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PHYSICO-CHEMICAL ASPECTS OF E.COLI MRE 600

DNA-DEPENDENT RNA POLYMERASE

by

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Presented for the degree of Doctor of Philosophy.

Institute of Biochemistry

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ERRATA

pp 273 and 274 are in reverse order,

p 278 has additional references.

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ABBREVIATIONS AND CONVENTIONS

The abbreviations, conventions and symbols recommended by Biochem. J. 116, 1. are used. Additional abbreviations are-

BSA	bovine serum albumin
CD	circular dichroism
CR	congo red dye
CT	calf thymus
DNase	deoxyribonuclease
equiv.	equivalent
GuHCl	guanidine hydrochloride
mA	milli-Amps
NTP	nucleoside triphosphates
ORD	optical rotatory dispersion
PC	phosphocellulose
PEP	phospho-enol pyruvate
RNase	ribonuclease
SS	salmon sperm
T7L	T7 Luria strain
T7M	T7 Meselson strain

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SUMMARY

In the introduction an outline of the currently known physical, chemical and enzymic properties of *E. coli* DNA-dependent RNA polymerase (E.C.2.7.7.6) is given. Some aspects of the regulation and control of RNA synthesis in *E. coli* with special reference to specific protein factors are mentioned.

Two purification procedures for the preparation and purification of RNA polymerase from *E. coli* MRE 600 are described, compared and discussed.

The subunit composition of *E. coli* MRE 600 RNA polymerase on analysis by SDS-polyacrylamide gel electrophoresis shows the presence of five major protein species- β' , β , α and σ already described in the literature, and a fifth subunit Θ , of molecular weight 58,000 daltons. Θ is shown to be strongly associated with *E. coli* MRE 600 RNA polymerase and it behaves in a physico-chemical manner similar to the σ subunit except that it has no σ -like activity determined by its inability to stimulate RNA synthesis on a T7 DNA template with core enzyme.

A crude fraction has been isolated from *E. coli* MRE 600 containing a protein factor which has the property of inhibiting both the initiation and rate of RNA synthesis by RNA polymerase holoenzyme in vitro. The inhibitor protein has a molecular weight of 320,000 daltons and has associated with it some proteolytic activity.

The amino acid composition of *E. coli* MRE 600 RNA polymerase,

core enzyme and holoenzyme, is presented. The ultra-violet spectrum of RNA polymerase has a maximum at 275.5nm and a minimum at 251nm. The enzyme has a specific absorbance of 0.63/mg/ml. A standard curve was prepared from the spectra of E. coli MRE 600 DNA and RNA polymerase to allow the calculation of nucleic acid contamination of the various fractions during the enzyme preparation. A formula was derived for the spectrophotometric determination of the concentration of RNA polymerase in the presence of any contaminating nucleic acid.

The ORD of native RNA polymerase is that of a typical protein containing right-handed helical and random coil (disordered) conformations. The helix content of RNA polymerase is around 32%. The presence of other conformations is also indicated. The conformation of E. coli MRE 600 RNA polymerase shows very little change with increasing ionic strength although the enzyme dissociates from the dimer to the monomer over the range of ionic strengths studied. The conformation of RNA polymerase in various denaturing solutions (8M-urea, 1% SDS and 6M-GuHCl) was also determined. There is a decrease in the $[\text{m}']_{232}$ of the ORD of RNA polymerase holoenzyme when it is bound to DNA indicating a conformational change in the enzyme. There is no detectable denaturation of the DNA. Thermal ORD studies indicate that RNA polymerase is unstable when bound to DNA. This thermal denaturation is irreversible.

The possible origin of the Θ subunit as a breakdown product of the σ subunit of RNA polymerase holoenzyme, its

role in the regulation of RNA synthesis and transcription, and the properties and function of the RNA polymerase inhibitor protein are discussed. The physical and chemical properties of the E. coli MRE 600 RNA polymerase are compared with those of other strains of E. coli.

SECTION 1

INTRODUCTION

1.1.

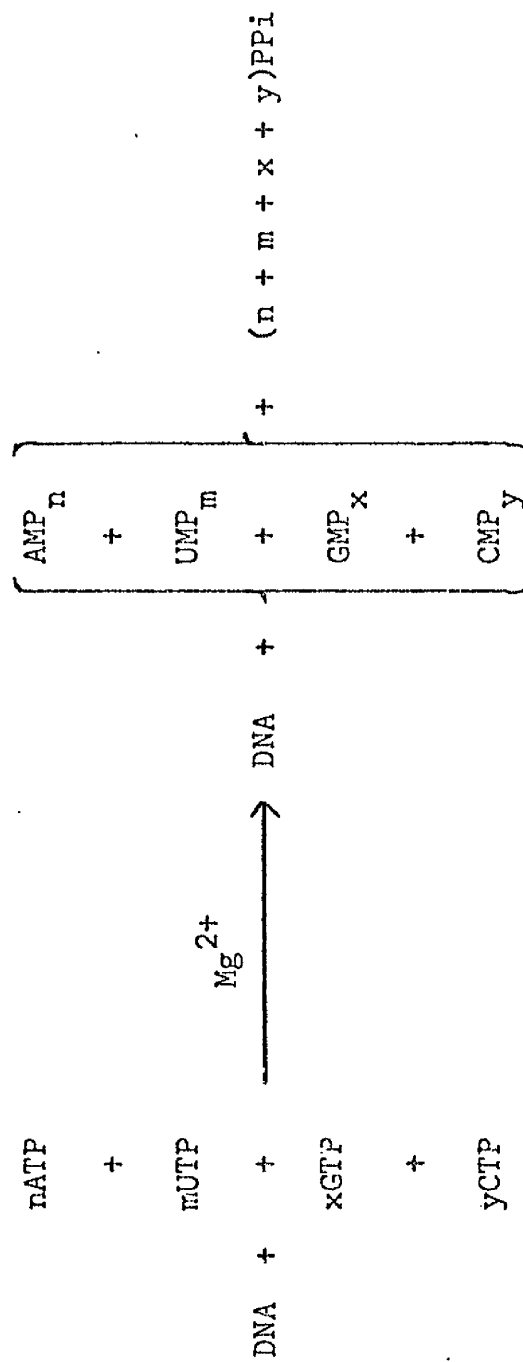
DNA-DEPENDENT RNA POLYMERASE

DNA-dependent RNA polymerase (systematic name: nucleoside triphosphate RNA nucleotidyl transferase; EC 2.7.7.6) is the enzyme which is believed to be responsible for the synthesis of all types of cellular RNA (messenger RNA, transfer RNA, ribosomal RNA, etc.). Under the direction of DNA, RNA polymerase catalyses the sequential assembly of ribonucleoside triphosphates into RNA molecules. Thus the genetic information stored in the nucleotide sequences of DNA can be transcribed into a form that can be used to direct the synthesis of specific proteins.

The existence of DNA-dependent RNA polymerase was first demonstrated by Weiss and Gladstone (1959) in rat liver nuclei. Since then it has been found in a wide variety of living organisms and tissues, and appears to exist in all cells where RNA synthesis occurs. RNA polymerase in its purest form has been isolated mainly from bacterial sources although T7 bacteriophage DNA-coded RNA polymerase and *Neurospora crassa* mitochondrial RNA polymerase have now been isolated and purified. Purification of the enzyme from mammalian cells has proceeded at a much slower rate due to the relatively lower specific activity of the mammalian enzyme; these purification difficulties now appear to have been partially resolved (Chamberlin, 1970).

Many excellent reviews on the properties of the bacterial RNA polymerase have appeared in the last two years (Richardson, 1969; Geiduschek and Haselkorn, 1969; Chamberlin, 1970; Travers, 1971).

FIGURE 1.1. REACTION CATALYSED BY E. COLI DNA-DEPENDENT
RNA POLYMERASE



1.2.

ESCHERICHIA COLI DNA-DEPENDENT RNA POLYMERASE

In *Escherichia coli* the synthesis of all types of cellular RNA is believed to be mediated by only one species of RNA polymerase. The highly purified enzyme catalyses the synthesis of RNA in vitro in the presence of DNA and the ribonucleoside triphosphates (figure 1.1.) When an intact double helical DNA is used as template, transcription in vitro is asymmetric - only one strand of the complementary DNA strands is transcribed (Hayashi, Hayashi and Spiegelman, 1964; Geiduschek, Tocchini-Valentini and Sarnat, 1964). This is characteristic of transcription in vivo (Tocchini-Valentini et al., 1963). The selective transcription in vitro of only certain regions of bacteriophage DNA suggest that RNA polymerase initiates RNA synthesis at specific sites on the DNA (Geiduschek, Snyder, Colvill and Sarnat, 1966; Cohen, Maitra and Hurwitz, 1967).

It had been assumed that "highly purified" RNA polymerase was a protein entity from which nothing further could be removed without destroying its enzyme activity, however in 1969 Burgess, Travers, Dunn and Bautz reported that apparently pure RNA polymerase (holoenzyme) could be split into two components. One of these is the basic enzyme itself, the core polymerase, and the other is a protein factor, sigma (σ) which adapts the enzyme to specific template DNA. Both RNA polymerase holoenzyme and core polymerase are active with dAT copolymer and calf thymus DNA as template but only the holoenzyme can efficiently trans-

scribe intact bacteriophage DNA. This discovery by Burgess et al. (1969) opened the way to an intensive study of the properties of E. coli RNA polymerase. The last two years have seen the determination of the subunit structure and function of RNA polymerase, the discovery of protein and other factors which influence its transcription properties and the elucidation of the enzyme's role after bacteriophage infection of E. coli. These discoveries have led to a much better understanding of the transcription process.

This thesis is an investigation into some of the physical and chemical properties of DNA-dependent RNA polymerase isolated from the bacterium *Escherichia coli* strain MRE 600 (an RNase 1⁻ strain).

1.2.

PURIFICATION OF RNA POLYMERASE

1.2.1.

BACTERIAL RNA POLYMERASE

DNA-dependent RNA polymerase has been isolated and extensively purified from several strains of *Escherichia coli* (Chamberlin and Berg, 1962 (*E. coli* B); Zillig, Fuchs and Millette, 1966 (*E. coli* K12); Maitra and Hurwitz, 1967 (*E. coli* ω); Babinet, 1967 (*E. coli* A-19); Burgess, 1969a (*E. coli* K12); Zillig, Zechel and Halbwachs, 1970 (*E. coli* K12); Bautz, Dunn, Bautz, Schmidt and Mazaitis, 1970 (*E. coli* B)).

RNA polymerase has also been purified from *Azotobacter vinelandii* (Krakow, Daley and Fronk, 1968), *Bacillus stearothermophilus* (Remold-O'Donnell and Zillig, 1969), *Bacillus subtilis* (Losick, Shorenstein and Sonenshein, 1970; Avila, Hermoso, Vinuela and Salas, 1970) and *Pseudomonas putida* (Johnson, de Backer and Boezi, 1971).

1.2.2.

PURITY OF RNA POLYMERASE PREPARATIONS

Before investigating the physical and chemical properties of any enzyme, it is important to consider the purity of the enzyme preparation.

The simplest criterion of purity would appear to be the specific activity of the enzyme. For RNA polymerase, unfortunately, this

RNA POLYMERASE PURIFICATION PROCEDURES

TABLE 1.1.

Purification Procedure	E. coli	yield mg/RNA polymerase per 100gm E. coli	DNA in assay	Specific activity [*]	A ₂₈₀ /A ₂₆₀
Zillig, Fuchs and Millette (1966)	K12	16	CT	574	1.25
Chamberlin and Berg (1962)	B	18	SS	1000	1.50-1.55
Maitra and Hurwitz (1967)	w	5	CT	1400	1.66
Babinet (1967)	A19	25	CT	1400	1.60
Zillig, Zechel and Halbwachs (1970)	K12	20-40	T ₄	3000	-
Bautz, Dunn, Bautz, Schmidt and Mazaitis (1970)	B	15	T ₄ CT	2000 900	-
Burgess (1969a)	K12	28	CT	600	-

* specific activity expressed as n moles NTP incorporated into RNA/10min/mg.

criterion is not reliable. (The specific activities of RNA polymerase preparations from different laboratories are given in different enzyme units as there is no generally accepted standard assay.) The specific activity of a preparation of RNA polymerase as determined by the incorporation of nucleotides into acid-insoluble material does not reflect a turnover rate of RNA chain elongation, as a result, there is currently no convenient way to measure the amount of active enzyme in a given preparation. Assays carried out with bacteriophage DNA (T2,T4,T7) give specific activities which reflect the amount of σ subunit in the enzyme preparation, while the specific activities obtained with calf thymus and salmon sperm DNA vary over a wide range depending on the source and the age of the DNA (Chamberlin, 1970). The synthetic copolymer poly dAT comes the closest of any known template to give a true reflection of the amount of RNA polymerase present since it is active with both core polymerase and RNA polymerase holoenzyme (Berg and Chamberlin, 1970). Most methods of RNA polymerase purification give mixtures of core polymerase and holoenzyme (Zillig et al., 1970b; Berg, Barrett and Chamberlin, 1970; Richardson, 1970). Where the holoenzyme is required it must be formed by reconstitution with purified σ subunit (Zillig et al., 1970b).

