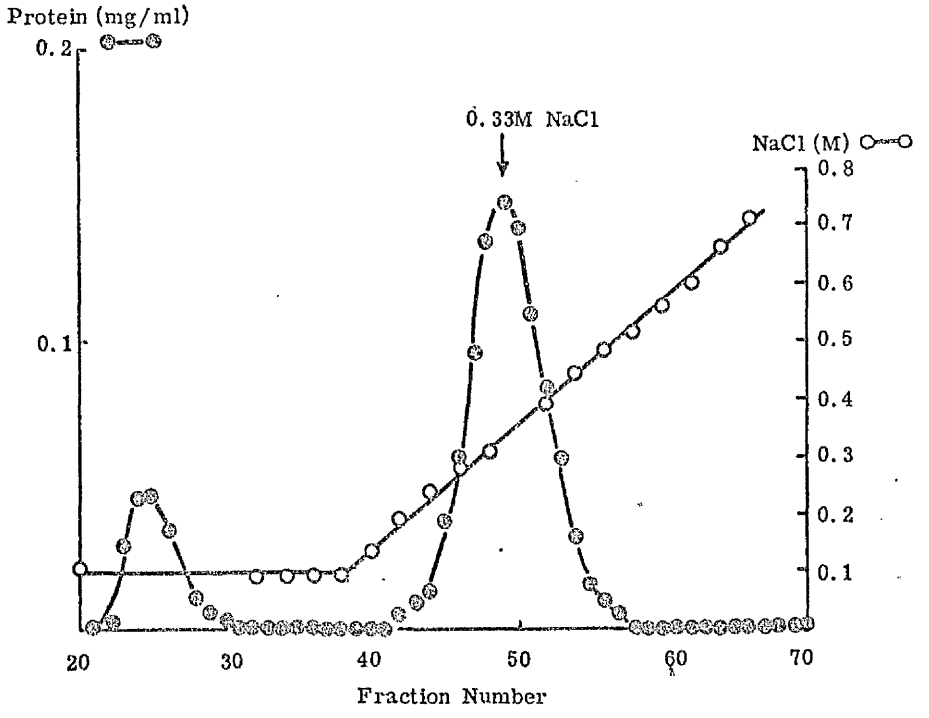
FIG 4.29 CIRCULAR DICHROIC SPECTRA OF NATIVE AND N-BROMOSUCCINIMIDE OXDISED RNA-POLYMERASE (CORE)

RNA polymerase, prepared as described in Fig 4.21, was modified with 0, 9, 25 and 50 fold excess of NBS for 5 min at 25°C. TNB was removed with 2 mM DTT, and the enzyme was dialysed into buffer A + 0.5 M KCl at 4°C. The CD spectra of these samples, and one which had not been reacted with DTNB, were recorded on a Cary 6001 at 27°C in 1 cm cells. No loss in activity occurred during the determination of the CD spectra.

NBS could have inhibited any of the catalytic activities of the enzyme (section 1.2.2). It was predicted, by analogy with other work on peptides and proteins (section 1.3), that the modification might have affected DNA binding, NTP binding, or unwinding of the double helix. DNA binding activity was determined by affinity chromatography

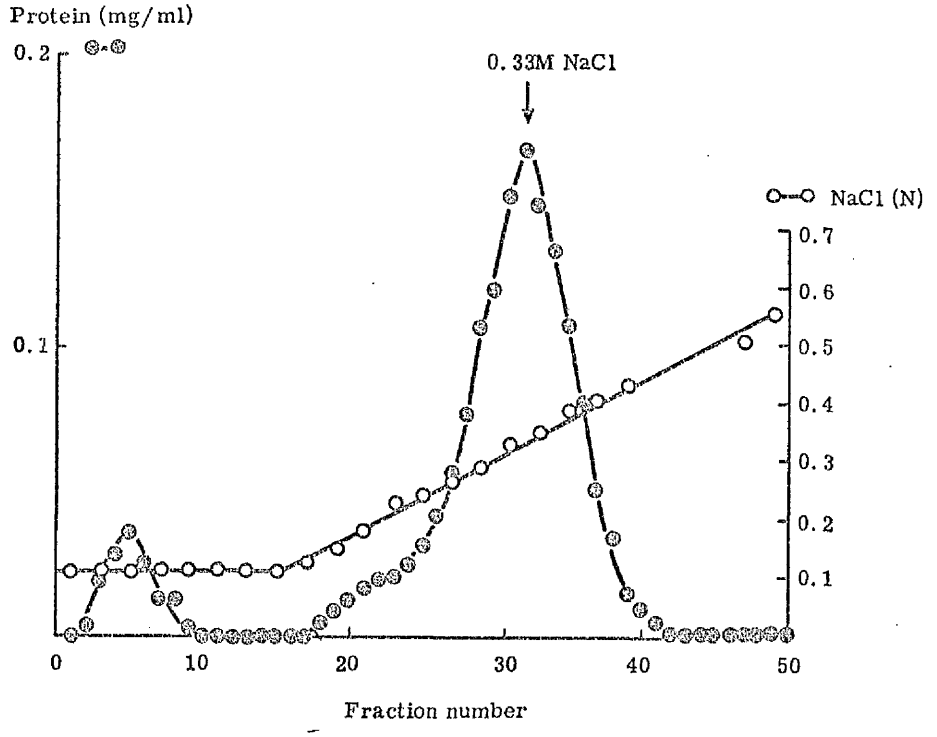
on DNA-agarose, which contains immobilised single stranded DNA. Both native and NBS inactivated enzyme bound to the column, and eluted at 0·33 M NaCl (Fig 4.30.1, 4.30.2). The results show that loss of DNA binding was not the cause of inactivation and that if the modified groups were involved in binding DNA they did not contribute the major part of the binding energy. Also the overall structure of the enzyme was not perturbed sufficiently to decrease the complementarity between the binding region and the DNA molecule.

FIG 4·30·1 BINDING OF NATIVE RNA-POLYMERASE TO DNA-AGAROSE



Core enzyme (~ 1 mg/ml) was precipitated with 1½ vol. buffer C + saturated ammonium sulphate, and centrifuged at 12,000 r.p.m. in an MSE 18, 8 x 50 rotor. It was dissolved in buffer N (10 mM Tris HCl pH 7·9, 1 mM EDTA, 0·1 mM DTT, 5 % glycerol)+0·1 M NaCl, and then chromatographed on a column of Sephadex G25 equilibrated with the same buffer. A DNA-agarose column (0·9 x 8 cm) was washed with buffer N + 1·0M NaCl, and then 20 vol. buffer N + 0·1M NaCl. The sample was applied at 5 ml/h, the DNA-agarose was washed with 10 ml buffer N + 0·1M NaCl, and eluted with a 50 ml linear gradient from buffer N + 0·1M NaCl to buffer N + 1·0M NaCl.

FIG 4·30·2 BINDING OF NBS OXIDISED RNA POLYMERASE (CORE) TO DNA-AGAROSE



TNB protected core enzyme, prepared as described in Fig 4.21, was modified with a 28 fold excess of NBS. After removal of the protecting groups with 2 mM DTT it was treated as described in Fig 4.30.1, except that the DNA-agarose column was eluted with a gradient from buffer N + 0.1M NaCl to buffer N + 0·7M NaCl.

A variety of methods have been used to measure the binding of small molecules to proteins. Three of these, fluorescence quenching, equilibrium dialysis, and column chromatography have been used for nucleoside triphosphates and RNA polymerase (Fig 4.31). There is no general agreement about the number and types of binding site present, or on the effects of σ on the process. Examples of contradictions are : Abrahams (1970) found that σ inhibited binding of NTPs to core enzyme, whereas Zillig et al. (1970b) found that it was essential for the binding to occur. Abraham (1970) could detect one binding site for ATP, Wu & Goldthwait (1969b) could detect two, Zillig et al. (1970) four, and Ishihama & Hurwitz (1969) eight. In general these authors found that pyrimidine nucleoside triphosphate binding was weak or undetectable. The enzyme might require a conformational change for pyrimidines to bind tightly.

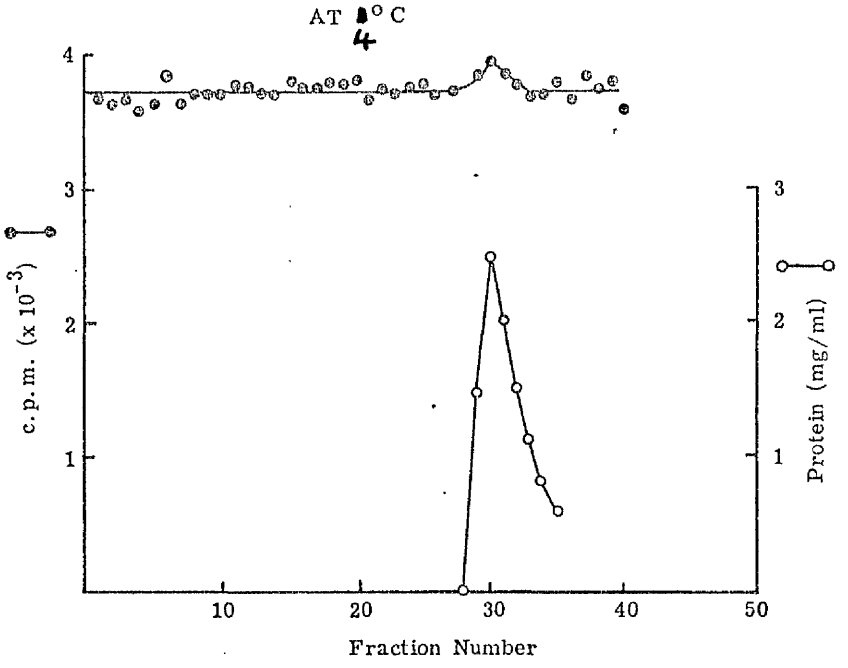
Fig 4.31 Binding constants for nucleoside triophosphates and RNA polymerase.

Technique	Dissociation constants (μM)				Enzyme	Temp(°C)	Authors
	ATP	GTP	CTP	UTP			
Fluorescence quenching	140(1)[1]	140(1)	-	-	holoenzyme	25	Wu & Goldthwait (1969a)
Equilibrium dialysis	17(1) 185(1)	15(1) 133(1)	230(1)	370(1)	holoenzyme	25	Wu & Goldthwait (1969b)
Column chromatography (Hummel & Dreyer, 1962)	34(1)[2]	-	-	-	core enzyme	-	Abraham (1970)
"	50(8)	-	-	-	holoenzyme	-	Ishihama & Hurwitz (1969)
"	ND[3,4]	ND	ND	ND	core enzyme	-	Zillig et al.(1970b)

1. The figures in brackets are the number of binding sites.
2. Binding could not be detected with holoenzyme.
3. Binding could not be detected with core enzyme.
4. ND = not detectable.

The binding of nucleoside triphosphates to core enzyme was determined by column chromatography and equilibrium dialysis (section 2.2.02). Assuming one binding site for ATP, column chromatography gave a value of 110 ($\pm$40) μM for the dissociation constant (Fig 4.32, Fig 4.33), whilst equilibrium dialysis gave values of 37 ($\pm$3) at 4°C, and 9 ($\pm$1) at 17°C (Fig 4.33). The weaker binding detected by column chromatography agreed with the finding of Zillig et al. (1970b) and may have resulted from the ability of Sephadex to retard preferentially aromatic compounds. A similar discrepancy has been found in other systems (Wood & Cooper, 1970). The dissociation constants obtained by equilibrium dialysis were similar, and agreed with values obtained by other groups. The small difference between the two values may have resulted from the difference in temperature. Inactivating the enzyme with NBS had little effect on the binding constants. Modified enzymes

FIG 4.32 ATP BINDING TO NATIVE RNA-POLYMERASE (CORE) AT 4°C



RNA polymerase was concentrated as described in Fig 4.30.1. The pellet was dissolved in 50 μl buffer A + 0·2M KCl + [8 -^{3}H] ATP (500 μCi/μM) and chromatographed as described in section 2.2.04, ii. The same result was obtained with a sample which was dialysed into buffer A + 0·2M KCl before chromatography.

are often more susceptible to denaturation (Means & Feeney, 1971) and the small difference in K diss found by the stirred method (section 2.2.02) may have been due to this effect. In a control experiment stirring the unmodified enzyme led to a 10 % loss of activity.

Fig 4.33 Binding constants for ATP and core enzyme[1].

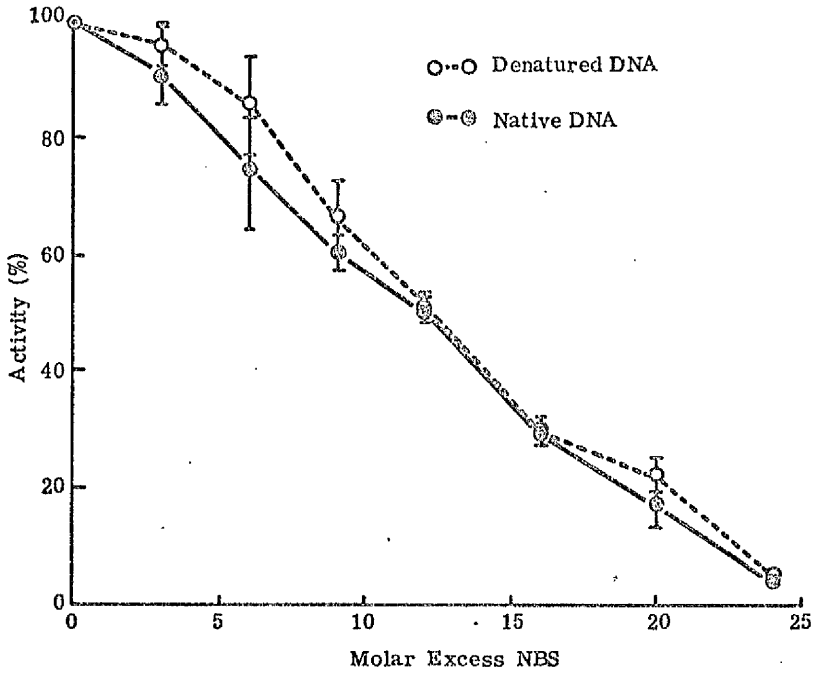
Technique	Dissociation constant (μM)[2]		Temperature
	Native	Modified[3]	
Column chromatography	110±40	-	4°C
Equilibrium dialysis[4] (non-stirred method)	37±3	34±3	4°C
Equilibrium dialysis[5] (stirred method)	9±1	36±4	17°C

1. The enzyme was prepared and modified as described in Fig 4.21 & Fig 4.23, concentrated as described in Fig 4.30.1, dialysed into buffer A + 0·2M KCl, and the binding constants determined as described in section 2.2.02.
2. Calculated assuming one binding site. Conditions were chosen so that binding at the weak site had a negligible effect on the dissociation constant.
3. 14 % remaining activity.
4. 10 mg/ml enzyme was dialysed against A + 0·2M KCl + 1 μM [8-^{3}H] ATP(10 μCi/μM). Samples after 24 and 30 h gave similar results.
5. 10 mg/ml enzyme was dialysed against A + 0·2M KCl + 50 μM [8-^{3}H] ATP (25 μCi/μM). Samples after 3 and 6 h gave similar results.

Thus inactivation was not due to a loss in affinity for ATP, and this was probably also true for GTP because ATP and GTP bind to the same site on the enzyme (Wu & Goldthwait, 1969 a, b). Inhibition may have been due to a loss of CTP or UTP binding activity, but they both have low association constants, and binding was difficult to detect. Of 14 experiments, binding of UTP could not be detected in 6 cases, and the other 8 gave a dissociation constant of about 300 μM. The failure to detect binding was not due to denaturation, since less than 10 % of the activity was lost during the determination.

The inhibition could have been an effect on the enzymes ability to denature the DNA template, by analogy with the properties of tryptophan and tyrosine peptides (section 1.3). When the modified enzyme was assayed with denatured DNA little or no recovery of activity could be detected (Fig 4.34). Therefore some activity other than (or as well as) unwinding was affected.

FIG 4.34 INACTIVATION OF DTNB-PROTECTED RNA-POLYMERASE (CORE) ASSAYED ON NATIVE AND DENATURED DNA TEMPLATE

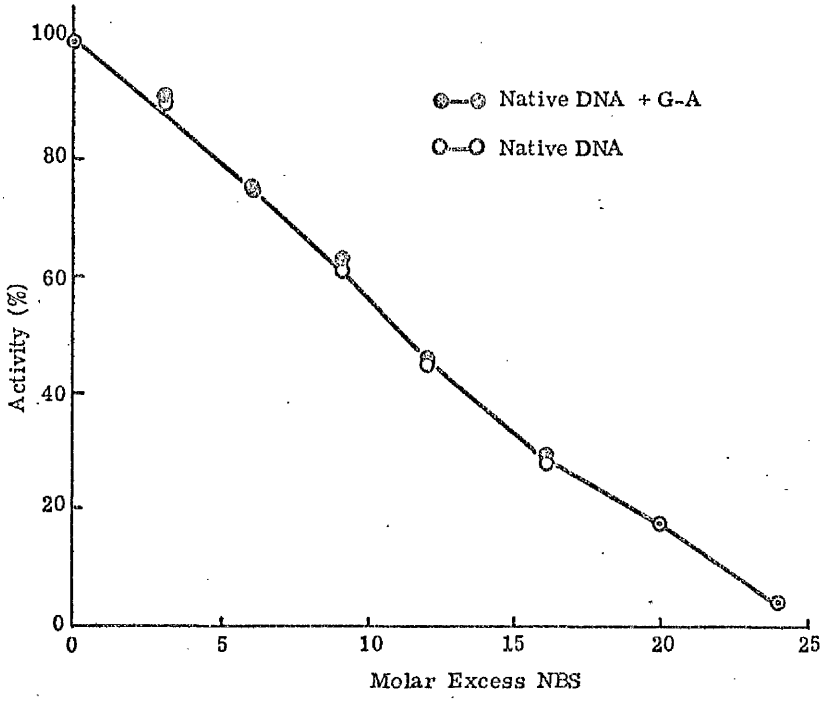


RNA polymerase (core) protected as described in Fig 4.21, and modified as described in Fig 4.25, was assayed in the usual manner, with native or denaturated DNA. Denaturated calf thymus DNA was prepared by heating to 100°C for 5 min, and cooling rapidly to 0°C. The activity on denatured DNA was 33 % of that on native DNA.

Wu & Goldthwait (1969 a) found that purine nucleotides quench the fluorescence of RNA polymerase by about 3 %, and they equated this site with the initiation site for RNA synthesis. If the quenching results from a direct interaction between indole and purine rings, then modifying these groups could lead to a block in initiation. Downey & So (1970) have shown that primers, such as G-A, circumvent the

initiation step, and stimulate transcription. A 30 % stimulation of activity was found with unmodified enzyme, and stimulation of partially modified enzyme was no greater than that expected from activation of unmodified species (Fig 4.35). Therefore the inhibition did not appear to be a block in initiation.

FIG 435 INACTIVATION OF DTNB-PROTECTED RNA POLYMERASE (CORE) ASSAYED WITH AND WITHOUT 100μM G-A



RNA polymerase (core) protected as described in Fig 4.21, and modified as described in Fig 4.25, was assayed in the usual manner with and without 100 μM G-A. Unmodified enzyme was stimulated by 30 % with G-A.

4.2.3 Further analysis to detect the amino acids oxidised by NBS.

Amino acid analysis has been used, by a number of groups, to study the selectivity of NBS oxidation (Williams, 1975; Kronman et al., 1967). The amino acid composition of core enzyme was determined (Fig 4.36), and found to be similar to that reported by other groups (Burgess, 1969 b; King & Nicholson, 1971; Fujiki & Zurek, 1975). An exception was the tryptophan content, which was found to be $10(\pm 1)$ as compared

Fig 4.36 Amino-acid composition of RNA polymerase (core).

Amino Acid	Methanesulphonic[5] Acid + Tryptamine		Hydrochloric Acid[6] + Mercaptoethanol	
	Number per Molecule[7]	Mole (%) frac.	Number per Molecule[7]	Mole (%) frac.
LYS	177	5·70	161	5·21
HIS	58	1·88	56	1·80
ARG	241	7·76	229	7·39
ASP	310	10·0	306	9·88
THR[1]	159	5·43	166	5·36
SER	181	5·84	174	5·62
GLU	391	12·7	401	12·9
PRO	135	4·37	155	4·93
GLY	244	7·89	243	7·84
ALA	228	7·36	226	7·29
CYS[2]	36	1·03	36	1·04
VAL[3]	283	9·23	267	8·21
MET	62	1·99	62	1·99
ILEU[4]	202	6·51	202	6·51
LEU	320	10·3	322	10·4
TYR	80	2·58	77	2·49
PHE	83	2·69	83	2·70
TRP[1]	11	0·37	9	0·29

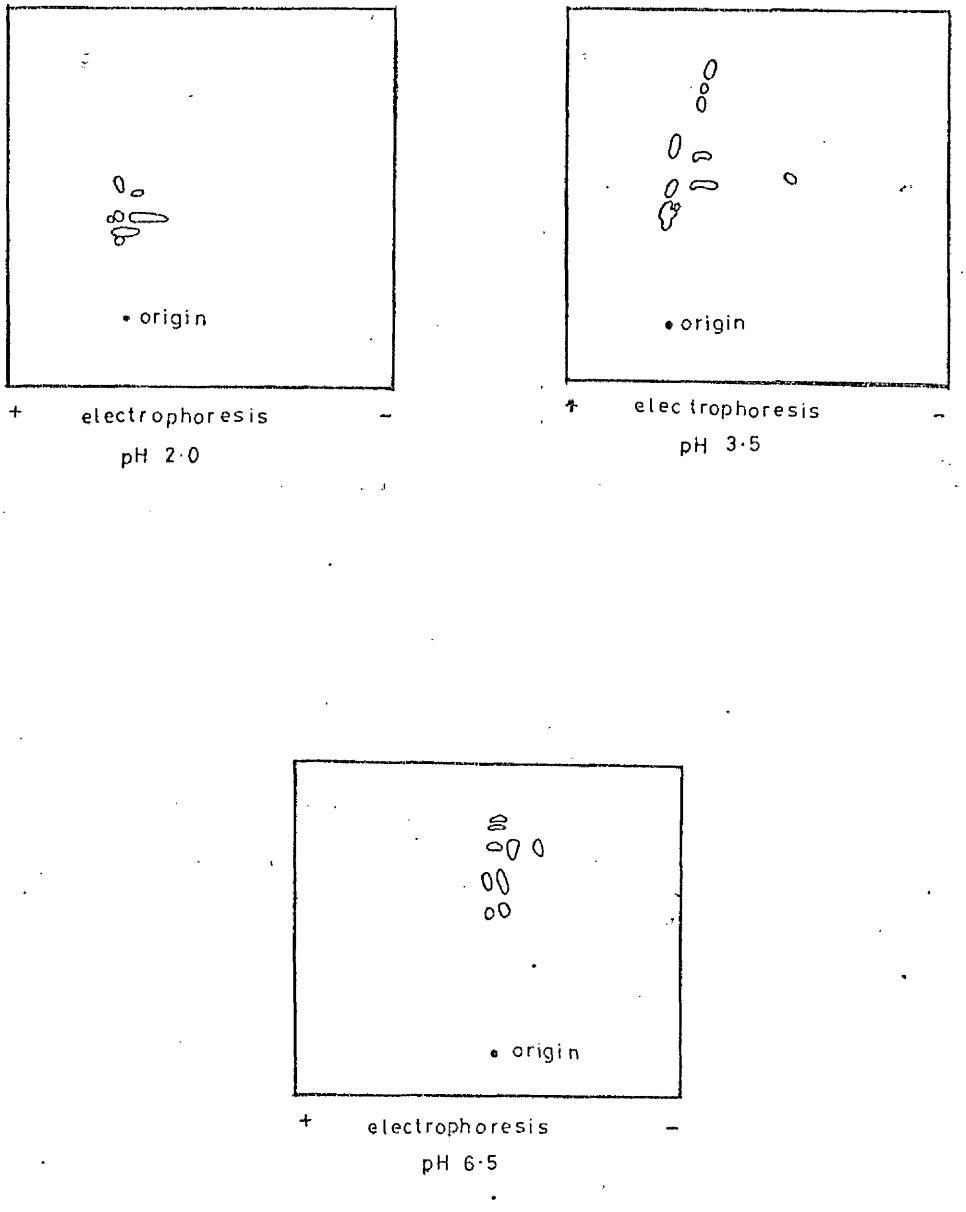
1. Value determined by extrapolation to zero time.
2. From titration of RNA polymerase (core) with DTNB.
3. Last time point value used.
4. Average of all values.
5. Duplicate samples, 48, 72, 96 hrs. at 110°C.
6. Duplicate samples, 24, 48, 72 hrs. at 110°C.
7. Using a mol. wt. of 400,000.

with 18 (Burgess, 1969 b), 19 (Fujiki & Zurek, 1975), and 23 (Nicholson, 1971). The method of Wetlaufer (1962) can be used to predict a probable range of tryptophan contents for the enzyme. Assuming there are 80 tyr, and 36 cys, and using $E^{1\%}_{280}$ values of 5.4 (King & Nicholson, 1971) and 6.5 (Richardson, 1966) probable tryptophan contents of between 14 and 21 and 21 and 31 respectively can be calculated. Thus it seemed that the tryptophan content was underestimated, but the value was reproducible in a number of experiments.

The tryptophan content could also be estimated by peptide mapping. When the enzyme was digested with several different proteases, up to 10 tryptophan spots could be detected (Fig 4.37, Fig 4.38, Fig 4.39). Schachner & Zillig (1971) could detect 16 using peptide mapping on the separated subunits. These numbers are only approximate because some peptides may have been generated in low yield, and others may not have been resolved. Peptide mapping may be useful in following tryptophan and other oxidations with NBS, because of the small molar quantities of sample required, and the small scale of the method. However it was difficult to resolve all the peptides of core enzyme and it was not used for modified enzyme.