There is some evidence that both the method of purification, the strain of *E. coli* and stage of growth of the *E. coli* may affect the properties of the enzyme. RNA polymerase prepared by the Zillig procedure (Zillig et al., 1966) and the Burgess glycerol gradient procedure (Burgess, 1969a) from the same *E. coli*, show different enzymic

properties in that in an unprimed synthesis reaction with ITP and CTP as the sole substrates, enzyme prepared by the Zillig method synthesised poly r(I-C), whilst enzyme prepared by the Burgess procedure synthesised poly rI. poly rC (Sternbach and Eckstein, 1970). The amount of σ factor is not involved in the reaction, therefore the differences between these two methods of enzyme purification appear to lead to differences in the purified RNA polymerase.

Morgan (1970) purified RNA polymerase from both E. coli B and ω and found there to be different qualitative and quantitative kinetic properties. Abraham (1970) has reported that even two enzyme preparations isolated by the same procedure from cells harvested at different stages of growth exhibit different template specificity.

Physical purity of a protein is usually indicated by its sedimentation and electrophoretic homogeneity. Under the appropriate conditions of ionic strength, e.g. 0.5M-KCl, RNA polymerase appears as one sedimenting species of approximately 13S. Electrophoretic homogeneity was harder to demonstrate, but now that the subunit composition of RNA polymerase is known (Burgess et al. 1969; Burgess 1969a; Burgess 1969b) SDS-polyacrylamide gel electrophoresis as described by Burgess (1969b) is an essential procedure in determining the purity of any enzyme preparation. With this technique very small quantities of contaminating protein can be detected.

The ratio of the absorbance at 280nm to that at 260nm of a purified preparation of RNA polymerase holoenzyme is 1.65 (Richardson, 1966a) this suggests a nucleic acid content of less than 0.1%, RNA polymerase core enzyme purified by phosphocellulose

chromatography by the method of Burgess (1969a) has an A_{280}/A_{260} of 1.8 to 1.9. For studies of the physical properties of the enzyme the above criteria may be sufficient. However for certain enzymatic studies there are other tests that are even more important. All highly purified RNA polymerase preparations have been shown to be free of ribonuclease and deoxyribonuclease (Richardson, 1969) and it can be concluded that it is possible to make preparations of RNA polymerase that are virtually free of the most important contaminating activities.

Ideally each RNA polymerase preparation should be prepared in large batches that can be analysed properly and checked by every possible criterion of purity and then used for many experiments as there tend to be variations between one enzyme preparation and the next even in the same laboratory.

1.3.

PROPERTIES OF RNA POLYMERASE

The physical properties of *E. coli* RNA polymerase have been under investigation for many years. It is only recently that the number of conflicting properties, especially the sedimentation behaviour of the enzyme, have become reconciled now that the subunit structure of the enzyme has been determined.

1.3.1.

SEDIMENTATION PROPERTIES

Early studies on the sedimentation behaviour of the enzyme led to conflicting results. The enzyme appeared to aggregate at low salt concentrations but there was no agreement as to the number and nature of sedimenting species encountered under these conditions. (See table 1.2). A dissociated form of the enzyme was generally obtained at high salt concentrations but reports for the sedimentation coefficient of this species varied from 11 to 13S. Berg and Chamberlin (1970) have performed an exhaustive study on the sedimentation properties of RNA polymerase and have determined the four major factors which affect the sedimentation behaviour of the enzyme and which serve to reconcile all previous reports. Those factors are (1) ionic strength, (2) subunit composition, (3) preferential hydration and (4) irreversible dissociation of the enzyme.

At high ionic strengths both core polymerase^{*} and RNA polymerase holoenzyme exist in the protomer form. As the ionic strength is lowered

TABLE 1.2

SEDIMENTATION COEFFICIENTS OF RNA POLYMERASE

Reference	E. coli	S-value			Other forms
		Monomer	Dimer		
Fuchs, Zillig, Hofschneider and Preuss (1964)	K12 Hfr	13	23.7		-
Stevens, Emery and Sternberger (1966)	B	13	22		-
Zillig, Fuchs and Millette (1966)	K12	12	23.7		32,39
Colvill, van Bruggen and Fernandez-Moran (1966)	-	-	25		18
Richardson (1966a)	B	13	21		-
Maitra and Hurwitz (1967)	ω	11	-		-
Pettijohn and Kamiyama (1967)	MRE600	11	21		-
Priess and Zillig (1967)	K12	13	-		-
Lubin (1969)	MRE600/B	12-13	18-19		-
Berg and Chamberlin (1970)*	B	core 12.6	-		44-48
		holo 15	23		

association to produce aggregates of various sizes occurs. At intermediate ionic strengths, sedimentation analysis gives S-values which appear to reflect sedimentation of reversible equilibrium mixtures of components e.g. 18S intermediates obtained by several authors (Zillig, Fuchs and Millette, 1966). The subunit composition of the enzyme can vary in the relative content of σ and ω components (Berg and Chamberlin, 1970). The amount of σ components in the enzyme affects the sedimentation behaviour of the enzyme both at high ionic strength, where it contributes to the mass of the sedimenting species, and low ionic strength where it also has the effect of limiting aggregation of the enzyme to a dimer. Preparations of RNA polymerase containing less than 1 equiv. of σ exhibit sedimentation behaviour intermediate to that of core polymerase and holoenzyme. Thus the subunit composition of an RNA polymerase preparation is important in the exact interpretation of sedimentation results. Thus differences in S-values obtained probably reflect different amounts of σ (and ω) in an RNA polymerase preparation. Different methods of purification of RNA polymerase from the same strain of *E. coli* give different S-values for the purified enzyme (Colvill, van Bruggen and Fernandez-Moran, 1966). The strain of bacteria too, may affect the sedimentation behaviour of RNA polymerase as different strains of *E. coli* appear to contain different amounts of σ component (Berg and Chamberlin, 1970) but this may also be due to the fact that different strains are used by different workers, each with their own method of purification and this again may lead to differences in the

relative amounts of σ and ω present in the preparation.

Preferential hydration of the protein plays a significant role only in solutions containing high concentrations of divalent salts (Berg and Chamberlin, 1970). Irreversible dissociation of the enzyme can occur (Berg and Chamberlin, 1970), the enzyme breaking down to an 8S product at a slow but constant rate.

1.3.2.

THE MOLECULAR WEIGHT OF RNA POLYMERASE

Burgess (1969b) determined the weight ratio of the polypeptide chains present in RNA polymerase and found that RNA polymerase core enzyme had the subunit composition $\beta'\beta\alpha_2$ and the holoenzyme $\beta'\beta\alpha_2\sigma$. Using the Burgess (1969b) values of 39,000, 155,000 and 165,000 for α , β and β' respectively the molecular weight of the core enzyme is $400,000 \pm 40,000$ daltons and the holoenzyme, with a full complement of σ (95,000 daltons), has a molecular weight of $495,000 \pm 40,000$ daltons.

1.3.2.1.

CORE POLYMERASE

The molecular weight of pure core polymerase has been determined by two different laboratories.

Berg and Chamberlin (1970) determined the molecular weight of core polymerase in high ionic strength buffer by the technique of low speed sedimentation equilibrium. This yielded a value of 380,000. Ruet, Sentenac and Fromageot (1970) determined a value for the molecular weight of $420,000 \pm 30,000$ by an electrophoretic technique using polyacrylamide gels. Both these values are comparable to that obtained by SDS

TABLE 1.3. MOLECULAR WEIGHT OF E. COLI RNA POLYMERASE

Reference	Molecular weight		Method
	Core enzyme	Holoenzyme	
Richardson (1966a)	-	440,000	Sedimentation equilibrium
Burgess (1969b)	400,000	495,000	SDS-polyacrylamide gel electrophoresis
Ruet, Sentenac and Fromageot (1970)	420,000	480,000	gel electrophoresis
Anthony and Goldthwait (1970)	-	390,000	Sedimentation equilibrium
Maitra and Hurwitz (1967)	-	370,000	"
Wu and Goldthwait (1969)	-	370,000	"
Priess and Zillig (1967)	-	360,000	Sedimentation and diffusion
Berg and Chamberlin (1970)	383,000	420,000	Sedimentation equilibrium

polyacrylamide gel electrophoresis.

1.3.2.2.

HOLOENZYME

The molecular weight of the holoenzyme has been determined by many authors (Table 1.3). Except for Ruet, Sentenac and Fromageot (1970) who obtain a molecular weight of $480,000 \pm 30,000$ by gel electrophoresis and Richardson (1966a) who obtained a value of $440,000 \pm 40,000$ by sedimentation equilibrium, the values of the molecular weight obtained in other laboratories are considerably lower than the expected molecular weight which is predicted from SDS-polyacrylamide gel electrophoresis or from the sedimentation equilibrium molecular weight of core polymerase to which one mole of σ component (molecular weight 80,000 - 95,000) has been added. These low values obtained for the sedimentation equilibrium and sedimentation and diffusion molecular weights can probably be attributed to several facts. Firstly, most preparations of RNA polymerase invariably contain less than one equivalent of σ subunit (Richardson, 1970; Zillig et al., 1970b; Berg and Chamberlin, 1970). Secondly partial dissociation of the holoenzyme occurs to give core polymerase and σ component during sedimentation (Berg and Chamberlin, 1970). Thirdly, because of the high ionic strength of the solution in which the sedimentation equilibrium molecular weights are determined, corrections may have to be made for the preferential binding of solvent components to the RNA polymerase to give the true molecular weight (Berg and Chamberlin, 1970).

1.3.2.3.

MOLECULAR WEIGHT OF RNA POLYMERASE SUBUNITS

The different methods used to purify RNA polymerase lead to enzyme preparations which have essentially the same subunit composition as determined by SDS-polyacrylamide gel electrophoresis.

There are four major kinds of protein subunits which appear to be common to all enzyme preparations. These have been designated as β' , β , σ and α (Burgess et al., 1969). For the E. coli RNA polymerase, these have molecular weights of 155,000 - 165,000, 145,000 - 155,000, 80,000 - 95,000 and 38,000 - 41,000 respectively. (Table 1.4) A value for a mixture of $\beta + \beta'$ of approximately 130,000 daltons have been obtained by Burgess by sedimentation equilibrium.

An additional minor subunit is present in most enzyme preparations and has been designated ω (Burgess et al., 1969). This has a molecular weight in the region of 10,000 to 12,000 (Burgess et al., 1969; Zillig, Fuchs, Palm, Rabussay and Zechel, 1970a). It is not clear, however, whether this is a true functional component of the enzyme or merely a tightly binding contaminant. Zillig et al. (1970a) have reported that β' and σ appear to be complex subunits. When a dry powder of σ is dissolved in 6M-urea at pH 10.5 to 11, besides the original σ , two new bands appear in SDS gel electrophoresis. Their molecular weight are around 15,000 for the lighter one, ν , and 60,000 for the larger one, μ . Two new bands appear also in 6M-urea cellogel electrophoresis. It would then appear that σ is a complex with the sum formula $\mu\nu_2$.

TABLE 1.4. MOLECULAR WEIGHT OF E. COLI RNA POLYMERASE SUBUNITS DETERMINED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Reference	E. coli	β'	β	σ	α	μ	ν	ω
Burgess et al. (1969)	K12	160,000	155,000	95,000	40,000	-	-	10,000
Zillig et al. (1970a)	K12 Hfr(λ)	160,000	145,000	85,000	40,000	60,000	15,000	12,000
Berg and Chamberlin (1970)	B	150,000	145,000	86,000	41,000	-	-	12,000
Ruet, Sentenac and Fromageot (1970)	-	-	-	80,000	-	-	-	-
Burgess (1969b)	K12	165,000	155,000	-	39,000 (38,400)*	-	-	9,000

* Sedimentation equilibrium.

μ has been reported as a component of an active RNA polymerase preparation purified by the method of Babinet (1967) from *E. coli* A-19 (Chelala, Hirschbein and Torres, 1971; Hirschbein, Dubert and Babinet, 1969). Krakow and von der Helm (1970) report a protein component which they term ϵ with a molecular weight of around 65,000, which is present in some preparations of *A. vinelandii* RNA polymerase.