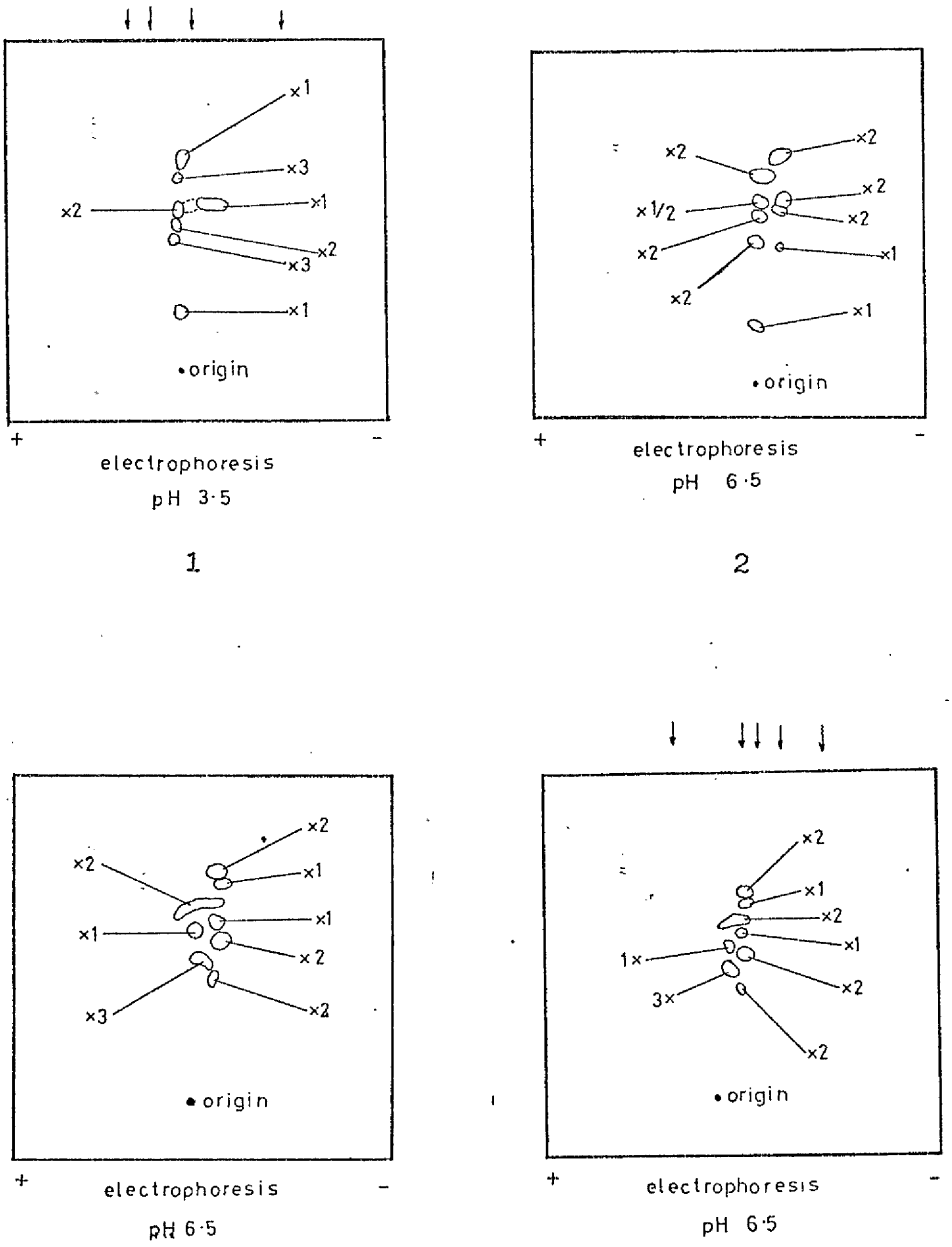
Amino acid analysis was used to follow the effects of NBS oxidation (Fig 4.40). When the results from 100 to 13 % activity were fitted to a straight line, by least squares analysis, it was found that there was no detectable loss of histidine, methonine, and tryptophan, and between 3 and 6 tyrosines were lost (Fig 4.41). Tyrosine oxidation affects the extinction method for calculating tryptophan loss (Kronman et al., 1967). The data of Schmir & Cohen (1961) were used to estimate that the increase in absorbance due to oxidation of 5 - 9 tyrosines was equivalent to the decrease from one tryptophan. Thus the tryptophan oxidation was underestimated by no more than one residue.

Fig 4.37 Peptide maps of RNA polymerase (core) digested with trypsin.



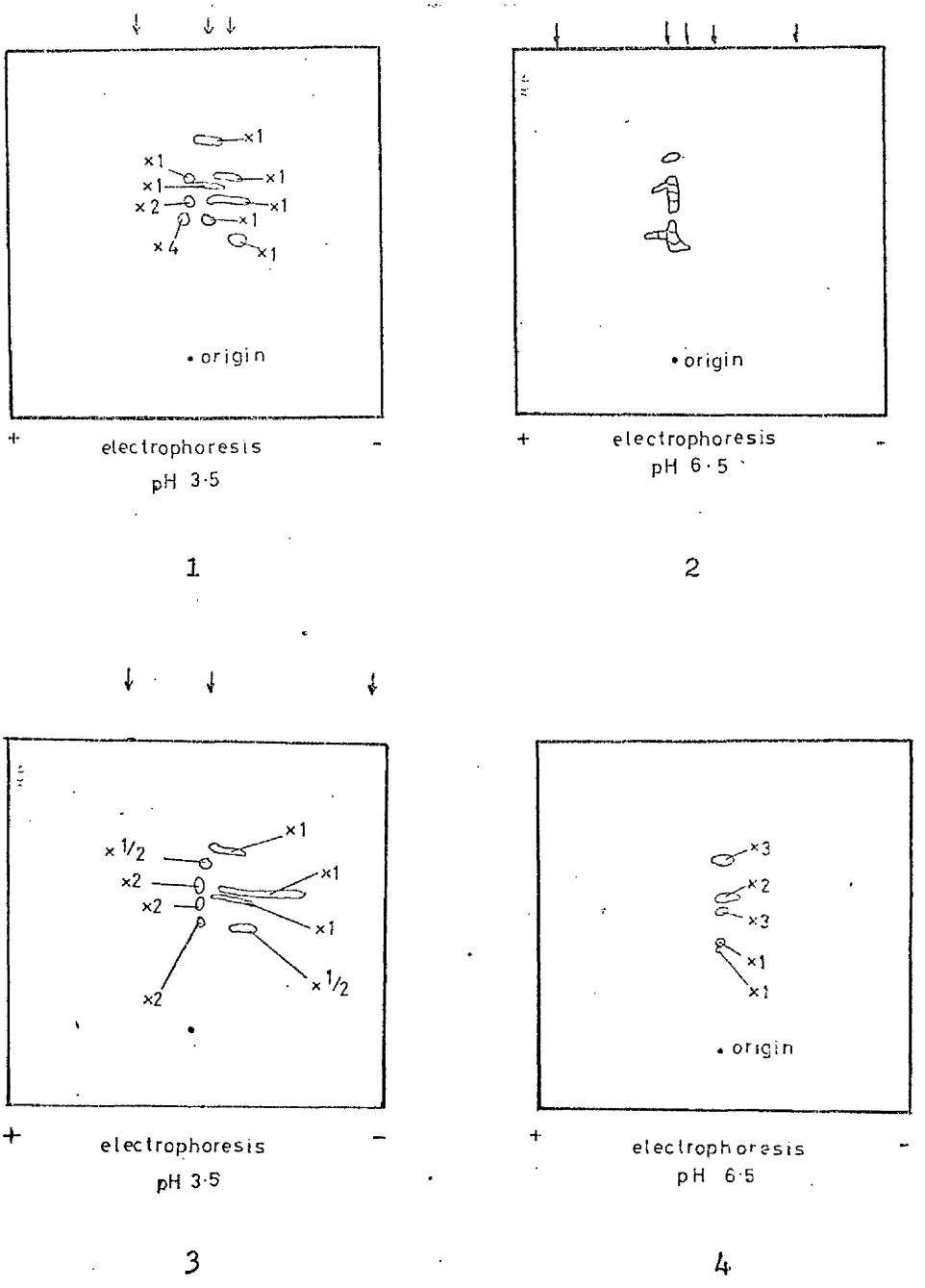
Peptide maps were run as described in section 2.2.09. 100 µg samples, digested with trypsin, were applied. Chromatography was from the bottom upwards. Tryptophan peptides were detected with ~~Ehrlichs~~ Ehrlick's reagent.

Fig 4.38 Peptide maps of RNA polymerase (core) digested with trypsin + chymotrypsin, and trypsin + thermolysin.



Peptide maps were run as described in section 2.2.09. 100 µg samples, digested with trypsin + chymotrypsin (1, 2), and trypsin + thermolysin (3, 4), were applied. Chromatography was from the bottom upwards. The tryptophan peptides were detected with ~~Ehrlich's~~ Ehrlich's reagent.

Fig 4.39 Peptide maps of RNA polymerase (core) digested with thermolysin and subtilisin.



Peptide maps were run as described in section 2.2.09. 100 µg samples, digested with thermolysin (1, 2), and subtilisin (3, 4), were applied. Chromatography was from the bottom upwards. Tryptophan peptides were detected with ~~Ehrlich's~~ Ehrlich's reagent.

Fig 4.40 Amino-acid composition of RNA polymerase (core) after N-bromosuccinimide oxidation of protected enzyme [1,2].

Amino acid	Activity (%)					
	100	76	70	33	13	0
LYS	167	167	170	167	167	173
HIS	52	51	60	55	55	55
ARG	222	218	227	223	228	224
ASP	310	312	314	306	307	306
THR	129	134	137	126	127	131
SER	149	167	173	143	142	154
GLU	406	407	413	411	408	405
PRO	101	125	111	114	115	119
GLY	247	253	256	248	243	245
ALA	232	233	237	235	227	232
VAL	275	252	264	281	266	268
MET	75	74	76	72	75	75
ILEU	202	189	197	207	202	199
LEU	327	327	321	321	325	314
TYR	78	78	79	75	75	75
PHE	82	81	83	81	81	80
TRP	11.4	11.4	11.6	9.6	12.0	-

1. Methanesulphonic acid + tryptamine hydrolysis for 48 h, duplicate samples.
2. The enzyme, protected and modified as described previously (Fig 4.21, Fig 4.25), was dialysed into 0·5 % ammonium bicarbonate, and analysed as described in section 2.2.01.

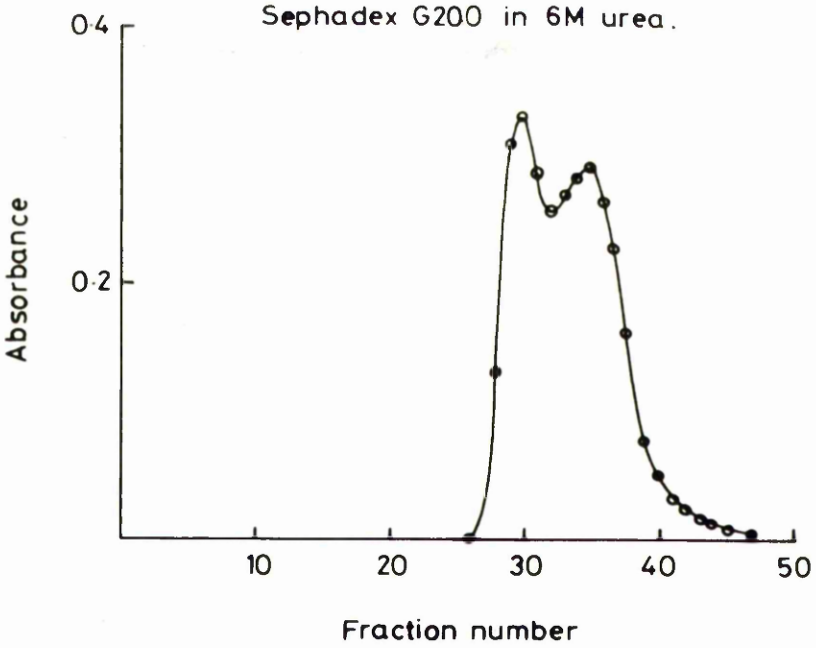
Fig 4.41 Loss of N-bromosuccinimide sensitive amino acids during inactivation of protected RNA polymerase (core)[1].

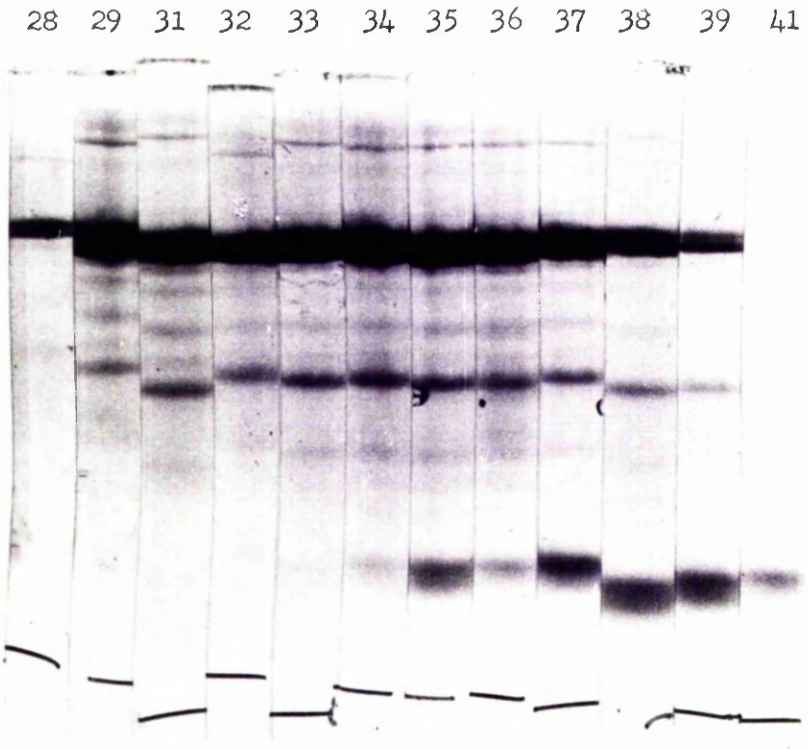
Amino acid	Loss over first 90 % of NBS inactivation
HIS	-2 ± 5
MET	0.3 ± 3
TYR	4.5 ± 1.5
TRP	0.35 ± 1.8

1. The number of amino acids remaining from 100 to 13 % activity were fitted to a straight line by least squares analysis.

The sensitivities of amino acid analysis and peptide mapping were limited by the size of the enzyme, and it could have been increased by studying the isolated subunits. Partial resolution of the subunits was achieved by chromatography on Sephadex G200 in 6M urea (Ishihama & Ito, 1972), which separated α from $\beta\beta'$ (Fig 4.42), and DEAE cellulose in 8 M urea (Burgess, 1969 b) which gave $\alpha + \beta$ (or β') and $\beta + \beta'$ (Fig 4.43). However better resolution was required before the separated subunits could be profitably analysed.

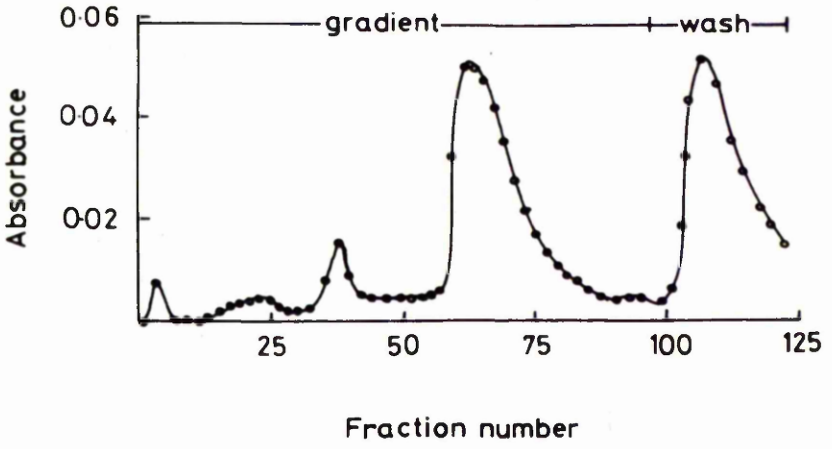
Fig 4·42 Separation of RNA polymerase subunits on Sephadex G200 in 6M urea.

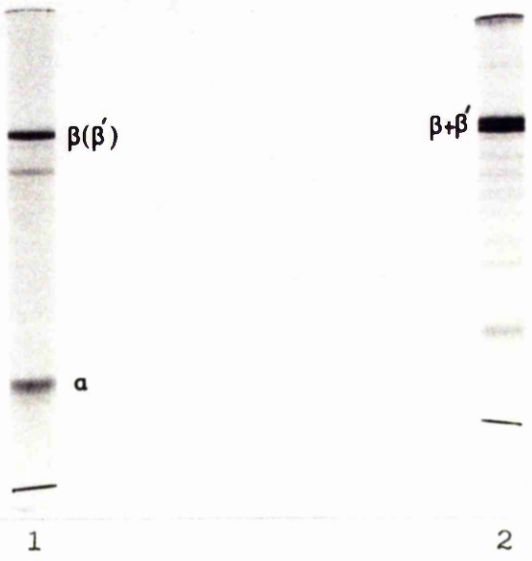




5 ml of 2 mg/ml holoenzyme in elution buffer (10 mM Tris HCl pH 7·9, 0·1 mM EDTA, 0·1 mM DTT, 100 mM KCl, 6M urea) was applied to a 2·5 x 100 cm column of Sephadex G200 which had been equilibrated with the same buffer. The subunits were eluted at 20 ml/h, 5 ml fractions were collected. SDS polyacrylamide gels were run of fractions 28, 29, 31, 32, 33, 34, 35, 36, 37, 38, 39, 41.

Fig 4·43 Separation of RNA polymerase subunits on DEAE cellulose in 8M urea.





5 ml of 2 mg/ml core enzyme in 5 mM Tris HCl pH 7·9, 0·1 mM DTT, 8M urea was applied to a 0·9 x 15 cm column of DEAE-cellulose equilibrated with the above buffer. The subunits were eluted with a 200 ml linear gradient, from 0·0 - 0·15 M KCl in the above buffer, at 20 ml/h, 2 ml fractions were collected. The column was washed with 0·5M KCl in the above buffer. SDS polyacrylamide gels were run of the pooled fractions 58 - 80 (gel 1) and 102 - 122 (gel 2).

4.3. Future experiments.

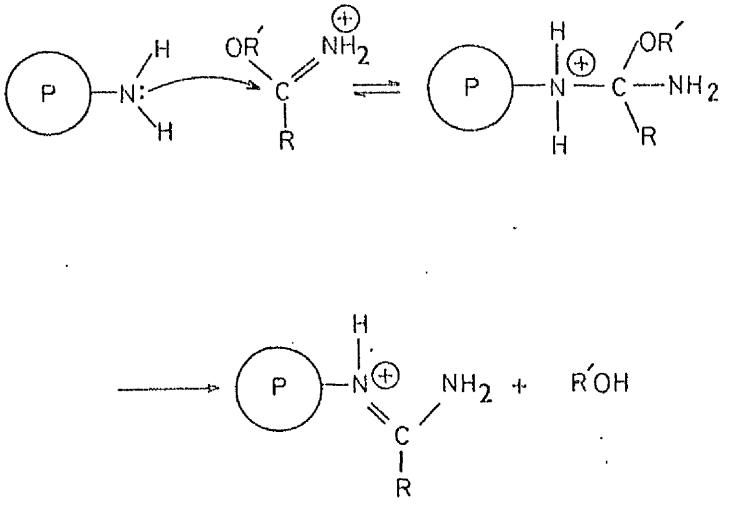
NBS modification of core enzyme did not appear to affect the binding of purine nucleoside triphosphates and DNA, nor initiation of RNA synthesis. A block in elongation could be detected by measuring unprimed synthesis of RNA (section 1.2.2.5). Hydrophobic amino acids contribute to the binding energy involved in the association of protein subunits (Clothia & Janin, 1975), and tryptophan appears to be involved in the monomer-dimer equilibrium of glutamate dehydrogenase (Witzemann et al., 1974). Hence the modification may have affected the association of the subunits of RNA polymerase, and this could be investigated by measuring the sedimentation value of the enzyme in low and high salt. Rifampicin binding to RNA polymerase quenches tryptophan fluorescence (Wu & Goldthwait, 1969a), and probably involves a hydrophobic site on the enzyme. A modification in this site could be detected by measuring the binding of rifampicin to the enzyme. Since quantitation and the exclusion of side reactions were difficult with NBS, the sensitivity of the methods used for detection need to be improved. This could be achieved by isolating the subunits and specific proteolytic fragments of the enzyme, or by using radioactive derivations of 2-hydroxy-5-nitrobenzyl bromide, and sulphenyl halides.

CHAPTER 5.

AMIDINATION OF CORE ENZYME.

5.1 Introduction.

Imidoesters react specifically, and under mild conditions, with the amino groups of proteins to give amidines (Hunter & Ludwig, 1972; Ludwig & Hunter, 1967; Means & Feeney, 1971). The reaction can be represented as:



The resulting amidines are stronger bases than the parent amines. The reaction is pH dependent and, with model compounds, a pH optimum occurs below which the breakdown of the tetrahedral intermediate is rate limiting, and above which the reaction with free base and the cationic form of the imidoester is rate limiting (Hand & Jencks, 1962). A competing reaction is a slower hydrolysis of the reagent, which is neither subject to general acid base catalysis, nor enhanced by the presence of protein (Means & Feeney, 1971).

Amidination should be useful in the study of RNA polymerase because of the mild reaction conditions, the specificity for amino groups, and the availability of radioactive reagents. Imidoesters with aromatic or charged side chains could be used to alter the selectivity for groups of lysines. In this study RNA polymerase core enzyme was modified with methyl- [^{14}C] acetimidate, in the presence and

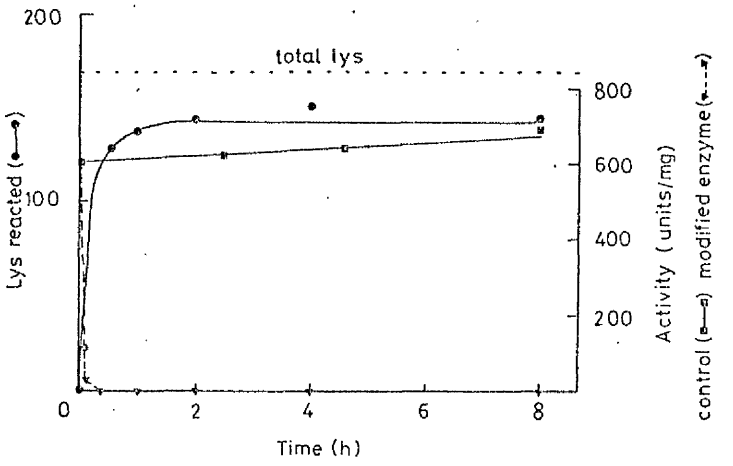
absence of substrates, to gain information on the structure and active site of the enzyme.

5.3 Results and Discussion.

Amidination of RNA polymerase (core), at a high reagent concentration, led to a modification of 147 of the available 169 lysines, and to a rapid loss of activity (Fig 5.1). At a lower concentration the reaction was limited to about 65 lysines (Fig 5.2). Modification of 5 lysines led to no loss of activity, wereas reaction of 50 gave 95 % inhibition. These essential lysines may be involved in maintaining the structural integrity of the enzyme, or may participate in the active site. Substrates were found to protect against the initial rate of inactivation (Fig 5.3), and, as expected, there was little difference between the final activities (Fig 5.2). The residual difference in activity may have been due to exhaustion of reagent by hydrolysis, and this may account for the smaller number of lysines reacted at the plateau regions for free enzyme (Fig 5.1, Fig 5.2).

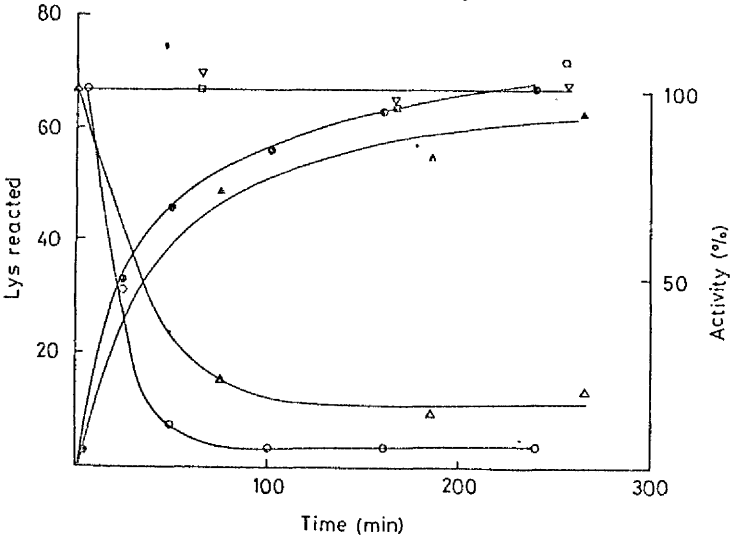
Protection against inactivation was greatest in the initiated complex of enzyme, DNA, ATP, GTP, and CTP, less when denatured DNA was used to protect, and only a small amount with purine nucleotides as protection (Fig 5.3). This was the same order as the association constants of the complexes, which would account for the observed protection. Alternatively it may have reflected a difference in the conformational states. Nicholson & King (1973) found that the initiated enzyme had an increased resistance to inactivation by thiol reagents, which they ascribed to its "tighter" conformation in this state. A similar effect could confer resistance on amino groups involved in maintaining the structure of RNA polymerase. This could be compared with the methyl acetimidate modification of bovine serum albumin, which affects the equilibrium between the N and F conformations, probably due

Fig 5.1 Amidination of RNA polymerase (core) with 0·085M methyl acetimidate.