β' , when reacted with heparin, appears to cleave into two parts, one with the same molecular weight and electrophoretic properties as β , and a small protein component which has been termed χ (Zillig et al., 1970a).

TABLE 1.5. MOLECULAR WEIGHT OF THE SUBUNITS OF RNA POLYMERASE FROM OTHER BACTERIA

Reference	Bacterium	β'	β	σ	μ	α
Krakow and von der Helm (1970)	A. Vinelandii	"similar to E. coli"			65,000	
Johnson, De Baker and Boezi (1970)	Pseudomonas putida	165,000	155,000	99,000		44,000
Losick, Shorenstein and Sonenshein (1970)	Bacillus subtilis (1) vegetative (2) sporulation	155,000 (mean)		57,000	-	45,000
		155,000	110,000	-	-	45,000
Avila, Hermoso, Vinuela and Salas (1970)	B. subtilis (vegetative)	150,000 (mean)		55,000	-	43,000

1.3.2.4.

ELECTRON MICROSCOPY

Since RNA polymerase is quite a large molecule, it would seem likely that something could be learned about its structure from electron microscope studies. So far, however, such studies have been disappointing. Objects with a very regular shape are observed in some preparations (Fuchs, Zillig, Hofschneider and Preuss, 1964; Slayter and Hall, 1966; Colvill, van Bruggen and Fernandez - Moran, 1966), and until recently a model for the enzyme consisting of two stacked hexagonal rings seemed appropriate (Zillig, Fuchs and Millette, 1966). Lubin (1969) questioned these findings and suggests that the hexagons and several other particles previously observed are contaminants of impure preparations. Lubin believes the enzyme to be 'porous' in nature without obvious planes of symmetry or regularly repeating units and this would appear to be the case now that the subunit composition of the enzyme has been determined (Burgess et al., 1969). None of the electron micrographs so far have been obtained with RNA polymerase whose homogeneity has been adequately demonstrated.

1.3.2.5.

AMINO ACID COMPOSITION

The amino acid compositions of core enzyme from *E. coli* B (Nicholson, 1969) and *E. coli* K12 (Burgess, 1969b) and of the holoenzyme from *E. coli* K12 (Priess and Zillig, 1967) and *E. coli* ω (Maitra and Hurwitz, 1967), have been reported. The amino composition of the RNA polymerases obtained from

these different strains of *E. coli* differ slightly, although the actual differences are quite small and may be due to variations in the purity of the preparation rather than to intrinsic differences between *E. coli* strains. Morgan (1970) has found that RNA polymerase purified from two different strains of *E. coli* (B and ω) show different qualitative and quantitative kinetic properties suggesting that there may be differences between the enzyme in at least these two strains.

1.3.2.6.

ULTRA-VIOLET SPECTROSCOPY

The ultra-violet spectrum of RNA polymerase has been reported by several authors (Neuhoff, Schill and Sternbach, 1969; Anderson and Abraham, 1970; Nicholson, 1971). The spectrum exhibits an absorption maximum in the region of 278nm and a minimum about 251nm. The $E_{280}^{0.1\%}$ has been determined by several authors. Fuchs, Zillig, Hofschneid and Preuss (1964) obtained a value of 0.82, Richardson (1966a), 0.65 and Nicholson (1971), 0.541.

1.3.2.7.

ORD AND CD OF RNA POLYMERASE

Novak and Doty (1970) have examined the far UV ORD and CD spectra of *E. coli* core polymerase. From the $[\theta]_{220}$ (13,000) and $[\eta]_{232}'$ (-3,900) they estimated that the enzyme contained 32% helix. Dissociation of the RNA polymerase in SDS resulted in no large change in conformation as detected by ORD. No change in the conformation of core polymerase when bound to native calf thymus DNA was detected by CD,

however, thermal ORD studies over the temperature range 25 to 60°C showed that the core polymerase was much less stable in the DNA complex than in the free state. This thermal instability was not found in an analogous complex formed with single stranded DNA or in the same complex after the start of RNA synthesis. Nicholson (1971) has determined a helix content of 13% for core polymerase isolated from *E. coli* B.

1.3.2.8.

PROPERTIES OF *E. COLI* RNA POLYMERASE SUBUNITS

1.3.2.8.1.

SEPARATION OF RNA POLYMERASE SUBUNITS

A promising approach to the study of subunit function in RNA polymerase involves the selective removal of one or more subunits from the native enzyme molecule. The first example of such a cleavage was the removal of the σ subunit from the *E. coli* polymerase by chromatography on phosphocellulose (Burgess et al., 1969) which led to the discovery of the role of σ in template promoter site recognition. Sigma subunit can be similarly removed from *A. vinelandii* (Krakow and von der Helm, 1970), *B. subtilis* (Losick, Schorenstein and Sonenshein, 1970), and *Pseudomonas putida* (Johnson, De Backer and Boezi, 1971). A continuation of this approach has been reported by Sethi, Zillig and Bauer (1970a, 1970b) who were able to partially cleave *E. coli* RNA polymerase into subunits by sucrose gradient centrifugation in 3M-LiCl. They were able to separate a mixture of β' + β subunits from the α subunit. On reconstitution only

25% of the original activity was recovered. Heil and Zillig (1970) have completely dissociated and separated the RNA polymerase subunits by cellulose acetate electrophoresis in 6M-urea. By codialysis of the separated subunits, they were able to recover up to 100% of the original activity. For a high yield reconstitution, the presence of σ during dialysis was required. The reconstituted enzyme was indistinguishable from the original RNA polymerase in sucrose gradient centrifugation. None of the subunits alone displayed any RNA polymerase activity. Burgess (1969b) has separated $\beta'+\beta$, α and ω by chromatography of *E. coli* core polymerase on Sephadex G-200 in the presence of 1%(w/v) SDS. He was also able to separate the core polymerase into two peaks, one containing β' and ω , the other α and β , on DEAE-cellulose equilibrated with 8M-urea.

Krakow and von der Helm (1970) have been able to partially dissociate *A. vinelandii* RNA polymerase with congo red dye (CR) to produce RNA polymerase fragments consisting of $\beta, 2\alpha$ -CR and β' -CR complexes.

1.3.2.8.2.

THE ROLE OF THE SUBUNITS IN TRANSCRIPTION

Zillig et al. (1970a) have shown that there is a striking immunochemical similarity between the different RNA polymerase subunits, but on the other hand the amino acid compositions of the subunits are significantly different (Zillig et al., 1970b, Burgess, 1969b). To test for the possibility that the different peptide chains shared sequences and also that β' and β were perhaps related to each other, as indicated by the conversion of β' into a product with a molecular weight and specific

electric charge very close to that of β by treatment with heparin, they compared fingerprints obtained by tryptic digest of the peptides β' , β and α . All three subunits yielded different peptide maps.

β' SUBUNIT: DNA BINDING

An α -free mixture of β' + β , obtained by sucrose gradient centrifugation of core enzyme in 3.4M-LiCl (Sethi, Zillig and Bauer, 1970b) is able to bind labelled T4 DNA when tested by the nitrocellulose filter technique of Jones and Berg (1966), α is almost inactive in this respect. Therefore either β' or β is the subunit responsible for this effect. Zillig et al. (1970b) have observed that when a complex of excess T4 DNA and holoenzyme is sedimented at high speed, a small amount of α and β remain in the supernate, but no β' , as shown by cellulose acetate chromatography. The interpretation is that the enzyme contained some β' free enzyme particles which remained in the supernate because they were unable to bind to DNA. Similarly after phosphocellulose chromatography Zillig et al. (1970b) report that α and β are found in the flow through along with σ . β' is the only subunit of RNA polymerase which binds to heparin (a polyanion) which is a potent inhibitor of the enzyme, and acts by competing with DNA for the RNA polymerase (Zillig et al. 1970b). Also among all the core polymerase subunits β' possesses by far the highest iso-electric point, between pH 8 and 9. The high basicity may possibly be connected to its binding to DNA and heparin.

Burgess (1969b) has shown that on applying core enzyme in 4M-urea to a column of phosphocellulose equilibrated with 4M-urea, α and ω appear in

the wash-through but β' and β bound strongly to the phosphocellulose suggesting that β' and β chains were able to bind very tightly to phosphate groups and thus may be involved in binding polynucleotides.

β SUBUNIT: INITIATION AND TRANSLOCATION

It is now known that σ is required for the correct initiation of RNA synthesis on native DNA templates (Bautz et al., 1970; Travers, 1970; Sugiura, Okamoto and Takanami, 1970). Since however the specific initiation inhibitor rifampycin does not bind to σ but to the core enzyme (di Mauro et al., 1969) the core enzyme is also clearly involved in initiation. Rabussay and Zillig (1969) have shown that in a rifampycin-resistant mutant of *E. coli*, the β subunit of the core enzyme has a different electrophoretic mobility from that of the wild type β , indicating a changed electric charge on the protein chain. Zillig et al. (1970b) have demonstrated directly by sucrose gradient centrifugation of the isolated subunits that rifampycin binds only to β . β subunit may also be involved in σ function. Sethi et al. (1970a) have shown that $\beta\sigma$ complex can be obtained on incomplete dissociation of holoenzyme in 4M-LiCl. Heil and Zillig (1970) prepared reconstitutes of RNA polymerase from subunits separated from both wild type (sensitive) and rifampycin-resistant RNA polymerase. The reconstitutes from the subunits of sensitive enzyme were fully sensitive, that from the subunits of resistant enzyme was fully resistant. Of the three mixed constitutes formed from either one of the subunits of the resistant enzyme with the others from the normal enzyme, those containing β from sensitive enzyme are fully sensitive, that containing β from resistant enzyme is resistant.

Thus the conclusion is that β is the subunit responsible for the interaction with rifampycin and is involved in the initiation step specifically inhibited by the drug. The same result has been obtained with a streptolydigin-resistant mutant. Thus streptolydigin-like rifampycin exerts its action on RNA polymerase over the β subunit. Since streptolydigin specifically blocks translocation, β is involved in this partial function as it is in initiation. It has been shown by other methods that β contains the binding sites for α and σ (Zillig et al., 1970a; Zillig et al., 1970b). Thus β exhibits multiple functions.

α SUBUNIT: CONTROL

The function of the α subunit is unknown but it can be modified by the addition of 5'-adenylate after T4 bacteriophage infection of E. coli (Goff and Weber, 1970). Thus α subunit probably plays a part in transcription specificity. Adenylation of RNA polymerase holoenzyme has been reported by Chelala, Hirschbein and Torres (1971), these observations suggest a possible control mechanism involvement for the subunit.

σ SUBUNIT : INITIATION

The role of σ subunit in initiation will be discussed in detail in the next section - RNA polymerase and transcription.

1.4.

RNA POLYMERASE AND TRANSCRIPTION

1.4.1.

STEPS IN RNA SYNTHESIS

RNA synthesis in vitro by purified bacterial RNA polymerase on a double-standed template DNA appears to proceed in a series of steps. These have been differentiated into:

1. binding of the enzyme to the DNA;
2. initiation, a number of consecutive events, the completion of which is prerequisite to the onset of RNA synthesis;
3. elongation of the RNA chain which consists of a continuous repetition of a number of sequential events required for the addition of each further nucleotide residue to the growing chain, and the concomitant translocation along the template by one nucleotide pair;
4. termination, which may or may not require the presence of a termination factor rho (ρ).

1.4.1.1.

DNA BINDING

The number of sites available on DNA for primary binding of RNA polymerase has been measured by saturation experiments based on the sedimentation of the enzyme as a stable complex with DNA in sucrose gradients (Richardson, 1966b) and also by a filter technique based on the finding that a complex of DNA and enzyme, in contrast to its free components, is fixed to nitrocellulose filters (Jones and Berg, 1966).

This binding is rapid and completely reversible and probably results from a fairly non-specific interaction between DNA and RNA polymerase (Richardson, 1966b; Jones and Berg, 1966). Measurement of the binding of RNA polymerase to DNA reveals that the number of enzyme molecules bound per unit of DNA varies with the ionic strength of the solution in a continuous manner. The values range from 24 μ g of RNA polymerase per μ g of DNA to zero as the ionic strength of the solution increases from 0.02 to 0.20 (Pettijohn and Kamiya, 1967; Ihler, reference in Chamberlin, 1970). The upper value represents essentially one RNA polymerase molecule bound for every 100 \AA of DNA helix, this approaches the diameter of the RNA ^{polymerase} molecule itself. Hence the total number of binding sites is probably limited only by the packing of enzyme molecule on the DNA a condition which would not obtain if the enzyme was binding specifically to the sparsely scattered true promoter sites. The decrease in binding with increasing ionic strength is attributable primarily to a decrease in the electrostatic free energy of interaction between RNA polymerase and DNA. σ is not required for this initial interaction for both holoenzyme and core polymerase bind to DNA in this manner with equal facility (Zillig et al., 1970b; Hinkle and Chamberlin, 1970; di Mauro, et al., 1969).