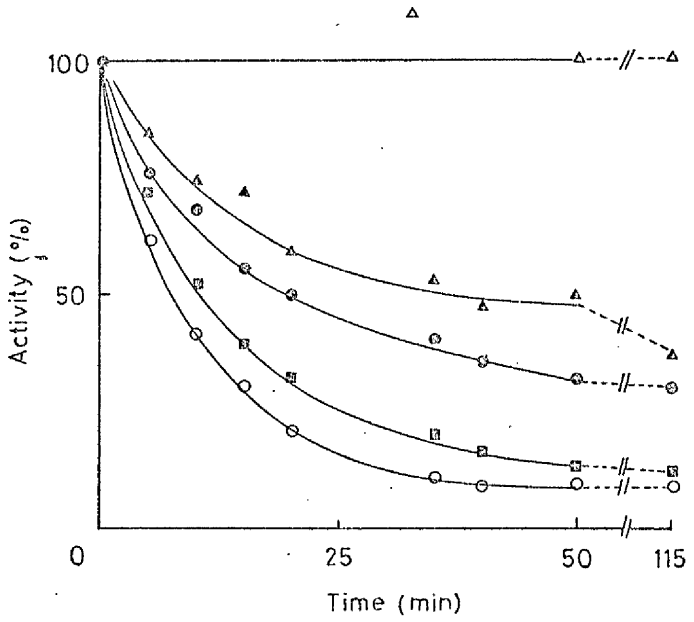
RNA polymerase (core), 0·8 mg/ml, in 180 mM triethanolamine HCl, 0·8 mM DTT, 0·8 mM EDTA, 4 % glycerol, pH 8·0, was modified with 0·085 M methyl-[^{14}C] acetimidate at 20°C. Samples were taken at intervals, cooled in ice, and chromatographed on Sephadex G25 equilibrated with 50 mM triethanolamine HCl, 0·1 mM EDTA, 0·1 mM DTT, 0·2 M NaCl, 5 % glycerol, pH 8·0. The number of reacted lysines was determined by comparison with RNA polymerase modified for 8 h in 6 M guanidinium hydrochloride. Samples were assayed for protein (section 2.2.07), radioactivity (section 2.2.07) and enzyme activity (section 2.2.12). Methyl-[^{14}C] acetimidate, prepared as described by Bates et al. (1975), was a gift from Dr. J.R. Coggins.

Fig 5.2 Amidination of RNA polymerase (core) ±DNA with 0·01M methyl acetimidate.



RNA polymerase (core), 1·0 mg/ml, in 100 mM triethanolamine HCl, 1 mM EDTA, 0·1 mM DTT, 5 % glycerol, pH 8·0, 20°C, with or without 0·7 mM denatured DNA, was modified with 0·01 M methyl-[^{14}C] acetimidate. Samples were chromatographed on Sephadex G25 equilibrated with 50 mM triethanolamine HCl, 0·1 mM EDTA, 0·1mM DTT, 0·2 M NaCl, 5 % glycerol, pH 8·0. Denatured DNA was prepared by sonication for 4 x 30s, boiling for 5 min, and rapid cooling. In a control experiment no incorporation of radioactivity into DNA could be detected. (□—□ control activity, free enzyme; ▽—▽ control activity, enzyme + DNA; ○—○ activity of enzyme; △—△ activity of enzyme + DNA; ●—● lysines modified, free enzyme; ▲—▲ lysines modified, enzyme + DNA).

Fig 5.3 Effect of substrates on the inactivation of core enzyme with methyl acetimidate.



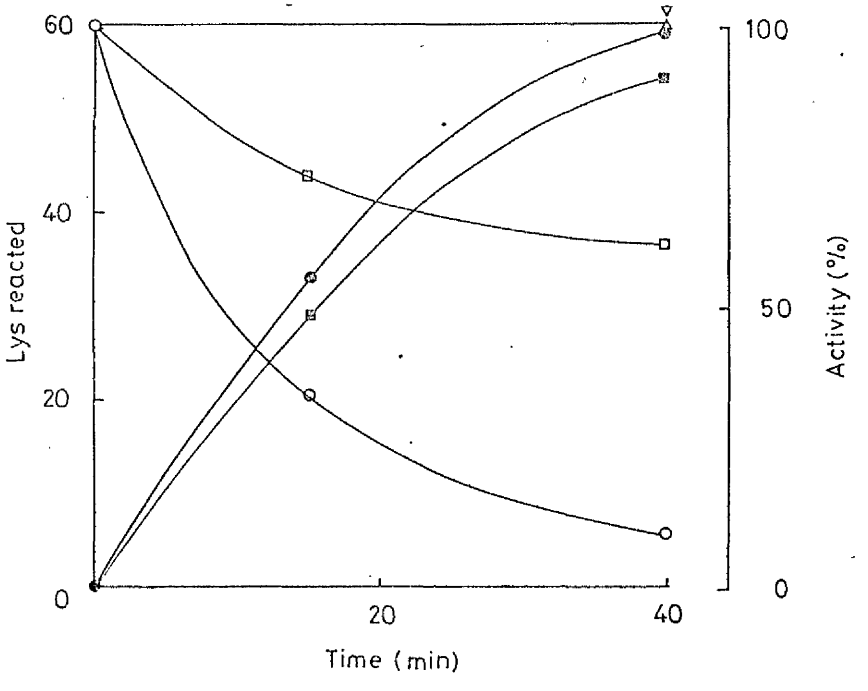
RNA polymerase (core) was modified, as described in Fig 5.2, in the presence of 0·7 mM denatured DNA, 0·7 mM denatured DNA + 0·4 mM ATP + 0·4 mM GTP + 0·4 mM CTP, and 0·4 mM ATP + 0·4 mM GTP. Samples were preincubated for 30 min, at 20°C, before addition of reagent. The reaction was stopped by diluting 10 µl samples in the normal assay solutions. Nucleoside triphosphates (J.R. Coggins, personal communications) and DNA do not react with the reagent under these conditions. (△—△ enzyme + DNA + ATP + GTP + CTP, ◎—◎ enzyme + DNA, ▣—▣ enzyme + ATP + GTP, ○—○ enzyme alone, △—△ control with enzyme and no reagent).

to disruption of $-COO^- \ldots {}^+H_3N-$ interactions (Avruch et al., 1969). The increased resistance due to nucleotides and DNA together could also be explained by inactivation occurring at two distinct sites.

There was a proportionally smaller protection of the rate of loss of amino groups compared with activity (Fig 5.2, Fig 5.4). Assuming that modification of a small group of fast reacting amino groups led to inactivation and that other groups reacted to the same extent, it could be concluded from Fig 5.4 that 8 - 10 lysines were involved in activity. If the assumptions were true it would be expected that the difference in activity would be proportional to the difference in amino groups modified. Further experiments are required to confirm this. The number of

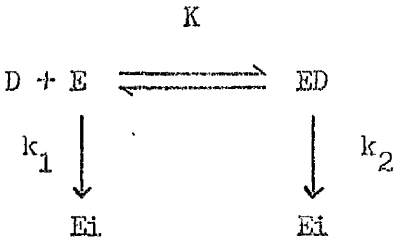
essential lysines could be compared to the size of the DNA binding site, the number of nucleotide binding sites, and the size of the open region during transcription. The nuclease resistant DNA fragments bound to RNA polymerase are 15-40 base pairs long (section 1.2.2.1), and complete charge neutralisation by lysine would require 30 - 80 amino groups, whereas two nucleotide binding sites would require 4 - 8 lysines, and the 8 base pair open region present during transcription about 16 lysines. Amidination of groups involved in purely ionic interactions would probably not have led to inactivation. The inactivation may be comparable with that observed with RNA ase (Reynolds, 1968). Modification with methyl acetimidate led to complete inactivation, although physical properties of the 97 % reacted enzyme were virtually unchanged. The inactivation was due to reaction with the essential lysine 41 (Hirs et al., 1965), and probably resulted from strict steric requirements around this group.

Fig 5.4 Amidination of RNA polymerase (core) ± DNA +ATP +GTP +CTP with 0·01M methyl acetimidate.



RNA polymerase (core) was modified, as described in Fig 5.2, with or without 0·7 mM denatured DNA + 0·4 mM ATP + 0·4 mM GTP + 0·4 mM CTP (▽—▽ control activity free enzyme, △—△ control activity with substrates, □—□ activity with substrates, ○—○ activity of enzyme, ◎—◎ lys modified free enzyme, ▣—▣ lys modified with substrates).

The rate of inactivation in the first 20 min of modification was found to follow pseudo first order kinetics, indicating that one group of lysines, (or possibly several groups with similar rate constants), was involved in the inactivation. The effect of varying the denatured DNA concentration on the pseudo first order rate constant was determined (Fig 5.5). The following simple model, in which the initial rate of inactivation depended upon the proportion of enzyme in the free and DNA bound state, was used to intercept the results:



where E, Ei = active and inactive enzyme respectively

D, ED = free and enzyme bound DNA

k_1, k_2 = rate constants for inactivation of free and DNA bound enzyme

K = dissociation constant = $[E][D]/[ED]$

This gives (Appendix 8):

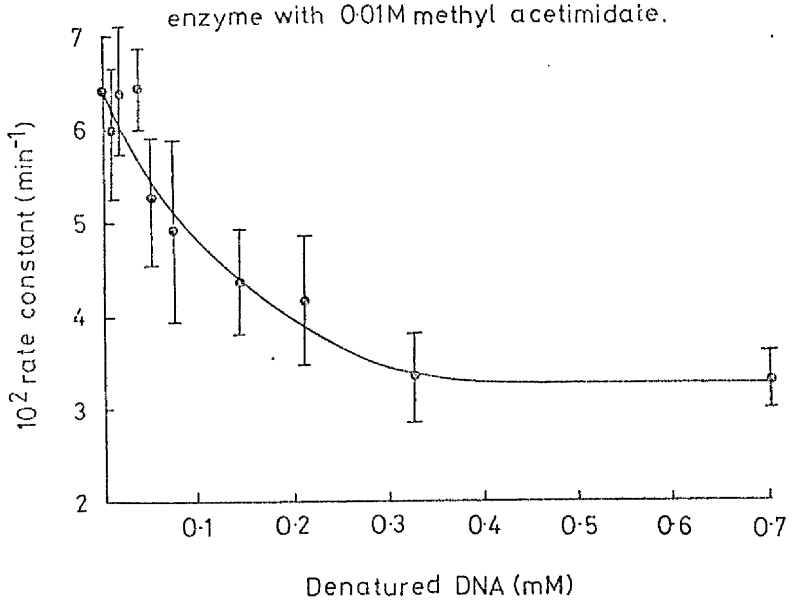
$$\frac{[D]t}{k_1-k_o} = \frac{K}{k_o-k_2} + \frac{[E]t}{k_1-k_2}$$

where $[D]t$, $[E]t$ = total concentrations of DNA and enzyme

k_o = observed rate constant.

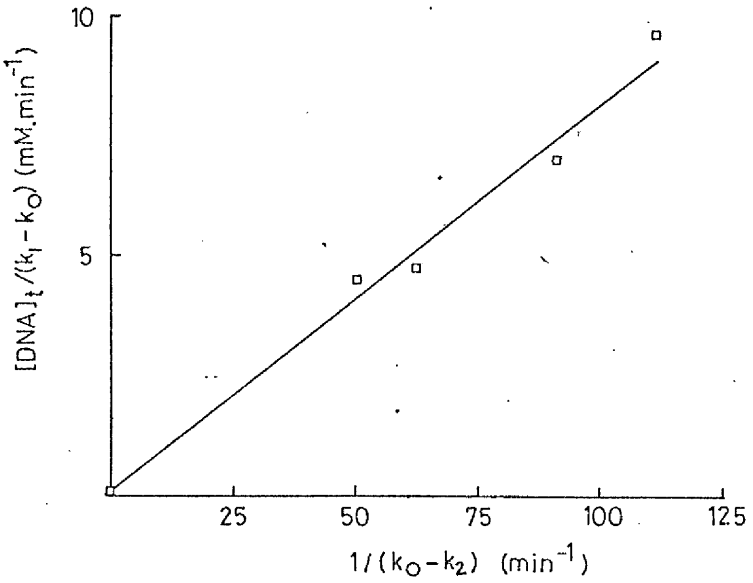
The data in Fig 5.5 gave $K = 8 \pm 2 \times 10^{-5}$M (Fig 5.6). This value was smaller than 5×10^{-11}M for core enzyme and native DNA (Hinkle & Chamberlin, 1972a) and suggests that core enzyme has less affinity for denatured DNA. The difference between the values is large and requires confirmation of the results by direct measurement of the dissociation constant for denatured DNA.