RNA polymerase binds at random sites on the DNA which it encounters during diffusion. This binding is in most cases rapidly reversible and the number of potential binding sites on DNA is large; probably any nucleotide sequence has an appreciable affinity for the enzyme. When however the holoenzyme binds at a true promoter site the

rapid dissociation no longer occurs, the enzyme forming a comparatively stable complex with DNA (Hinkle and Chamberlin, 1970; Zillig et al., 1970b). This specific binding site on the DNA is termed a promoter site (promoter site is a specific binding and initiation site which has an *in vivo* function). The number of such sites on any *E. coli* bacteriophage DNA molecule is believed to be small. Binding at such a promoter site leads to a specific reaction with RNA polymerase in which the strands of the double-helical DNA are opened over a short local region (Zillig et al., 1970b; Hinkle and Chamberlin, 1970). The affinity of the enzyme for DNA in this complex is enhanced by several orders of magnitude over that for random binding. The initiation of specific RNA chains appears to occur without the release of σ from this complex. Evidence for this model for specific DNA binding comes from direct studies of the binding of RNA polymerase to DNA (Zillig et al. 1970; Hinkle and Chamberlin, 1970) and from studies of the sensitivity of RNA polymerase to the inhibitors heparin and rifamycin (Zillig et al., 1970b; Bautz and Bautz, 1970). RNA polymerase bound to DNA in a promoter complex has an extremely low rate of dissociation from DNA and is relatively resistant to the inhibitors heparin and rifamycin.

With an intact phage DNA as template, formation of an active promoter complex occurs at temperatures over 20°C and requires the presence of the RNA polymerase subunit σ . Even at 37°C, RNA polymerase dissociates from such a complex very slowly, the complex itself having a half life of 60 hours (Travers, 1971). The requirement for σ subunit and elevated temperatures in the formation of a complex resembling the

promoter complex can be replaced by using a single-stranded or broken DNA, or a DNA with an open structure such as dAT copolymer. The complex formed with these templates is active in subsequent RNA synthesis (but is not formed at specific promoter sites.)

RNA polymerase holoenzyme transcribes bacteriophage DNAs asymmetrically and in many cases initiates only early 'phage mRNA (Geiduschek and Haselkorn, 1969; Richardson, 1969). In addition the *E. coli* RNA polymerase can carry out asymmetric transcription of DNAs from bacteriophage specific for other hosts. In contrast, the core polymerase shows no apparent strand selectivity with a variety of DNA templates and fails to synthesise detectable amounts of specifically initiated RNA chains. (Takanami et al., 1970). Two important conclusions can be drawn from these observations. First, the nature of promoter sequences for bacterial RNA polymerase located on different *E. coli* bacteriophage DNAs, and on the DNAs of other bacteriophage seem to be similar. This suggests that at least part of the promoter site structure is kept relatively constant in bacteria. Secondly, σ subunit is intimately involved in the functional interaction of the RNA polymerase with such promoter sites.

Chamberlin (1970) has proposed two general models to account for the mechanism by which RNA polymerase holoenzyme recognises a specific promoter site; both of these assume that the promoter site is a specific sequence of nucleotides -

(1) Sigma subunit identifies a specific nucleotide sequence (promoter) and directs the core polymerase to bind at this site. By this hypothesis, the specific information for site selection is localised mainly in σ subunit. A major fraction of the binding energy is provided by the core polymerase since σ itself, does not bind to DNA (Darlix et al., 1969).

(2) Core polymerase identifies a specific nucleotide sequence but must bind σ in order to enhance its affinity. By this hypothesis the specific information for site recognition is localised in the core polymerase and σ subunit serves as a kind of allosteric effector to promote tight binding.

These are extreme models for the recognition of promoter sites by RNA polymerase more probably, both σ and core polymerase both contain specificity determinants which are essential for promoter recognition. It is known that σ subunit a component of core polymerase, is required for initiation (Zillig et al., 1970b) and also that the initiation specificity of core polymerase can be altered by substituting one type of σ -like activity for another (Travers, 1969) showing that σ subunit itself must contain at least some information necessary for promoter recognition. Therefore it would appear that both sigma factor and core polymerase contain promoter recognition determinants.

1.4.1.2.

CHAIN INITIATION

Chain initiation by RNA polymerase involves the oriented binding of two nucleoside triphosphates to the enzyme followed by elimination of

inorganic pyrophosphate to form a nucleotidyl nucleoside - 5' - triphosphate. Polymerisation of RNA chains in vivo and in vitro occurs with a 5' → 3' chemical polarity. It is quite likely that the binding of the first nucleotide is different from the binding of the rest because there is no 3' hydroxyl group to which the first nucleotide can be added. It has been suggested by Anthony, Zeszotek and Goldthwait (1966) that the rate limiting step in initiation may be the binding of the first nucleotide. When the nucleoside triphosphates are present in low concentrations there is a notable lag in initiation which is most pronounced when purine nucleoside triphosphates are limiting. Since RNA chains are known to be initiated preferentially with a purine nucleotide, this observation would be consistent with initiation being more sensitive to the substrate concentration than subsequent steps in synthesis. The 5'-terminal nucleoside triphosphate is predominantly ATP or GTP when native phage DNAs are used as templates in vitro (Maitra, Lockwood, Dubnoff and Guha, 1970). There is probably a unique starting sequence associated with each specific DNA binding site. In the case of transcription with fd bacteriophage RF DNA as template, each of the four RNA products has a discrete size and seems to begin with a unique sequence of nucleotides at the 5'-terminus. (Takanami, et al., 1970). This suggests that the selection of the initial nucleotide sequence to be transcribed is dictated exactly by the positioning of the RNA polymerase on the promoter site. However in the case of T7 DNA, only a single promoter site was

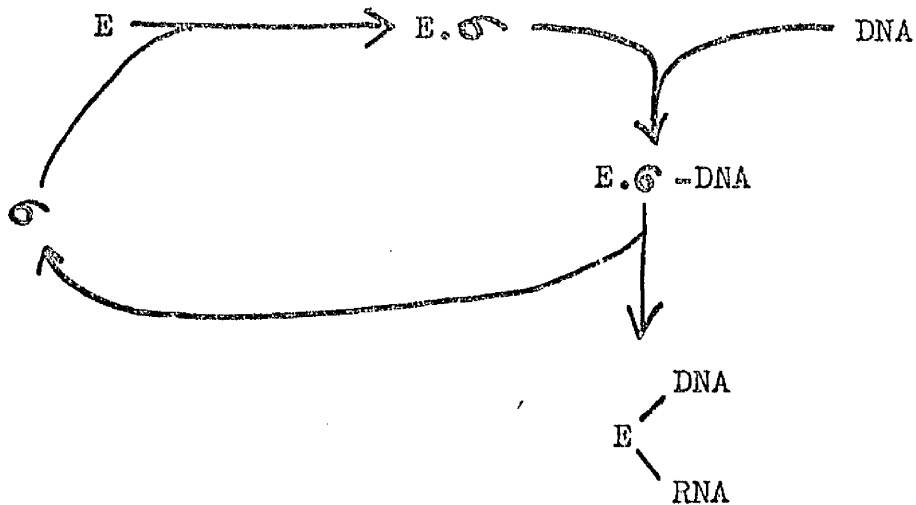
visualised by Davis and Hyman (1970) in their electron micrographs of in vitro transcription, yet both ATP and GTP initiated RNA chains have been reported (Maitra et al. 1970). This could be explained by non-specific initiation of T7 RNA species with GTP and there is some evidence for this. (1) core polymerase transcribes only GTP - initiated RNA chains with T7 DNA as template (Goff and Minkley, 1970; Chamberlin, 1970), (2) at raised salt concentrations, GTP incorporation into the 5'-terminal nucleotide is much reduced (Maitra et al. 1970), (3) RNA polymerase from a template-sensitive RNA polymerase mutant is deficient in ATP, but not GTP initiation with T7 DNA as template (Jacobson and Gillespie, 1970).

Rifampycin, an antibiotic which binds specifically to the β subunit of bacterial RNA polymerase blocks RNA chain initiation. The drug does not prevent the binding of the enzyme to DNA or the formation of the highly stable enzyme-promoter complex (Chamberlin, 1970). Bautz and Bautz (1970) and Lill and Hartmen (1970) have shown that the enzyme bound to DNA is inactivated at a much slower rate than the free enzyme. Bautz and Bautz (1970) have employed this observation to estimate the number of promoter sites on a variety of phage DNAs to which the enzyme will bind in a rifampycin-resistant complex.

What is the role of σ in initiation? It is known that σ acts catalytically in the initiation of RNA chains (Travers and Burgess, 1969; Berg et al., 1969) but is not required for chain elongation (Darlix et al. 1969). In accordance with this conclusion was the observation that σ

is released from the DNA-RNA polymerase complex in vitro during or shortly after the process of initiation itself (Travers and Burgess, 1969; Krakow, Daley and Karstadt, 1969) and that the core polymerase continues chain elongation. The free σ is able to combine with another core enzyme molecule and RNA synthesis can be initiated. This has led Travers and Burgess (1969) to propose the 'sigma cycle' (figure 1.2). Pettijohn, Stenington and Kossman (1970) have isolated a DNA-RNA polymerase complex from *E. coli* which is actively synthesising rRNA. No σ is present in this complex suggesting that σ release probably occurs in vivo as well as in vitro. It is not yet clear what triggers the release of σ or at what step in initiation this release occurs. Krakow and von der Helm (1970) have noted that with poly dAT as template, using UTP and a low concentration of ATP as substrates, RNA polymerase (*A. vinelandii*) will catalyse active pyrophosphate exchange but no observable σ release occurs nor is σ released with DNA templates when initiation is limited by adding only three of the four ribonucleoside triphosphates (Fromageot in Chamberlin, 1970). On current evidence it is not possible to pinpoint the timing of σ release with any great accuracy. In vitro σ release can be mediated by binding single-stranded polynucleotides to the holoenzyme (Krakow, Daley and Karstadt, 1969). If the reaction mimics σ release during initiation, it follows that σ will be released when a single-stranded polynucleotide structure is generated. Such a structure could either be the product RNA, in which case σ release

FIGURE 1.2 THE SIGMA CYCLE



E is the core RNA polymerase $\beta'\beta\alpha_2$

Travers and Burgess (1969) proposed this model for the involvement of σ in the transcription process. Sigma initially forms a complex (E. σ) with core enzyme (E) which then binds to DNA (E. σ -DNA) to initiate RNA synthesis. After initiation σ is released from the complex and becomes available for re-use by another core enzyme molecule.

would occur shortly after the initiation of an RNA chain, or single-stranded DNA formed by melting of the double helix.

To elucidate the role of σ , the nature of the lesion in the initiation process as manifested by core polymerase must be determined. Single-stranded breaks in the DNA template stimulate initiation by core polymerase (Vogt, 1969) and open structured DNA species such as poly dAT (Chamberlin, 1970) and fd RF DNA (Sugiura, Okamoto and Takanami, 1970) are also efficient templates for core polymerase suggesting that core is only able to initiate RNA synthesis when a certain receptive DNA structure, possibly a local melting, occurs fortuitously in the template. Goff and Minkley (1970) have observed that with T7 DNA as template a large fraction of the RNA chains initiated by core polymerase are hybridised to the template DNA at their 5'-terminus, whereas no such effect is observed when holoenzyme transcribes T7 DNA. This suggests that initiation by core polymerase may occur at sites with unusual structural features. Random initiation by the core polymerase could then be simply a consequence of the random occurrence of a receptive DNA structure, the core enzyme differing from the holoenzyme in being unable to form the receptive DNA structure of the promoter complex itself.

1.4.1.3.

ELONGATION

Growth of RNA chains occurs by the sequential addition of nucleoside monophosphates to the 3'-terminus of the nascent RNA chain. The rates of

RNA chain growth in vivo and in vitro are comparable (Geiduschek and Haselkorn, 1969). Davis and Hyman (1970) determined the in vitro rate of RNA synthesis to be 45 nucleotides per second into an RNA chain, using T7M DNA as template. Bremer (1970) has obtained a maximum in vitro rate of 36 nucleotides per second using T4 DNA as template; with T7 DNA as template the RNA chains grow 2.3 times faster than on T4 DNA. The in vivo rate of RNA chain growth in T4-infected *E. coli* is 28 nucleotides per second (Bremer and Yuan, 1968a). In uninfected *E. coli* Bremer and Yuan (1968b) have determined the rate of chain growth to be 55 nucleotides per second.