Fig 5·5 Effect of denatured DNA on inactivation of core enzyme with 0·01M methyl acetimidate.



RNA polymerase (core) was modified as described in Fig 5.3, with different denatured DNA concentrations.

Fig 5·6 Determination of K for denatured DNA.



The data in Fig 5.5 was plotted using the equation derived in Appendix 8. The gradient gave K = 80 ± 20 μM.

In summary modification of RNA polymerase (core) with methyl acetimidate led to loss of activity. Protecting the enzyme with substrates prevented the modification of 8 - 10 lysines. Krakow (1973) obtained similar results with Azotobacter vinlandii core enzyme. He found that trinitrobenzenesulphonic acid modified about 50 lysines with complete loss of activity, and that poly d(A-T) protected against inactivation and the loss of about 8 lysines.

5.3 Further work.

Methyl acetimidate may affect the structure of core enzyme, and a variety of techniques could be used to study the conformation of the active and inactive molecules. The inhibition may be at any of the distinguishable steps in the activity of RNA polymerase. A likely condidate is DNA binding, and the template binding ability of modified and modified-protected enzyme could be compared. Analysis of the subunit distribution of label in free enzyme, substrate protected enzyme, and after modification at low and high ionic strength, could give information on the relative exposure of subunit lysines, the subunit distribution of substrate binding sites, and the interactions involved in the association of the enzyme.

CHAPTER 6.

FORMALDEHYDE MELTING AS A PROBE OF RNA POLYMERASE DNA INTERACTIONS.

6.1. Introduction.

Kosaganov et al. (1971) showed that RNA polymerase, in a ternary complex of DNA - protein - RNA, affected the kinetics of formaldehyde melting. They analysed their results by the method of Trifonov et al. (1968), which predicts that formaldehyde melting follows the equation:

$$-\frac{\ln(n)}{t} = 2vc + pvt$$

where n = fraction of base pairs hydrogen bonded at time t

c = concentration of defects (regions permanently accessible to reaction with formaldehyde)

p = rate constant for the formation of regions capable of irreversibly reacting with formaldehyde

v = rate constant for the growth of denatured regions.

This equation was derived assuming that random regions of the double helix opened spontaneously, and if they were a certain size they reacted irreversibly with formaldehyde. These areas increased in size, along the helix, until the molecule was denatured. Defects provided extra regions from which denaturation could extend, and so increased the rate of melting. The concentration of defects was obtained by plotting $-\ln(n)/t$ versus t, the intercept gave 2vc. v was determined using shear degraded DNA of known molecular weight.

This method was used by Trifonov and his co-workers to determine the number of defects introduced into DNA by pancreatic DNA ase (Bannikov & Trifonov, 1969) and uv irradiation (Trifonov et al., 1968). They were able to show that the results agreed with independent determinations of the number of defects. RNA ase, bound to DNA, was also found to affect the kinetics in a similar manner, and hence the number of defects could be determined (Kosaganov et al., 1969). In this case, at least,

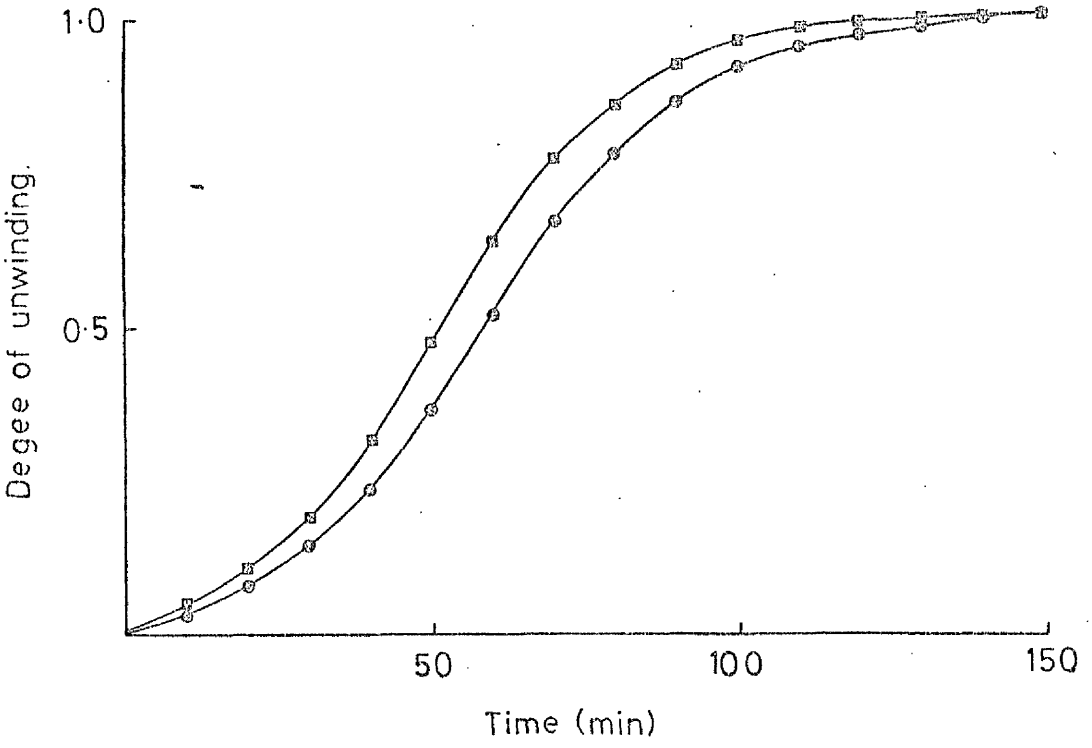
the reaction of protein with formaldehyde (Means & Feeney, 1971) did not affect the kinetics. Thus Kosaganov et al. (1971) argued that the kinetic formaldehyde method could be applied to a protein such as RNA polymerase. They found that the transcription complex produced defects, presumably due to unwinding of the double helix. However, there were no detectable defects with the preinitiation complex, which also unwinds the helix (section 1. 2. 2.1).

In this study the kinetic formaldehyde method was investigated as a spectrophotometric assay for RNA polymerase. A spectrophotometric assay would be more convenient than the normal assay, which measures the incorporation of radioactivity into acid precipitable RNA. It might also have provided information on the number of slow regions present during transcription, the number of independent polymerase molecules which can transcribe a gene, and the size of the transcribing region.

6.2. Results and Discussion.

Formaldehyde melting experiments were performed as described by Kosaganov et al. (1971) except that T7 DNA and 54°C were used, instead of T2 DNA and 48°C. T7 is smaller than T2 DNA (mol. wts. 24 & 160 x 10^6 respectively), and hence more easily prepared in a native form. Also more is known about its transcription (Chamberlin, 1974b). 54°C was used to decrease the reaction time from about 200 to 100 min, and make it comparable to that for T2 DNA. Melting curves in the presence and absence of RNA polymerase holoenzyme (Fig 6.1) were analysed by the method of Trifonov et al. (1968). The results were fitted to a straight line by the least squares method, weighted to compensate for the greater errors involved in determining the rate of absorbance changes at the beginning and end of the melting (Appendix 7).

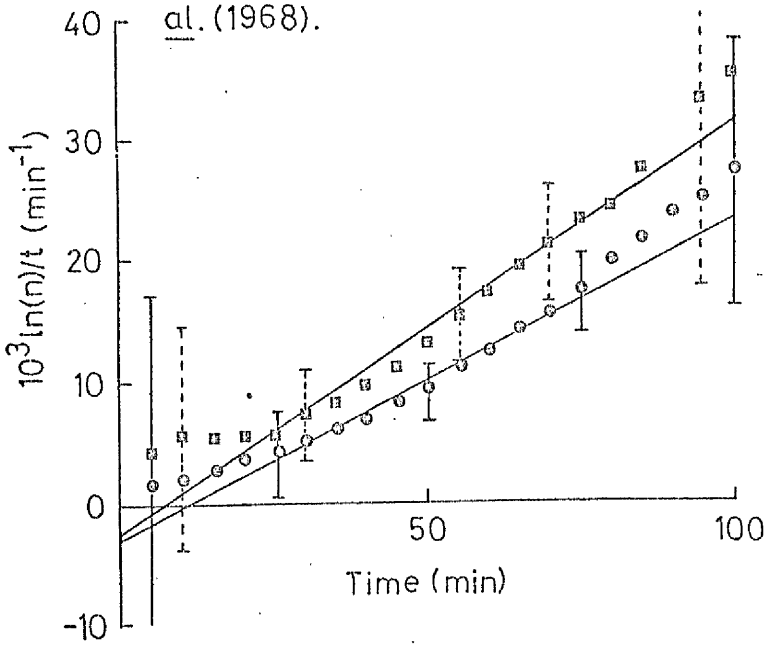
Fig 6.1 Denaturation of T7 DNA by 5·6 % formaldehyde.
(wavelength = 255 nm, temp. = 54°C)



T7 DNA, 60 µg in 0·5 ml of 5 mM triethanolamine HCl pH 7·7, 4·5 mM $MgCl_2$, 43 mM KCl, 1·4 mM 2-mercaptoethanolamine, 4 µM ATP, 4 µM GTP, 4 µM CTP, 4 µM UTP, with or without RNA polymerase (holoenzyme) was preincubated for 15 min at 54°C in a 1 ml, teflon stoppered, jacketed spectrophotometer cell. 0·5 ml of 11% (w/w) formaldehyde in 5 mM triethanolamine HCl, pH 8·4, preincubated for 15 min at 54°C, was added from a preheated pipette tip. The solutions were mixed and absorbance changes recorded with an SP8000 spectrophotometer and Heathkit recorder (⊙—⊙ T7 DNA, ⊡—⊡ T7 DNA + 3 enzyme units of holoenzyme).

The plot for DNA gave a negative value for the intercept, and the presence of enzyme made this value less negative (Fig 6.2). These "negative defects" may have been an artefact of the experimental conditions, or they may have resulted from a lag in DNA melting (also equivalent to renaturation compensating for denaturation). Altering a number of experimental conditions did not affect the negative intercept. Taking precautions to eliminate dust particles, and varying the time of preincubation from 5 to 120 min, did not affect the results. The isosbestic point used to follow denaturation, without interference from

Fig. 6·2 Analysis of T7 DNA-formaldehyde denaturation by the method of Trifonov et al. (1968).

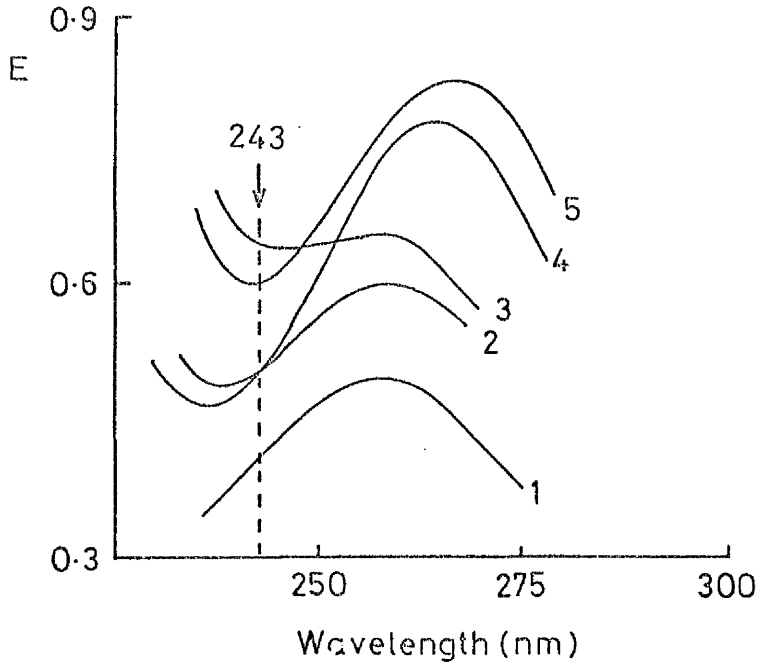


The results in Fig 6.1 were analysed by the method of Trifonov et al. (1968). They were fitted to a straight line by the weighted least squares method. Error bars are included on several points to indicate the relative sizes of the weighting factors (⊙—⊙ T7 DNA; ⊡—⊡ T7 DNA + 3 enzyme units holoenzyme).

the reaction with formaldehyde, was redetermined. Kosaganov et al. (1971) used a wavelength of 255 nm, determined by comparing the absorption spectrum of denatured DNA in the presence and absence of formaldehyde at 100°C. Denatured DNA at 54°C gave an isobestic point at 243 nm (Fig 6.3), but analysis of the kinetics at this wavelength also gave a negative intercept (Fig 6.4). The negative intercept was probably not due to impurities in the formaldehyde, since purifying the formaldehyde (section 2.2.11) did not affect the results (Fig 6.4).

Jonker & Blok (1975) obtained a negative intercept for T2 DNA, about one-fifth of that for T7 DNA, despite taking a number of precautions. They corrected for errors in determining the initial absorbance changes by using a differentiated form of the normal equation. Other precautions they took were storing formaldehyde at room temperature for two days before use, increasing the preincubation time to 1 h, and

Fig 6·3 Isosbestic point for formaldehyde and heat denatured T 7 DNA.