It has been suggested that the rate of RNA chain growth may vary during the synthesis of a specific RNA chain, perhaps due to variations in the sequence of the RNA transcript (Chamberlin, 1970). In support of this T4 and T7 RNA seem to differ twofold in their rate of elongation (Bremer, 1970) although this could be due to the absence of glucosylation in T7 DNA. The extreme case of such a dependence of chain growth rate on sequence would be sequence where chain growth rate stopped. Such sequences would provide control points for chain termination. Chain elongation is blocked by the antibiotic streptolydigin (Cassani, Burgess and Goodman, 1970) which, like rifampycin, acts by binding to the RNA polymerase molecule and not DNA.

Kosagonov et al. (1971) have produced some physical evidence for the unwinding of DNA during transcription.

1.4.1.4.

TERMINATION

Termination of an RNA chain can occur by at least four possible mechanisms. Although probably only two of them have any in vivo significance. The four types of RNA chain terminations are (1) DNA sequence-induced termination, (2) ρ -induced termination, (3) salt-induced termination and (4) hybrid induced termination.

(1) DNA-induced termination.

Termination can occur during RNA synthesis with native bacteriophage DNA as template when the RNA polymerase encounters a specific termination site (DNA sequence). This has been demonstrated for fd RF, ϕ 80, T7, T2 and T4 bacteriophage DNAs (Takanami et al., 1970; Millette, Trotter, Herrlich and Schweiger, 1970; Maitra, Lockwood, Dubnoff and Guha, 1970; Maitra and Barash, 1969; Richardson, 1970). This termination results in the release of RNA molecules of defined length and a unique 3'-terminus, at the same time the RNA polymerase is released. It is not certain whether this form of termination is of strict physiological significance as RNA molecules synthesised in vitro are generally larger than those synthesised on the same template in vivo (Travers, 1971). Morgan (1970) has shown that transcription of synthetic DNA polymers terminates soon after initiation when the transcribed DNA stand contains either UAA (ochre) or UAG (amber codons). This DNA sequence-induced termination occurs in the absence of protein factors other than purified bacterial RNA polymerase and occurs at both low and high salt concentrations although termination is greatly

enhanced by high (0.2M-KCl) salt concentrations.

There is a difference in termination between the two commonly used strains of T7 bacteriophage DNA. T7L DNA contains a DNA termination site at the end of the 'early' T7 genes (Millette et al., 1970) whereas T7M DNA contains no such DNA termination signal and RNA polymerase can transcribe the entire length of the T7 DNA r-strand (Davis and Hyman, 1970).

(2) Rho-induced termination.

A second mechanism of RNA chain termination involves the protein factor rho (ρ) (Roberts, 1969). This protein is isolated from *E. coli* and exists as a tetramer whose individual polypeptide chains have a molecular weight of 50,000. Termination in the presence of ρ occurs at sites in addition to DNA termination sites. Takanami et al. (1970) have reported that ρ factor leads to a reduction in the size of fd RF RNA. The in vitro transcription of T4, T7 and λ DNAs is limited to early mRNA when ρ is present, but in the absence of ρ , delayed early mRNA appears after a time lag (Richardson, 1970; Roberts, 1969). For T7M DNA where only one active promoter is present, RNA synthesis in the presence of ρ leads to the formation of several RNA species. (Davis and Hyman, 1970) instead of one. The mechanism of ρ -induced termination is not known. Rho-induced termination appears to require more than the stoichiometric amount of ρ protein, suggesting that its mechanism of action does not require a tight permanent binding at its binding site. The ρ protein does not bind appreciably to DNA or to RNA polymerase but does bind slightly

to RNA (Richardson, 1970). Rho is not a simple ribonuclease (Goldberg, 1970).

(3) Salt-induced termination.

Termination in the absence of ρ is enhanced in the presence of elevated salt concentrations. Salt-induced termination leads to the release of both active enzyme and RNA molecules and there is reinitiation of RNA synthesis. (Maitra, Lochwood, Dubnoff and Guha, 1970; Millette et al., 1970; Bremer, 1970). There is evidence that, in the presence of elevated salt, much of the RNA released with T4 and T7 DNA as templates consists of species of unique size (Millette et al., 1970; Maitra et al. 1970). One explanation of this effect is that elevated salt leads to a general decrease in the binding affinity of RNA polymerase for DNA. It may also be that the high salt concentrations increase the recognition of RNA polymerase for DNA termination signals.

(4) Single stranded DNA termination.

Termination of RNA chains is strongly enhanced with single stranded DNA as template (Maitra and Hurwitz, 1967). Chamberlin (1970) has suggested that this is due to the formation of DNA-RNA hybrids in the reaction. The possible biological significance of this type of termination is unknown.

1.4.2.

THE RATE OF TRANSCRIPTION

The relative rates of the various steps in the overall reaction sequence for the synthesis of a specific RNA molecule by *E. coli* RNA polymerase can be summarised. Binding of RNA polymerase holoenzyme to DNA is known to be complete within a few seconds. Chain initiation is completed with

one or two minutes. The growth of RNA chains is rapid from 40 to 100 nucleotides per second. Enzyme release after chain termination is slow, even in the presence of elevated salt, about half of the enzyme is released and restarts in 60 minutes. Thus in vitro RNA polymerase spends a large portion of its time in chain initiation and termination (Chamberlin, 1970).

1.5.

CONTROL OF TRANSCRIPTION IN BACTERIA

Transcription of the bacterial genome is regulated in response to the environmental conditions imposed on the cell. At any one time only a certain set of all possible RNA transcripts will be synthesised, these to be succeeded by another set with changes in the growth conditions of the bacteria. The production of a defined RNA molecule from a DNA template requires that synthesis of the molecule be initiated at a specific site on the template, then that the coding DNA strand be copied in accordance with classic Watson-Crick rules of base pairing before synthesis is finally terminated at a specific site on the template.

At what stage of this transcription process could control be exerted? Only control of initiation appears to provide the necessary flexibility for transcriptional control (Travers, 1971). Other suggested mechanisms invoking regulation of chain elongation (Stent, 1966) or of chain termination (Roberts, 1969; Brody, Sederoff, Bolle and Epstein, 1970) are probably of less importance.

Control of transcription can be exerted at various molecular levels. As the basic component of the transcription machinery the core polymerase ($\beta'\beta\alpha_2$) does not have sufficient specificity for accurate promoter recognition (section 1.4), such necessary specificity is provided by σ factor which is the primary determinant for promoter recognition and is probably required for all in vivo initiations. Although RNA polymerase holoenzyme ($\beta'\beta\alpha_2\sigma$) transcribes most bacteriophage DNA species efficiently it has long been known that bacterial DNA is a poor template for this

enzyme (Travers, Kamen and Schleif, 1970). It has been suggested that the transcription of many bacterial operons requires additional positive control elements termed ψ (Ψ) factors (Travers, Kamen and Schleif, 1970) of which there would exist several types in the bacterial cell, and which would themselves be regulated by low molecular weight effectors, providing a system of control that is readily reversible allowing the bacterial cell to respond rapidly to environmental changes and exerting their control by regulating the initiation of transcription of a class of transcriptional units. An example of such a postulated class is the rRNA and tRNA cistrons which are under the control of a protein factor ψ_r (Travers, Kamen and Schleif, 1970). ψ_r activity is inhibited by ppGpp, guanosine tetraphosphat (Travers, Kamen and Cashel, 1970), a nucleotide whose appearance in vivo is correlated with the stringent response (Cashel and Gallant, 1969). If there were several types of ψ factor in the bacterial cell it would be predicted that they would compete for the RNA polymerase and thus the level of RNA synthesis of each class of transcriptional units would depend on the relative affinities of these ψ factors for the enzyme or their availability..

It has been known for some time that a positive control factor is required for the expression of the arabinose operon (Englesberg, Squires and Meronk, 1969). Chamberlin (1970) has suggested that the protein factor M (Davidson, Pilarski and Echols, 1969) may be relevant in this context. This factor stimulates RNA synthesis by both core polymerase and holoenzyme with λ DNA as template. M thus appears to combine the properties of a ψ and σ factor. Evidence has been obtained that M also acts by binding to RNA polymerase (Davidson, Brookman, Pilarski and Echols 1970).

In the control of transcription of the lac operon, a protein factor, cyclic AMP receptor protein, CRP (Emmer, de Crombrugghe, Pastan and Perlman, 1970) which is probably identical to the "catabolite gene activation protein" (CAP) isolated by Zubay, Schwarz and Beckwith (1970) is a necessary protein factor. Cyclic AMP binds to and activates CRP. Travers (1971) has proposed that CRP be classified as a ψ factor but this classification has now turned out to be incorrect.

Control of transcription can be mediated by another category of regulatory proteins which interact directly with DNA at or near promoter sites and thus either potentiate or inhibit initiation. Examples of such regulatory elements would be the λ and lac repressors. Any individual regulatory element of this type would probably control only a small number of transcriptive units which may or may not be under ψ control.

CRP appears to be another such regulatory protein. Pastan et al. (1971) (reference in de Crombrugghe et al. 1971) have demonstrated that CRP binds tightly to lac-containing DNA and not to RNA polymerase. De Crombrugghe et al. (1971) have suggested that the promoter region on the lac genome is subdivided into an RNA polymerase binding site and a CRP binding site. The formation of a complex between CRP and lac DNA is probably required for the binding of RNA polymerase to its site on the DNA molecule. De Crombrugghe et al. (1971) have shown that three proteins only control the transcription of the lac operon: RNA polymerase (+ σ), lac repressor and CRP. All three interact with DNA. The latter

two, in conjunction with small effector molecules, regulate the activity of the first by controlling the rate of transcription.

Modifications to the structure of RNA polymerase itself may change the initiation specificity of the enzyme. Martelo, Woo, Reimann and Davie (1970) have shown the phosphorylation of σ factor stimulates RNA synthesis by the holoenzyme. Chelala, Hirschbein and Torres (1971) have shown that adenylation of RNA polymerase holoenzyme results in an inhibition of RNA synthesis. How either of these modifications could control specific gene expression is not yet known.

Changes of a more irreversible nature such as those associated with sporulation in *B. subtilis* (Losick, Shorenstein and Sonenshein, 1970) or with T4 phage development (Walter, Seifert and Zillig, 1968) involve such phenomena as modification to the polypeptide chains of core polymerase, the appearance of a phage induced transcription initiation factor (Travers, 1970) or even the denovo synthesis of a new RNA polymerase (Chamberlin, McGrath, Waskell, 1970).

SECTION 2 MATERIALS AND METHODS

2.1.

MATERIALS

2.1.1.

NUCLEIC ACIDS AND NUCLEOTIDES

ATP, GTP, UTP and CTP were obtained from P-L Biochemicals and used without further purification. 8-¹⁴C-ATP and ³H-GTP were purchased from Schwarz Bio Research. Calf thymus DNA (Worthington) and E. coli DNA (Mann) were used without further purification. E. coli MRE 600 DNA was a gift from D. Donnelly. T7 DNA, prepared by the method of Rush et al. (1967) was a gift from Ailsa M. Campbell and D.J. Jolly.

2.1.2.

ENZYMES AND OTHER PROTEINS

Deoxyribonuclease (electrophoretically pure), alkaline phosphatase, pyruvate kinase, trypsin, pepsin, ovalbumin, lysozyme and ribonuclease were purchased from Sigma. Sheep γ -globulin and bovine serum albumin were Armour products. For all the proteins purchased only the most pure available were obtained.

2.1.3.

DYES

Coomassie blue and amido black were purchased from Gurr. Ethidium bromide was a Calbiochem product.

2.1.4.

BIOCHEMICALS

Phosphoenol pyruvate was purchased from Sigma. Actinomycin D (a Merck Sharpe and Dohme product) was a gift from R. Billing. Rifampicin was bought from Mann.

2.1.5.

CHROMATOGRAPHIC MATERIAL

Phosphocellulose (PC 11) and DEAE-cellulose (DE 52) were Whatman products. Biogel A5M, A1.5M and HTP (hydroxylapatite) were purchased from Bio-Rad. Sephadex G-25 and Sepharose 6B were bought from Pharmacia.

2.1.6.

CHEMICALS

Ludox was a gift from E.I. Du Point De Nemours Inc. Casamino acids were purchased from Difco. Guanidine hydrochloride, urea and sucrose (ribonuclease-free) were Mann Ultrapure reagents. 2-mercaptoethanol and dithiothreitol were obtained from Koch-Light. All other chemicals were BDH Analar grade.

2.1.7.

MISCELLANEOUS

Dialysis tubing (Visking) was boiled twice in 5% (w/v) Na_2CO_3 , rinsed

with water, boiled in 0.05M-EDTA, pH 7, and then stored at 4°C in 0.05M-EDTA, pH 7.