T7 DNA, 0·5 ml in 5 mM triethanolamine HCl, pH 7·7, 4·5 mM $MgCl_2$, 43 mM KCl, 1·4 mM 2-mercaptoethanol, was diluted with 0·5 ml 5 mM triethanolamine HCl, pH 8·4, alone or containing 11 % formaldehyde. The absorption spectra of 1) native DNA at 54°C, 2) DNA, denatured at 100°C for 5 min and cooled to 54°C (the spectrum at 54°C was constant for 5 min), 3) denatured DNA at 100°C, 4) denatured DNA, in the presence of 5·6 % formaldehyde, at 54°C, and 5) denatured DNA, with formaldehyde, at 100°C, were recorded on an SP8000 spectrophotometer. Repeated heating to 100°C and cooling to 54°C had no effect on the spectra of 2 - 5. The isosbestic points were: denatured DNA with and without formaldehyde at 54°C = 243 ± 0·2 nm, denatured DNA with and without formaldehyde at 100°C = 247 nm, denatured DNA at 100°C and denatured DNA + formaldehyde at 54°C = 252 nm.

Fig 6.4 Effect of wavelength, purity of formaldehyde, and enzyme, on the melting kinetics of T7 DNA[1].

Wavelength (nm)	Enzyme (units)	10^3 gradient (min^{-2})	10^2 intercept (min^{-1})	formaldehyde
255	-	0·17(1)[2]	- 0·62	commercial
255	-	0·22±0·02(3)	- 0·48±0·10	purified
243	-	0·29±0·04(5)	- 0·56±0·07	purified
255	6	0·47±0·03(2)	- 0·22±0·01	commercial
255	3	0·36±0·04(2)	- 0·51±0·4	purified
243	12	0·32(1)	+ 0·76	purified

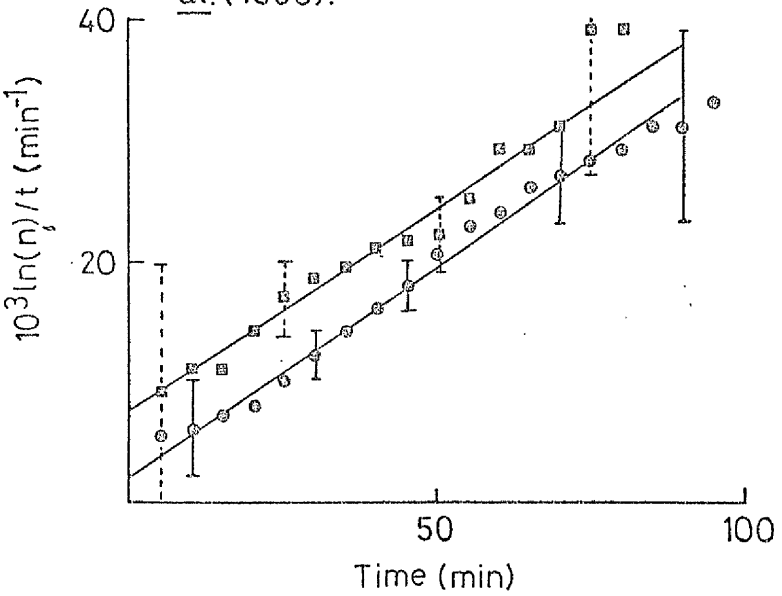
1. Conditions were as described in Fig 6.1.
2. Figures in brackets are the number of experiments performed.

ensuring there was little change in temperature during the mixing of formaldehyde and DNA. Thus a negative intercept seems to be an inherent error in the method, and is worse for some types of DNA than others.

RNA polymerase increased the value of the intercept, as predicted by the theory (Fig 6.4). Since the effect was small, and similar in size to the negative intercept observed with DNA alone, interpretation of the results was greatly influenced by the explanation for a negative incept.

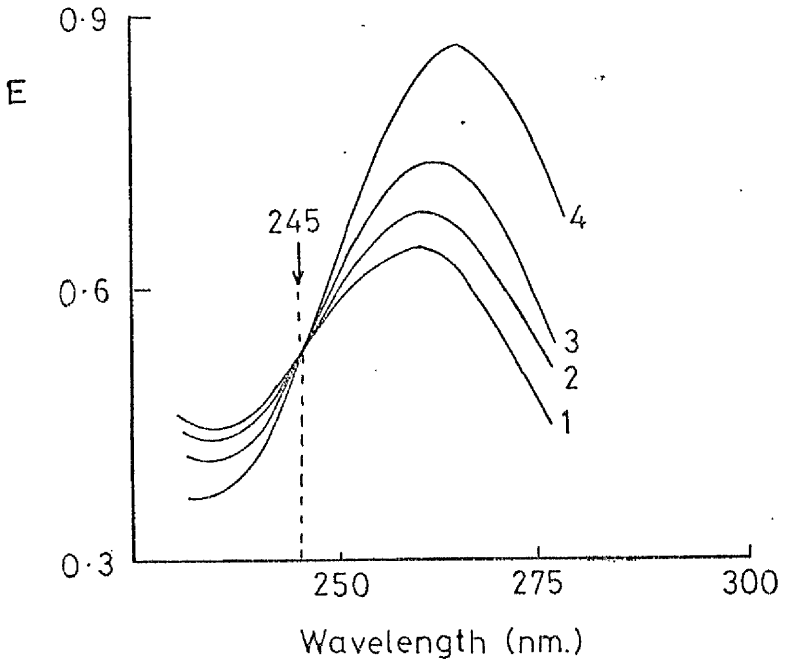
Calf-thymus DNA gave a positive intercept under similar conditions (Fig 6.5), both at 255 nm, and at 245 nm (the "isosbestic" point at 54°C, Fig 6.6). This DNA normally contains nicks and single stranded regions which affect the melting, and complicate interpretation of the results in the presence of RNA polymerase.

Fig.6·5 Analysis of CT DNA-formaldehyde dena= turation by the method of Trifonov et al. (1968).



Conditions were as described in Fig 6.1, except that calf-thymus DNA, 245 nm, and 6 enzyme units were used. (⊙—⊙ calf-thymus DNA, ⊡—⊡ calf-thymus DNA + 6 enzyme units).

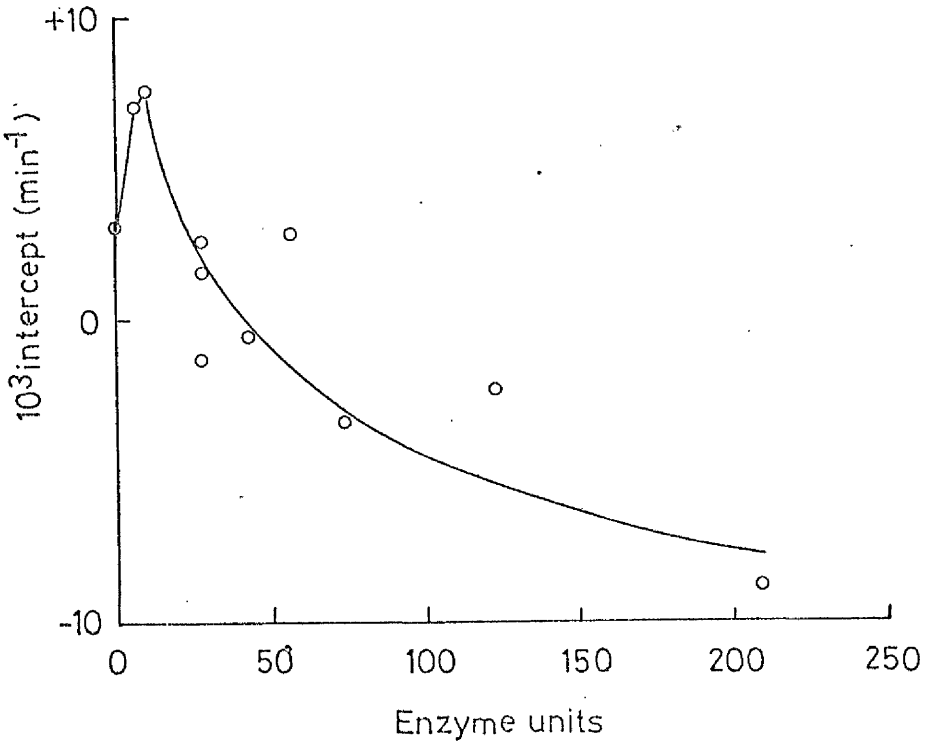
Fig.6·6 Isosbestic point for formaldehyde and heat denatured calf-thymus DNA (54°C).



Calf-thymus DNA, in the buffer described in Fig 6.3, was heated to 100°C for 5 min, cooled to 54°C, and the absorption spectrum recorded. The formaldehyde concentrations were: 1. none, 2. 0·011 %, 3. 0·55 %, 4. 5·6 %. Reheating to 100°C and cooling had no effect on the spectra. Isosbestic point = 245 ± 1 nm.

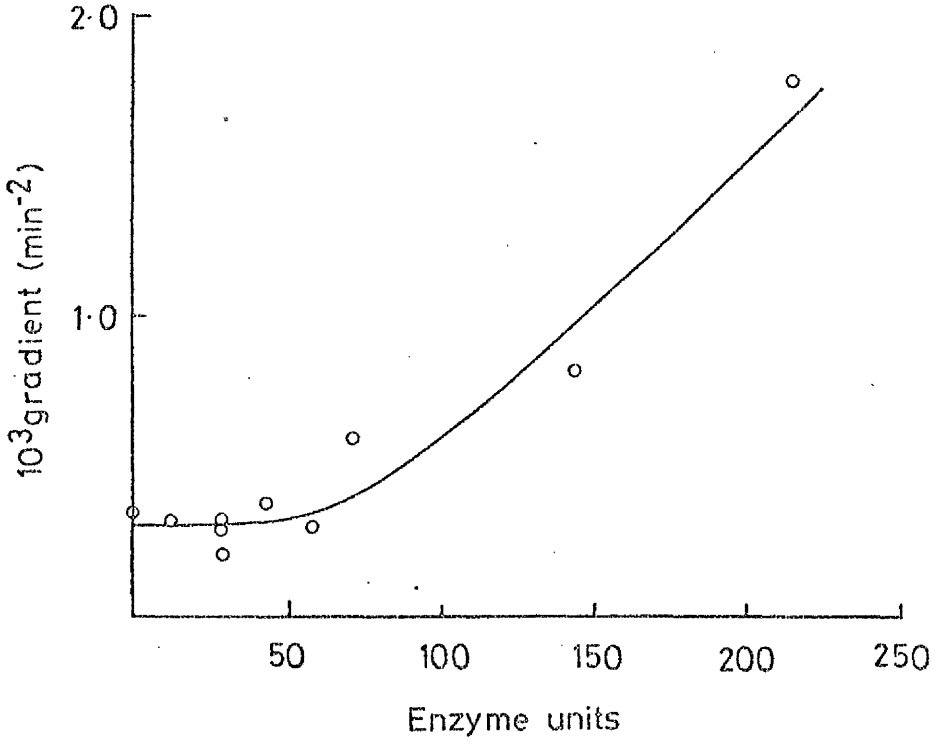
At low concentrations of enzyme there was an increase in the intercept, indicating an increasing number of defects (Fig 6.7). Further enzyme reduced the intercept, eventually to a negative value. The gradient remained at a constant value up to approximately 50 units of enzyme, and then increased (Fig 6.8). These results suggested that as well as producing defects RNA polymerase also affected the rate of both denaturation and extension of denatured regions. Siomonov et al. (1974) observed a comparable effect with amine containing compounds. They showed that amino-acids, nucleotides, and histones, accelerated the rate of reaction between DNA and formaldehyde. At a low concentration RNA polymerase was mainly in initiated complexes and this may have affected the term c. As the concentration was increased the

Fig.6·7 Effect of enzyme concentration on the intercept of the Trifonov et al.(1968) plot (calf-thymus DNA).



Formaldehyde melting of calf-thymus DNA, with various concentrations of holoenzyme, was followed at 245 nm, as described in Fig 6.1. The enzyme was assayed on a calf-thymus DNA template.

Fig.6·8 Effect of enzyme concentration on the gradient of the Trifonov et al.(1968) plot (calf-thymus DNA).



Conditions were as described in Fig 6.7.

ratio of protein to DNA and nucleoside triphosphates, and hence the ratio of DNA bound to initiated enzyme, increased. This may have had an increasing effect on the gradient of the plot. The behaviour of the intercept was more difficult to explain, but it may have resulted from a lag in the onset of the acceleration effect.

This method, in its present form, was felt to be unsuitable as an assay for RNA polymerase because the melting kinetics with a small double helical DNA (from phage T7) was not adequately described, and because of the poorly understood effects of RNA polymerase. However, since some of the effects appeared to be on the later stages of denaturation, it might be possible to use the initial kinetics of denaturation as an assay for the enzyme.

Appendix 1.