Table 2.1. E. COLI GROWTH MEDIUM

COMPONENT	g/litre
Glucose	1.8
Casamino acids	15.25
KH_2PO_4	5.442
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.493
$(\text{NH}_4)_2\text{SO}_4$	1.32
Fe_2SO_4	0.0028

The pH was adjusted to 7.1 by the addition of NaOH.

The growth medium was that of Holms, Bennet and Robertson (1969).

2.2.

METHODS

2.2.1.

PREPARATION OF ESCHERICHIA COLI 15224

E. coli 15224 were grown at 37°C with vigorous aeration in 10 litre flask to the end of the logarithmic growth phase (10^9 cells/ml). The growth of the bacteria was followed by taking aliquots of the medium at 20 min time intervals after inoculation and measuring the turbidity at 420 nm on a Cary 15 spectrophotometer. The medium was that of Holms, Bennet and Robertson (personal communication, 1969) (table 2.1). The cells were harvested after 225 min by pouring onto an excess of crushed ice and collected by centrifugation at 15,000 rev/min (10,000g) in a Sharples continuous flow centrifuge. The bacterial sediment was washed with TMA buffer (0.01M-tris acetate, pH 7.5, 0.022M-NH₄Cl, 0.01M-Mg acetate and 0.001M-2-mercaptoethanol). The washed bacterial sediment was stored at -70°C.

2.2.2.

PURIFICATION OF RNA-POLYMERASE BY THE ZILLIG PROCEDURE

RNA polymerase was originally prepared by a method based on that of Zillig, Fuchs and Millette (1964).

COLUMN CHROMATOGRAPHY

Whatman DEAE-cellulose (DE52) was suspended in 5 volumes of TMA buffer and titrated to pH 7.5 with 1M-HCl at 20°C. The DEAE-cellulose was equilibrated with an excess of TMA buffer.

PROTEIN DETERMINATION

The protein concentration of fractions 1 to 3 was determined by the Biuret method (Layne, 1957). Fractions 4 to 6 were assayed by the method of Lowry, Roseborough, Farr and Randall (1951). Both procedures used crystalline bovine serum albumin as a standard.

RNA POLYMERASE ASSAY

The assay measured the incorporation of ^{14}C -ATP into acid insoluble RNA.

One unit of enzyme is that amount of enzyme that, in excess of DNA template, incorporates one nmole of ATP into RNA in 10 min at 37°C , under the conditions described. The assay mixture contained in a total volume of 0.5 ml;

0.03M - tris acetate pH 7.9

0.13M - NH_4Cl

0.03M - magnesium acetate

0.001M - 8- ^{14}C -ATP (specific activity approx. 0.3 c/mole)

0.001M - GTP, UTP, CTP

0.01M - phosphoenolpyruvate

10 μg pyruvate kinase

50 μg calf thymus DNA

1-10 units of enzyme.

In the standard assay, incubation was carried out at 37°C for 10 min.

ASSAY PROCEDURE

The reaction was started by the addition of enzyme to the pre-incubated reaction mixture. After 10 min incubation the reaction was stopped by the addition of 1 ml of ice water (where the amount of protein in the incubation was less than 200 μ g, 200 μ g of BSA was added with the ice water). The acid insoluble product (RNA) was precipitated by the addition of 1.5ml of ice-cold 10% (w/v) TCA. After cooling on ice for 10 min, the precipitates were collected on membrane filters (Millipore HA45, 450nm pore size), washed four times with 5ml of ice-cold 5% (w/v) TCA, placed in a scintillation vial and dried in a hot oven. Both TCA solutions contained 1% (w/v) tetra-sodium pyrophosphate to help prevent binding of labelled ATP to the filter. The assay blank either contained no enzyme or was not incubated. The incorporation of ^{14}C -ATP into the acid insoluble precipitate was determined in a Nuclear Chicago liquid scintillation counter with 8ml of a toluene solution containing 0.5% PPO.

PURIFICATION OF E. COLI RNA POLYMERASE

This procedure was performed at 0 to 4 $^{\circ}\text{C}$. All centrifugation was at 4 $^{\circ}\text{C}$.

PREPARATION OF CRUDE EXTRACT

400g of washed E. coli MRE 600 were suspended in 600ml of TMA buffer (0.01M-tris acetate pH 7.5, 0.022M- NH_4Cl 0.01M magnesium acetate, 0.001M-2-mercaptoethanol) and homogenised in 90ml portions in the presence of 135ml of dry cold acid-washed glass beads (diameter

$0.11 \pm 0.012\text{mm}$). Homogenisation was performed by shaking at 50Hz for 2 min at 0 to 4°C in the 200ml stainless steel vessel of the Zillig vibrational cell-mill (Zillig and Holzel, 1958). The bacterial extract was separated from the glass beads by squeezing it through two layers of muslin by hand. The beads were suspended in 400ml of TMA buffer and re-extracted. The beads were further re-extracted with TMA buffer until the volume of the bacterial extract (fraction 1) was 1 litre. The bacterial debris was removed by centrifugation at 30,000 rev/min (78,000g in Spinco 30 or MSE 30 rotors. The supernate is the crude extract (fraction 2).

PREPARATION OF DNA-PROTEIN FRACTION

The crude extract was centrifuged at 78,000g for 20h in the Spinco 30 rotor and the supernate was discarded. The sediment consisted of two distinct layers. The upper layer, DNA and protein, which contains the enzyme, was removed from the lower layer, ribosomes, with a spatula. The combined upper layers were suspended in a final volume of 400ml of TMA buffer by gentle homogenisation in a Potter-Evejhem homogeniser (fraction 3).

DEAE-CELLULOSE CHROMATOGRAPHY

400g of wet weight TMA-equilibrated DEAE-cellulose was added to the DNA-protein fraction and stirred slowly for 20 min. The excess TMA buffer was then removed by suction on a Buchner funnel.

To elute a large part of the unwanted protein the DEAE-cellulose was washed three times with 300ml of 'prewash' buffer (0.05M-tris acetate pH 7.5, 0.11M-NH₄Cl, 0.01M-magnesium acetate, 0.001-β-mercapto-ethanol, 0.001M-EDTA), suspended in 300ml of this buffer and finally de-aerated by suction in a Buchner flask.

The DEAE-cellulose with the absorbed DNA and protein was poured onto a 4cm column of DEAE-cellulose and washed with 'prewash' buffer in a 5 cm × 60cm glass column. The adsorbed proteins and nucleic acids were eluted from the column by a linear gradient of 0.11M to 0.7M-NH₄Cl in 'prewash' buffer (figure 2.2). Each of the closed mixing vessels contained 600ml of buffer. The flow rate through the column was 90ml/h. The extinction of the effluent at 280nm was recorded with an LKB Uvicord. 15ml fractions were collected and assayed for enzyme activity. The enzyme containing fractions were pooled (fraction 4).

AMMONIUM SULPHATE FRACTIONATION

Fraction 4 was adjusted to a protein concentration of 5-10mg of protein per ml by the addition of TMA buffer. Ice-cold saturated (NH₄)₂SO₄ in TMA (pH 7.5) was added slowly to give 40% (NH₄)₂SO₄ saturation. After standing in ice for 30 min, the precipitate was removed by centrifugation at 20,000 rev/min (35,000g) for 10 min in the 30 rotor of the MSE ultracentrifuge. The supernate was then brought to 50% (NH₄)₂SO₄ saturation. After standing in ice for 30 min the precipitate was further collected by centrifugation and dissolved in 30ml of TMA buffer. The crude enzyme particles were collected by centrifugation at 50,000

rev/min (151,000g) for 5h in the Spinco 50 rotor. The sediment was dissolved in 6ml of TMA buffer (fraction 5).

SUCROSE GRADIENT CENTRIFUGATION

Three Spinco SW 25.2 tubes were each filled with 56ml of a linear sucrose gradient of 10 to 30% (w/v) sucrose in TMA buffer (Glycerol 5-10% (v/v) was added to stabilise the enzyme) (figure 2.3). (This sucrose gradient was later replaced by a 16 to 50% (v/v) glycerol gradient (figure 2.4)). The gradients were each layered with 2ml of fraction 5 and centrifuged at 25,000 rev/min (75,500g) for 20h. 2.2ml fractions were collected from the top of the tubes and the extinction at 280nm was followed by an LKB Uvicord II.

The enzyme containing fractions were combined, diluted two-fold with TMA precipitated by 55% $(\text{NH}_4)_2\text{SO}_4$ saturation and collected by centrifugation. The precipitate was dissolved in 6ml of TMA (fraction 6).

STORAGE OF ENZYME

Fraction 6 enzyme was stored either at 0°C in ice or at -10°C diluted 1 in 2 with glycerol.

2.2.3.

PURIFICATION OF RNA POLYMERASE BY THE BURGESS PROCEDURE

This purification procedure is based on that of Burgess (1969a) and was used to obtain the RNA polymerase for the physical studies.

BUFFERS

All buffer solutions (Table 2.2) were prepared from Analar grade chemicals.

TABLE 2.2 BURGESS RNA POLYMERASE PREPARATION BUFFERS

Buffer	pH	Tris-HCl (M)	MgCl ₂ (M)	EDTA (M)	Dithiothreitol (mM)	Glycerol (%)
G	7.5	0.10	0.01	0.10	0.10	5
A	7.9	0.10	0.01	0.10	0.10	5
C	7.9	0.05	-	0.10	0.10	5
2 x S	7.9	0.02	0.02	0.20	0.20	-

Buffer S was prepared by adding an equal volume of glycerol.

KCl was added to buffers as indicated in the text.

Distilled water was used for all solutions. All buffers were made from stock solutions and KCl was added as indicated in the purification procedure.

COLUMN CHROMATOGRAPHY

DEAE-cellulose (DE 52) was suspended in 2 volumes of 0.05M-Tris-HCl pH 7.9 with 1M-HCl. The column was poured at 20°C (room temperature) and equilibrated at 4°C with at least four column volumes of buffer A before use.

Biogel A5M, 100 to 200 mesh, was mixed with 2 volumes of buffer C at room temperature and allowed to settle. The supernate was discarded. Another 2 volumes of buffer C was added, and the slurry de-aerated under vacuum before pouring into a column. The column was equilibrated at 4°C with at least two column volumes of buffer C. After use the column was washed twice with buffer C + 0.02% (w/v) sodium azide as a bacteriostatic agent. Before re-use the column was washed three times with buffer C. Biogel A1.5M, 100 to 200 mesh, was treated similarly with buffer A + 1M-KCl.

PROTEIN DETERMINATION

Protein in fractions 1 to 3 was measured by the Biuret procedure (Layne, 1957). Protein concentration in fractions 4 to 6 was measured by the method of Lowry et al (1951). Protein estimation was also carried out by a method modified from that of Warburg and Christian (1941). (See Section 3.6).

RNA POLYMERASE ASSAY

RNA polymerase activity was assayed by two procedures:

- (1) a radio-active assay similar to that described by Burgess (1969a),
- (2) a fluorescence assay modified by Roach (personal communication, 1970) from that of Krakow et al. (1969). This assay utilises the increase in fluorescent intensity of the fluorescent dye ethidium bromide when bound to nucleic acids (Le Pecq et al. 1964, Le Pecq et al. 1966). The standard assay mixture contained in a total volume of 0.5ml:

0.04M-trisHCl pH 7.9 (20°C)

0.01M-MgCl₂

0.1mM-EDTA

0.1mM-dithiothreitol

0.15M-KCl

1.5mM-GTP, UTP, CTP

1.5mM-¹⁴C-ATP

0.05-0.15 mg/ml DNA (calf thymus or T7)

0.01M-PEP*

20µg/ml pyruvate kinase*

* omitted when pure enzyme was assayed.

(1) Radio-active assay: This assay mixture also contains

¹⁴C-ATP (specific activity 0.3 c/mole). Incubation is at 37°C for 10 min and the rest of the procedure is as described in the Zillig assay procedure (Section 2.2.2.).

(2) Fluorescence assay: This required an assay volume of 1 ml. RNA polymerase was added to start the reaction, 0.1 ml aliquots were removed at various time intervals and added to 1.9 ml of an ethidium bromide solution (5 μ g/ml ethidium bromide, 0.01M-tris-HCl pH 7.9, 0.05M-NaCl, 1mM-EDTA). Excitation was at 300nm and the fluorescent intensity at 590nm was measured. The fluorescent intensity increases with increasing RNA concentration. Fluorescence was measured on an Aminco-Bowman Spectrophotofluorometer. RNA polymerase units measured by the fluorescence assay were corrected (from μ g RNA synthesised/10 min to n moles of nucleotide monophosphate (ATP) incorporated into RNA per 10 min.

PURIFICATION OF RNA POLYMERASE

All operations were performed at 0 to 4°C.