Programme to analyse formaldehyde melting curve in terms of degree of unwinding, and fit data to a straight line by the method of Trifonov et al. (1968).

C-FOCAL, 1969

```
Ø1.1Ø T "FORM. MELTING CURVE"
Ø1.2Ø A !,"OD AT START"A
Ø1.3Ø A "OD AT END"B
Ø1.4Ø A "TIME INTERVAL"T1
Ø1.5Ø A "TOTAL TIME"T2
Ø1.55 S T3 = T2-T1
Ø1.6Ø A "NOISE WIDTH"D
Ø1.7Ø S S1=Ø;S S2=Ø; S S3=Ø;S S4=Ø;S S5=Ø;S S6=Ø
Ø1.8Ø F X=T1,T1,T3;DO 2.Ø
Ø1.9Ø GO TO 4.1

Ø2.1Ø A "OD"C
Ø2.2Ø S Y1=(B-C)/(B-A);S Y2=FLOG(Y1)/X;S Z1=2*D/(B-C)*X;S Z2=1/Z1↑2
Ø2.3Ø T %,1-Y1," ",Y2," ",Z1," ",X,!
Ø2.4Ø DO 3.Ø

Ø3.1Ø S S1=S1+Z2
Ø3.2Ø S S2=S2+Z2*Y2
Ø3.3Ø S S3=S3+Z2*X
Ø3.4Ø S S4=S4+Z2*X↑2
Ø3.5Ø S S5=S5+Z2*X*Y2

Ø4.1Ø S G1=S4*S1-S3↑2
Ø4.2Ø S G2=S1*S5-S2*S3
Ø4.3Ø S G3=S2*S4-S5*S3
Ø4.5Ø S G5=G3/G1
Ø4.7Ø T !!!"GRADIENT"G4
Ø4.8Ø T !!!"INTERCEPT"G5
```

Appendix 2.

Programme to convert data from the amino acid analyser to mole fraction and number of amino acids per molecule of protein.

C-FOCAL, 1969

```
Ø1.1Ø T "P. TO CALC. NO. & MOLE % FROM AA ANAL.",!
Ø1.15 C P CONVERTS NM/ML MOLE FRAC % + NN NF /MOLE, WITH CYS=25
Ø1.21 S X=Ø;S Y=Ø;S A(1)=146;S A(2)=155;S A(3)=175;S A(4)=133
Ø1.22 S A(5)=119;S A(6)=1Ø5;S A(7)=147;S A(8)=115;S A(9)=75;S A(1Ø)=89
Ø1.23 S A(11)=117;S A(12)=149;S A(13)=131; S A(14)=131;S A(15)=181
Ø1.25 S A(16)=165;S A(17)=2Ø4;S A(18)=121
Ø1.3Ø T !,"LYS  :","HIS",!,"ARG",!,"ASP",!,"THR"!,"SER",!,"GLU",!
Ø1.31 T "PRO",!,"GLY",!,"ALA",!,"VAL",!,"MET",!,"ILEU",!,"LEU"
Ø1.32 T !,"TYR",!,"PHE",!,"TRP",!
Ø1.4Ø F N=1,17;DO 2.Ø
Ø1.5Ø GO TO 3.1

Ø2.1Ø A "     "B(N)
Ø2.2Ø S X=X+B(N)
Ø2.3Ø S Y=Y+B(N)*A(N)

Ø3.1Ø S P=((25*Y)/(4ØØØØØ-25*A(18)))
Ø3.2Ø S X=X+P
Ø3.3Ø S Y=Y+P*A(18)
Ø3.4Ø T  ,"          MOLE%              NO",!
Ø3.5Ø F N=1,17;DØ 3.7
Ø3.6Ø QUIT
Ø3.7Ø T %,"   ",1ØØ*B(N)/X,"        ",4ØØØØØ*B(N)/Y,!
```

Appendix 3.

Programme to fit data to a straight line by the least-squares method, and calculate the standard error of the intercept and gradient.

C-FOCAL, 1969

```
Ø1.1Ø T "PROGRAMME TO FIND L.S. LINE Y=AX+B"!
Ø1.2Ø S S1=Ø;S S2=Ø;S S3=Ø;S S4=Ø; S S5=Ø
Ø1.3Ø A "HOW MANY DATA POINTS DO YOU HAVE"N
Ø1.4Ø F M=1,N;DO 2.Ø
Ø1.5Ø GO TO 3.1

Ø2.1Ø A "X"X
Ø2.2Ø A "Y"Y
Ø2.3Ø S S1=S1+X
Ø2.4Ø S S2=S2+X↑2
Ø2.5Ø S S3=S3+Y
Ø2.6Ø S S4=S4+Y↑2
Ø2.7Ø S S5=S5+X*Y

Ø3.1Ø S A=N*S2-S1↑2
Ø3.2Ø T %,"A",(N*S5-S1*S3)/A,!
Ø3.3Ø T "B" ,(S2*S3-S1*S5)/A,!
Ø3.4Ø S B=N*S4-S3↑2-(N*S5-S1*S3)↑2/A
Ø3.5Ø T "STANDARD ERROR OF A",FSQT(B/ (N-2)*A),!
Ø3.6Ø T "STANDARD ERROR OF B" FQST(B*S2 /N*(N-2)*A)
*
```

Appendix 4.

Programme to fit data to a quadratic equation using the least-squares method.

C-FOCAL, 1969

```
Ø1.1Ø T "TO FIT POINTS TO QUADRATIC Y=A+BX+CX↑2 BY L.S." !!!
Ø1.2Ø S C1=Ø;S C2=Ø;S C3=Ø;S C4=ØS;S  D1=Ø;S D2=Ø; S D3=Ø
Ø1.3Ø A "NO. OF DATA POINTS"N
Ø1.4Ø F X=1,N;DO 2.Ø
Ø1.5Ø GO TO 3.1

Ø2.1Ø A "CONC,GM/1ØØGM.,X"G
Ø2.2Ø A "REF. INDEX DIFFERENCE,Y"H
Ø2.3Ø S C1=C1+G
Ø2.4Ø S C2=C2+G↑2
Ø2.5Ø S C3=C3+G↑3
Ø2.6Ø S C4=C4+G↑4
Ø2.7Ø S D1=D1+H
Ø2.8Ø S D2=D2+H*G
Ø2.9Ø S D3=D3+G↑2*H

Ø3.1Ø S S1=C2-C1↑2/N
Ø3.2Ø S S2=C3-C2*C1/N
Ø3.3Ø S S3=C4-C2↑2/N
Ø3.4Ø S S4=D2-C1*D1/N
Ø3.5Ø S S5=D3-D1*C2/N
Ø3.6Ø S S6=S1*S3-S2↑2
Ø3.7Ø S S7=S3*S4-S2*S5
Ø3.8Ø S S8=S1*S5-S2*S4
Ø3.9Ø S B=S7/S6
Ø3.91 S C=S8/S6
Ø3.92 S A=D1/N-B*C1/N-C*C2/N
Ø3.93 T %,"A"A,"B"B,"C"C
```

Appendix 5.

Programme to correct the absorbance at 280 nm of a protein for light scattering by linear extrapolation of log (absorbance) v log (wavelength) from the 320 - 450 nm region of the spectrum.

C-FOCAL, 1969

```
Ø1.1Ø T "L.S. LINE Y=AX+B,OD 28Ø"!
Ø1.2Ø S S1=Ø;S S2=Ø;S S3=Ø;S S4=Ø; S S5=Ø
Ø1.3Ø A "HOW MANY DATA POINTS DO YOU HAVE"N
Ø1.4Ø F M=1,N;DO 2.Ø
Ø1.5Ø GO TO 3.1

Ø2.1Ø A "X"X1
Ø2.15 S X=FLOG(X1)
Ø2.2Ø A "Y"Y1
Ø2.25 S Y=FLOG(Y1)
Ø2.3Ø S S1=S1+X
Ø2.4Ø S S=S2+X↑2
Ø2.5Ø S S3=S3+Y
Ø2.6Ø S S4=S4+Y↑2
Ø2.7Ø S S5=S5+X*Y

Ø3.1Ø S A=N*S2-S1↑2
Ø3.2Ø T %,"A",(N*S5-S1*S3)/A,!
Ø3.3Ø T "B" ,(S2*S3-S1*S5)/A,!
Ø3.4Ø S B=N*S4-S3 2-(N*S5-S1*S3)↑2/A
Ø3.5Ø T "STANDARD ERROR OF A",FSQT<B/(N-2)*A>,!
Ø3.6Ø T "STANDARD ERROR OF B" FSQT (B*S2/N*(N-2)*A)
Ø3.7Ø S C=(N*S5-S1*S3)/A
Ø3.8Ø S D=(S2*S3-S1*S5)/A
Ø3.9Ø T "OD 28Ø",FEXP ((FLOG(28Ø)-D) /C)
*
```

Appendix 6.

Programme to calculate mean, standard deviation, and standard error.

```
*C-FOCAL, 1969
*
*Ø1.1Ø T "PROGRAM TO CALC. STAN. DEV.",!
*Ø1.2Ø A "NO. OF POINTS",N
*Ø1.3Ø S Y=Ø
*Ø1.35 S A=Ø
*Ø1.4Ø F Z=1,N;DO 2.Ø
*Ø1.5Ø T %,"AV.",Y/N,!,"ST. DEV.",FSQT(A/N-(Y/N)↑2)
*Ø1.55 T "ST. D. OF MEAN",(FSQT(A/N-(Y/N)↑2))/N
*Ø1.6Ø QUIT
*
*Ø2.1Ø A "X",X
*Ø2.2Ø S Y=X+Y
*Ø2.3Ø S A=X↑2+A
```

Appendix 7.

Calculation of weighting factor for the analysis of formaldehyde melting curves.

Errors in absorbance determinations, δA, affect the expression

$$-\ln\left[1 - \left[\frac{A \pm \delta A - Ai \pm \delta A}{Af \pm \delta A - Ai \pm \delta A}\right]\right] \quad \ldots i$$

where A = absorbance at some time during experiment.

Ai = initial absorbance.

Af = final absorbance.

in the analysis of Trifonov et al. (1968).

$$\therefore 2\delta A \ll Af - Ai$$

$$\therefore i \simeq -\ln\left[1 - \left[\frac{A - Ai}{Af - Ai} \pm \frac{2\delta A}{Af - Ai}\right]\right]$$

$$\simeq -\ln\left[1 - \left[\frac{A - Ai}{Af - Ai}\right]\right] \pm \frac{2\delta A}{Af - A}$$

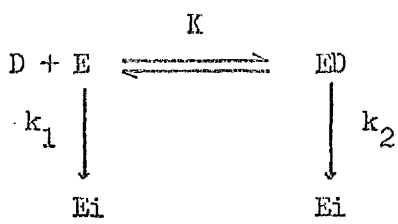
Assuming the error is normally distributed then the weighting factor for

$$-\ln\left[1 - \left[\frac{A - Ai}{Af - Ai}\right]\right] \text{ becomes } \frac{1}{\left\langle \frac{2\delta A}{Af - A} \right\rangle^2}$$

Appendix 8.

Model for denatured DNA protection of RNA polymerase against inactivation by methyl acetimidate.

Assuming that both the free and DNA bound enzyme could be inactivated by methyl acetimidate, then the observed initial rate of inactivation, at a given DNA concentration, is determined by the rate of inactivation of the free and DNA bound enzymes. From :



where E, Ei = active and inactive enzymes respectively

D, ED = free and enzyme bound DNA respectively

k_1, k_2 = rate constants for the inactivation of free and DNA bound enzymes respectively

k_o = observed rate constant

K = dissociation constant = $[E][D]/[ED]$ . . . 1

then $k_o [E] t = k_1 [E] + k_2 [ED]$ . . . 2

$[E] t = [E] + [ED]$ . . . 3

$[D] t = [D] + [ED]$ . . . 4

where $[E] t$, $[D] t$, = total concentration of enzyme and DNA respectively.

from 2 & 3, $$[ED] = \frac{(k_o - k_1)}{(k_2 - k_1)} \cdot [E]t \quad \ldots 5$$

from 1, 4, & 5, $$K = \frac{[E][D]}{[ED]}$$

$$= \frac{\left\langle [E]t - \frac{(k_o - k_1)}{(k_2 - k_1)} \cdot [E]t \right\rangle \left\langle [D]t - \frac{(k_o - k_1)}{(k_2 - k_1)} \cdot [E]t \right\rangle}{\frac{(k_o - k_1)}{(k_2 - k_1)} \cdot [E]t - }$$

$$\therefore \quad K = \frac{((k_2 - k_1) - (k_o - k_1))([D]t\,(k_2 - k_1) - (k_o - k_1)\,[E]t)}{(k_2 - k_1)\,(k_o - k_1)}$$

$$\therefore \quad K\,(k_2 - k_1)(k_o - k_1) = (k_2 - k_o)([D]t(k_2 - k_1) - [E]t(k_o - k_1)$$

$$\therefore \quad \frac{K}{k_o - k_2} + \frac{[E]t}{k_1 - k_2} = \frac{[D]\,t}{k_1 - k_o}$$

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