CELLS

E. coli MRE 600 were obtained from MRE Porton. These cells had been grown in continuous culture at 37°C under conditions of carbon limitation and processed as described by Elsworth et al. (1968). The cells had been washed with a buffer containing 0.01M-magnesium acetate, 0.01M-tris-HCl pH 7.4. The cells were stored at -70°C.

GRINDING AND DNASE TREATMENT

The frozen pellets (2 × 100g) of *E. coli* MRE 600 were broken into small pieces and suspended in 300ml of buffer G in an Atomix blender. 90ml portions of the bacterial suspension were homogenised in the presence of 135ml of cold dry glass beads. Homogenisation was performed by shaking at 80Hz for 2 min at 4°C in the 200ml stainless steel vessel of the Zillig vibrational homogeniser. The combined extracts and glass beads were poured into a beaker and a further 300ml of buffer G added. 2ml of a freshly prepared solution of 1.25mg/ml DNase was added with mixing and stirred slowly for 30 min. The glass beads were allowed to settle and the supernate was then decanted into a flask through a funnel loosely plugged with glass wool. The beads were then re-extracted several times with buffer G. Gentle suction was applied to draw the bulk of the solution from the beads. Excessive foaming was avoided. The filtrates were combined to give fraction 1 (1 litre).

ULTRACENTRIFUGATION

Fraction 1 was centrifuged at 78,000g for 4 hours. This removes cell debris and ribosomes in one step. About 800ml of clear amber supernate is obtained (fraction 2).

AMMONIUM SULPHATE FRACTIONATION

23.1g of solid ammonium sulphate per 100 ml of fraction 2 was slowly added with stirring to give a 33% saturated solution. The solution was stirred slowly for 30 min and the precipitate removed by centrifugation at 5,200 rev/min (5,100g) for 45 min in 750ml polypropylene bottles in an MSE 6L centrifuge. 10.75g of ammonium sulphate was added per 100ml of 33% saturated supernate to give a 50% saturation. The precipitate contained the RNA polymerase and was stirred and centrifuged as above. The precipitate was suspended in 260ml of 42% saturated solution of ammonium sulphate in buffer A, stirred 30 min and centrifuged for 70 min at 5,200 rev/min. The pellet, which contains the enzyme was dissolved in buffer A and diluted with approximately 500ml of buffer A until the specific conductivity of the solution was 9.6mmhos/cm (fraction 3).

DEAE-CELLULOSE CHROMATOGRAPHY

Fraction 3 was applied to a DEAE-cellulose column (100ml) which was equilibrated with buffer A. The column was washed with 40ml of buffer A then 300ml of buffer A + 0.13M-KCl (figure 2.5). The RNA polymerase was then eluted with buffer C + 0.23M-KCl(300ml). The fractions containing

the enzyme activity were pooled (fraction 4).

AGAROSE GEL FILTRATION (1)

Fraction 4 was precipitated by the addition of $1\frac{1}{2}$ volumes of saturated ammonium sulphate in buffer C, centrifuged 70 min at 5,200 rev/min, suspended in a total volume of 20ml of buffer C and dialysed 5 hours against the same buffer. A column of Biogel A5M (5×95 cm) was equilibrated with buffer C. The dissolved enzyme was applied, washed into the column with buffer C and eluted with this same buffer at a flow rate of 60ml/h (figure 2.6). The tubes containing RNA polymerase activity were pooled to give (fraction 5).

AGAROSE GEL FILTRATION (2)

Fraction 5 was precipitated by the addition of $1\frac{1}{2}$ volumes of saturated ammonium sulphate in buffer C, centrifuged 70 min at 5,200 rev/min and suspended in 4ml of buffer A + 1.0M-KCl. A column of Biogel A1.5M (2.5×95 cm) was equilibrated with buffer A. 1.0M-KCl and eluted with the same buffer at a flow rate of 24ml/h (figure 2.7). The tubes containing RNA polymerase activity were pooled, precipitated by the addition of $1\frac{1}{2}$ volumes of saturated ammonium sulphate in buffer C, centrifuged at 12,000 rev/min (11,000g) for 40 min in an MSE 18 centrifuge. The pellet was suspended in 3ml 2 \times storage buffer ($2 \times S$), and an equal volume of ice-cold glycerol was added (fraction 6).

STORAGE OF ENZYME

RNA polymerase in storage buffer does not freeze at the storage temperature.

-10°C and is stable for at least six months.

2.2.4.

ANALYTICAL COLUMN CHROMATOGRAPHY

2.2.4.1.

PHOSPHOCELLULOSE CHROMATOGRAPHY

Phosphocellulose was stirred with 5 volumes of 0.5M-NaOH for 30 min then rinsed with distilled water until the rinse was about pH 8. The phosphocellulose was then suspended in 5 volumes of 0.5M-HCl, stirred for 30 min then rinsed with distilled water until the rinse was about pH 4. The phosphocellulose was then resuspended in 4 volumes of 0.05M-trisHCl, pH 7 stirred for 15 min and titrated with 6M-KOH on a Radiometer PHM 52 digital pH meter. The column was poured and equilibrated at 4°C with buffer C + 0.05M-KCl until the pH and conductivity of the flow through was identical with that of the equilibration buffer. RNA polymerase (fraction 1) was dialysed overnight (12h) at 4°C against buffer C + 0.05M-KCl, applied to the column, and washed in with the buffer C + 0.05M-KCl. Protein peaks were eluted batch-wise by the procedure of Bautz et al. (1970) with buffer C + 0.25M-KCl and 0.4M-KCl. Washing the column with buffer C + 1.0M-KCl led to the elution of no further protein peaks.

2.2.4.2.

DEAE-CELLULOSE CHROMATOGRAPHY

DEAE-cellulose was prepared as described in Section 2.2.3. The column was poured at 20°C and equilibrated at 4°C with buffer A. Protein was equilibrated with buffer A by overnight dialysis at 4°C before application to the column. Protein was eluted either by a salt gradient or by a stepwise

procedure.

2.2.4.3.

HYDROXYAPATITE CHROMATOGRAPHY

A column of hydroxylapatite was equilibrated at 4°C with 500ml of a buffer containing 0.04M-sodium phosphate, pH7, 5% (v/v) glycerol, 1mM-dithiothreitol. RNA polymerase (fraction 6) was dialysed at 4°C against this same buffer and applied to the column and eluted with a 0.04M to 0.4M-sodium phosphate, pH 7, gradient.

2.2.4.4.

SEPHADEX CHROMATOGRAPHY

Sephadex G-25 was allowed to swell in the appropriate buffer solutions for at least three days, then equilibrated further with at least 5 column volumes of buffer before use.

2.2.5.

AMINO ACID ANALYSIS

Samples of both RNA polymerase and core polymerase in storage buffer were dialysed against 0.01M-phosphate buffer pH 7.9 at 4°C for 24h then transferred to conical tubes and lyophilised. The lyophilised protein was suspended in 6M-HCl (constant-boiling HCl) and the tubes were evacuated for 10 min with vigorous vortexing, sealed, then placed in a 110°C oven for 24h and 60h. The hydrolysates were taken to dryness by lyophilisation. The amino acids were dissolved in 1ml of 0.2N-citrate buffer, pH 2.2 and analysed by the method of Spackman, Moore and Stein (1958) on a Locarte amino acid analyser with automatic loading (85% amino acid recovery). Tryptophan was estimated spectrophotometrically

by the method of Edelhoch (1967).

2.2.6.

POLYACRYLAMIDE GEL ELECTROPHORESIS

2.2.6.1.

'ORNSTEIN AND DAVIS" POLYACRYLAMIDE GELS

Polyacrylamide gels at pH 8.9 were prepared by the general method of Ornstein and Davis (1964) as described by Brewer and Ashworth (1969). The 'standard' analytical system consisted of 7.5% (w/v) acrylamide upper gel, pH 8.9, and a tris-glycine electrode buffer, pH 8.3. RNA polymerase samples for disc electrophoresis were prepared by dialysing against the tris-glycine buffer. The dialysed samples were made 10% (w/v) in sucrose before layering onto the upper gels. About 50 to 100µg of protein was applied to the gel. A current of 1.5mA per tube was applied. The gels were stained for 1 to 3 hours in 1% (w/v) amido black in 7% (w/v) acetic acid.

2.2.6.2.

8M-UREA POLYACRYLAMIDE GELS

7.5% (w/v) polyacrylamide gels at pH 8.9 containing 8M-urea were prepared as described by Jovin, Chrambach and Naughton (1964). RNA polymerase samples were prepared by dialysing overnight (18h) against 8M-urea, 1% (v/v) 2-mercapto-ethanol.

2.2.6.3.

SDS-POLYACRYLAMIDE ELECTROPHORESIS

This was the major procedure used in the study of RNA polymerase by the technique of disc electrophoresis in polyacrylamide gels.

PREPARATION OF PROTEIN SOLUTIONS

All standard proteins were incubated at 37°C for 6h in a buffer solution containing 0.1M-sodium phosphate, pH 7.1, 0.1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. RNA polymerase samples were prepared by overnight (16h) dialysis at 20°C against the above buffer.

PREPARATION OF SAMPLES

For each gel, 5µl of tracking dye (half-saturated Bromophenol blue in 80% (v/v) glycerol) is mixed with 50µl of the protein solution. 50µl of this solution was applied to the gel. Each sample usually contained 10 to 50µg of protein. Where the protein sample was very dilute, 200µl was applied.

PREPARATION OF GELS

The glass gel tubes were 10cm long with an internal diameter of 6mm. Before use they were soaked in cleaning solution (Decon), rinsed in distilled water and dried. 1.5ml of freshly prepared gel solution was added to each tube. The gels contained 5% (w/v) polyacrylamide and were prepared as described by Shapiro et al. (1967). Before the gels hardened, a few drops of water were layered on top of the gel solution. Just before use the water was removed. The sample was then applied to the gel and the electrophoresis buffer (0.1M-sodium phosphate, pH 7.1, 0.1% (w/v) SDS) was carefully layered on top to fill the tubes. The two compartments of the electrophoresis apparatus were filled with buffer.

Electrophoresis was performed at a constant current of 8ma per gel. The positive electrode was in the lower chamber. Under these conditions the marker dye, Bromophenol blue, moved through 5/6 of the gel in 3.5h.

After electrophoresis the gels were removed from the tubes by rimming under water with a syringe needle through which water was flowing. The length of the gel and the distance moved by the dye were measured on a Vitatron scanning densitometer.

FIXING, STAINING AND DESTAINING

The gels were fixed overnight in 20% sulphosalicylic acid, and stained for 2.5h in either 1% (w/v) Amido black or Coomassie blue in 7% (v/v) acetic acid. The staining solutions were filtered before use to remove any insoluble material. The gels were destained overnight (16h) in acetic acid.

The gels can alter length in 7% (v/v) acetic acid, therefore the calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and of the Bromophenol blue. Assuming even swelling of the gels, mobility was calculated as

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \cdot \frac{\text{length before staining}}{\text{distance of dye migration}}$$

2.2.7.

QUALITATIVE AND QUANTITATIVE ESTIMATIONS

2.2.7.1.

ASSAYS OF ENZYMIC IMPURITIES

RNase assay was performed as described by Zillig, Fuchs and Millette (1966).

The DNase assay conditions were based on those of Lehman, Roussos and Prasad (1962). The alkaline phosphatase assay was that of Richardson (1966b).

2.2.7.2.

DNA, RNA AND PROTEIN ESTIMATION

DNA estimation was performed by the procedure of Burton (1968). RNA estimation was the orcinol procedure of Kerr and Seraidarian (1945).

Protein concentrations were measured by several different methods. The concentration of protein in crude extracts was usually determined by the Biuret method of Layne (1957). The method of Lowry, et al. (1951) or the improved method of Warburg and Christian (1941) as described in section 3 were generally used to measure most protein concentrations.

2.2.8.

ANALYTICAL ULTRACENTRIFUGATION

A Spinco model E analytical ultracentrifuge equipped with both schlieren optics and a Beckman DU spectrophotometer with scanner attachment (Schachman et al., 1962; Schachman, 1963) was employed.

2.2.8.1.

SEDIMENTATION VELOCITY

Velocity sedimentation was usually performed at 20°C using 12mm aluminium filled Epon double sector centrepieces in an An-D rotor.

Sedimentation was followed with either absorption optics at 280nm or with schlieren optics. The schlieren patterns were photographed on Ilford G.30 chromatic plates. Sedimentation coefficients $S_{20,w}$ were calculated from the observed S values by correcting for the viscosity and density of the solution (Schachman, 1959) which were obtained from International Critical Tables (1926).

$$\frac{S \cdot \omega^2}{2.303} = \frac{d \log_{10} r}{dt}$$

where S = sedimentation constant, r = distance from axis of rotation (cm), t = time (sec) and ω = angular velocity (radians/sec) $2\pi \times \text{rev/sec}$. A plot of $\log_{10} r$ against t (sec) is a straight line, the slope of which is $\omega^2 S / 2.303$.

$$S = \frac{\text{slope} \cdot 2.303}{\omega^2}$$

$$S_{20,w} = S \cdot \frac{\eta}{\eta_{20,w}} \cdot \frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)}$$

Where $S_{20,w}$ is the sedimentation coefficient under standard conditions, η is the viscosity of the experiment and $\eta_{20,w}$ is the viscosity of water at 20°C. The other correction term in the equation involves the partial specific volume of the solute, and the density of the solution and accounts for the difference between the buoyancy term, $(1 - \bar{v}\rho)$, in the experiment and the value had the experiment been performed in water at 20°C.

2.2.8.2.

SEDIMENTATION EQUILIBRIUM

Sedimentation equilibrium experiments were performed by using absorption optics at 280nm and the method described by Schachman and Edelstein (1966). Experiments were usually performed at 20°C. Equilibrium was achieved within 48h. The distribution of protein was determined at several time intervals to ensure that equilibrium had been attained. Double sector cells were used containing sample and buffer in the two chambers. Samples were prepared by dialysis against the appropriate buffers. The protein concentration was evaluated in centimetres of pen deflection of the scanner. After the equilibrium distribution had been determined, the base line of the sample was obtained by centrifuging at 40,000 rev/min to sediment the protein to the bottom of the cell (Berg and Chamberlin, 1970). The 70 to 80% of the sample column was used to calculate the weight average molecular weight (M_w) of the protein. Partial specific volume (\bar{v}) was calculated from the amino acid composition. Densities and viscosities of solutions were obtained from International Critical Tables (1926).

$$M_w = \frac{2RT}{(1-\bar{v}\rho)^2} \cdot \frac{d \ln C}{d(r^2)} \quad (\text{Schachman, 1959})$$

where R = gas constant = 8.312×10^7 ergs/°C/mole, T = temperature (°K),
 C = concentration in mg/ml. A plot of $\ln C$ versus r^2 yields $d \ln C / dr^2$.

2.2.9.

OPTICAL ROTATORY DISPERSION

2.2.9.1.

INSTRUMENTATION

Optical rotatory dispersion (ORD) was measured in a Bendix/Bellingham and

Table 2.3 SUCROSE CALIBRATION CONSTANTS

Reference	K	λ^2_c
National Bureau of Standards (U.S.) based on data at 589 and 546nm	21.615	0.0223
Harris, Hirst and Wood (1932)	21.676	0.0213
Tommimatsu and Gaffield (1965)	21.520	0.0221

Stanley Polarmatic 62 spectropolarimeter fitted with a 250 Watt Xenon lamp. The X-Y recorder was an Advance Electronics HR-96. Refractive indices of solutions were determined in either a Bellingham and Stanley Abbé refractometer or in a Brice-Phoenix differential refractometer at 546 and 436nm.

2.2.9.2.

CALIBRATION

The spectropolarimeter was calibrated on the basis of $\alpha_{589}^{\circ} = 50m^{\circ}$ for a 0.6378% (w/v) sucrose solution (Bendix Instruction Manual based on Harris Hirst and Wood, 1932). Slits were set at 0.6mm. The ORD of sucrose was measured over the wavelength range 600 to 250nm. The resulting ORD of sucrose satisfied the one term Drude equation

$$[\alpha]_{\lambda} = \frac{K}{\lambda^2 - \lambda_c^2}$$

Plotting $\lambda^2 [\alpha]_{\lambda}$ versus $[\alpha]_{\lambda}$ yields a straight line and the constants K and λ_c^2 can be determined. These calibration constants compare favourably with those in the literature. (Table 2.3).

2.2.9.3.

QUANTITATIVE MEASURES OF ROTATION

Optical activity of nucleic acids is usually reported in terms of the specific rotation $[\alpha]_{\lambda_1}$

$$[\alpha]_{\lambda} = \frac{100 \alpha}{l c}$$

where λ is the wavelength, α the observed rotation in degrees, l the cell path length in decimetres, and c is the concentration of optically active solute in g/100ml. The optical activity of proteins is usually expressed

as $[\alpha']$, the reduced mean residue rotation,

$$[\alpha'] = [\alpha]_{\lambda} \cdot \frac{\text{MRW}}{100} \cdot \left(\frac{3}{n^2 + 2} \right)$$

where MRW is the mean residue weight of the protein and $(3/n^2 + 2)$ is the Lorentz correction factor, where n is the refractive index of the solvent at wavelength λ . To compare observed rotations in a variety of solvents the rotations are reduced to a value they would have in a vacuum by means of the Lorentz correction factor. Dispersion of the refractive index of the solvent is taken into account for accurate measurements. When these values are not available they can be approximated by use of the Sellmeier equation

$$n^2 = 1 + \frac{a \lambda^2}{\lambda^2 - \lambda_v^2}$$

where λ_v^2 and a are coefficients to be determined. The equation can be solved for λ_v^2 and a by measurement of the refractive index at two wavelengths. There is only a very small change of refractive index of water with temperature and this usually can be neglected. The value of n , however, varies significantly with high concentrations of salt such as 6M-guanidine hydrochloride and 8M-urea solutions. The physical units for $[\alpha']$ are degree centimetres² per decimole.

2.2.9.4.

ANALYSIS OF ORD RESULTS

$$[\alpha]_{\lambda} = \frac{K}{(\lambda^2 - \lambda_c^2)}$$

This can be rearranged to the form $[\alpha]_{\lambda} \lambda^2 = K + [\alpha]_{\lambda} \lambda_c^2$.

λ_c^2 can be obtained from the slope of the plot versus $[\alpha]_{\lambda} \lambda^2$ and K can be obtained from intercept.

THE MOFFITT-YANG EQUATION

This equation was first formulated by Moffitt and Yang (1956) and takes the form of a two term Drude equation.

$$[m']_{\lambda} = \frac{a_0 \lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

This can be rearranged to a linear form such that on plotting

$[m']_{\lambda}(\lambda^2 - \lambda_0^2)/\lambda_0^2$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, a_0 is the intercept and b_0 the slope. A λ_0 of 212nm was assumed.

THE SCHECHTER-BLOUT EQUATION

To evaluate the coefficients $A_{(\alpha, \rho)193}$ and $A_{(\alpha, \rho)225}$ (designated A_{193} and A_{225} respectively below) in the Schechter-Blout modified two term Drude equation (Schechter and Blout, 1964),

$$[m']_{\lambda} = \frac{A_{193} \lambda_{193}^2}{(\lambda^2 - \lambda_{193}^2)} + \frac{A_{225} \lambda_{225}^2}{(\lambda^2 - \lambda_{225}^2)}$$

$[m']_{\lambda}(\lambda^2 - \lambda_{193}^2)/\lambda_{193}^2$ was plotted against $\lambda_{225}^2/(\lambda^2 - \lambda_{225}^2)$

$$A_{225} = \text{gradient} \cdot (\lambda_{193}^2/(\lambda_{225}^2 - \lambda_{193}^2))$$

$$\text{and } A_{193} = \text{intercept} - A_{225} \cdot \frac{\lambda_{225}^2}{\lambda_{193}^2}$$

2.2.9.5.

ESTIMATION OF HELICAL CONTENT

From the Moffitt-Yang plots for $\lambda_0 = 212\text{nm}$, the helix content calculated by the method of Urnes and Doty (1961) is

$$\% \text{ helix} = \frac{-b_0}{-6.30}$$

Schechter, Carver and Blout (1964) suggest that

$$\% \text{ helix} = \frac{-(b_0-100)}{8.00}$$

gives a better estimation of helix content.

From the A coefficients in the Schechter-Blout modified two term Drude equation, per cent helical contents (Blout, Carver and Schechter, 1967) are given as

$$H_{193} = \frac{(A_{193}+750)}{36.5}$$

and

$$H_{225} = \frac{-(A_{225}+60)}{19.9}$$

with the mean per cent helix expressed as

$$H_{\text{mean}} = \frac{(A_{193}-A_{225}+650)}{55.8}$$

From the value of the 233nm trough of the Cotton effect the per cent helix can be estimated (Blout, Schmier and Simmons, 1962)

$$\% \text{ helix} = \frac{([\text{m}']_{233}+2000)}{119}$$

FIG. 2.1

FIGURE 2.1. GROWTH CURVE OF E.COLI 15224

This is the mean of seven growth curves. The mean generation time was 31min. 5ml aliquots were removed every 30min and two drops of formalin were added before reading the absorbance at 420nm on a Cary 15 recording spectrophotometer. Where the absorbance was greater than 0.4 the aliquots were diluted with distilled water before reading.

/ 1.2 1.3 1.4

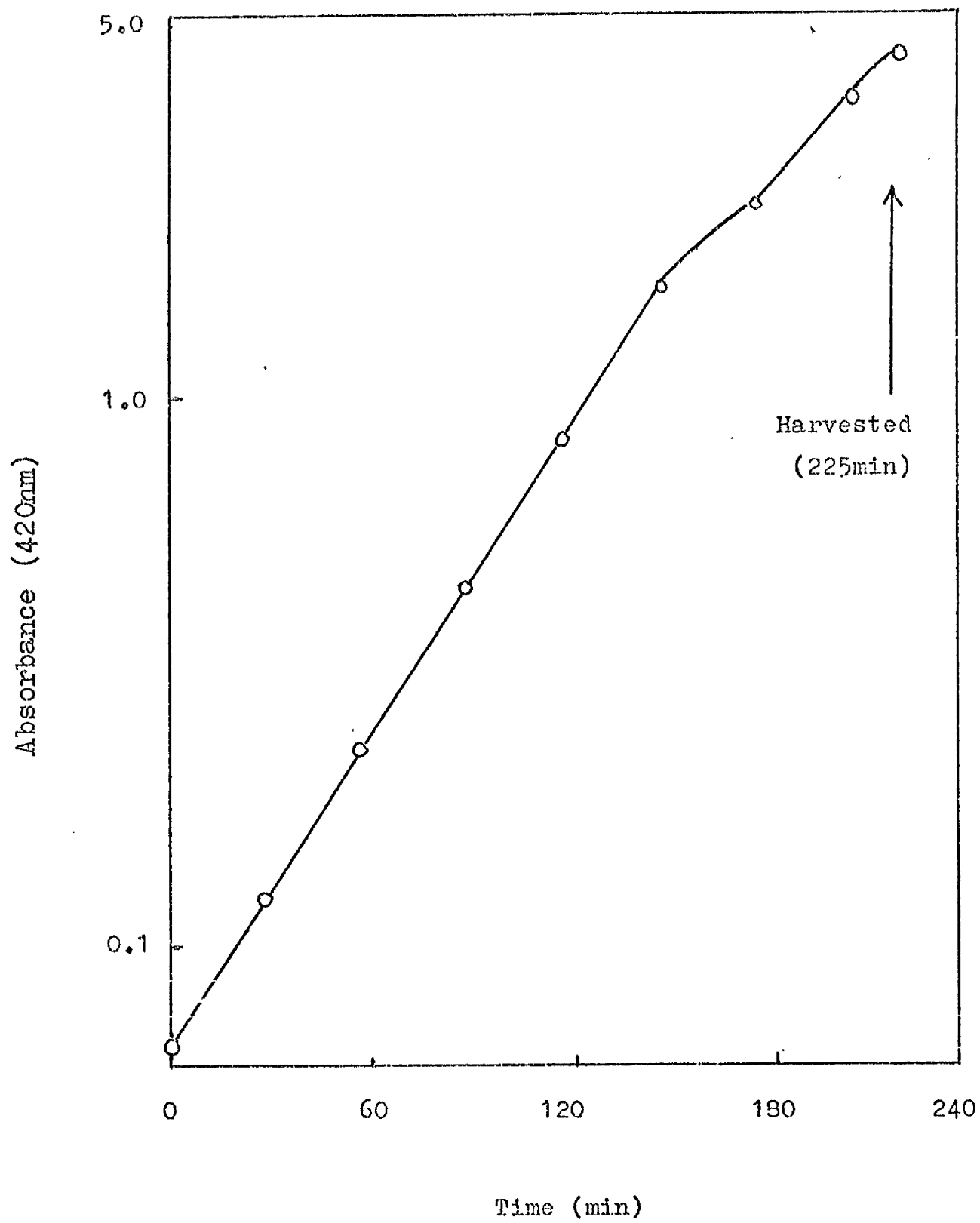


FIG. 2.2

FIGURE 2.2. PREPARATIVE DEAE-CELLULOSE CHROMATOGRAPHY
GRADIENT ELUTION PROFILE

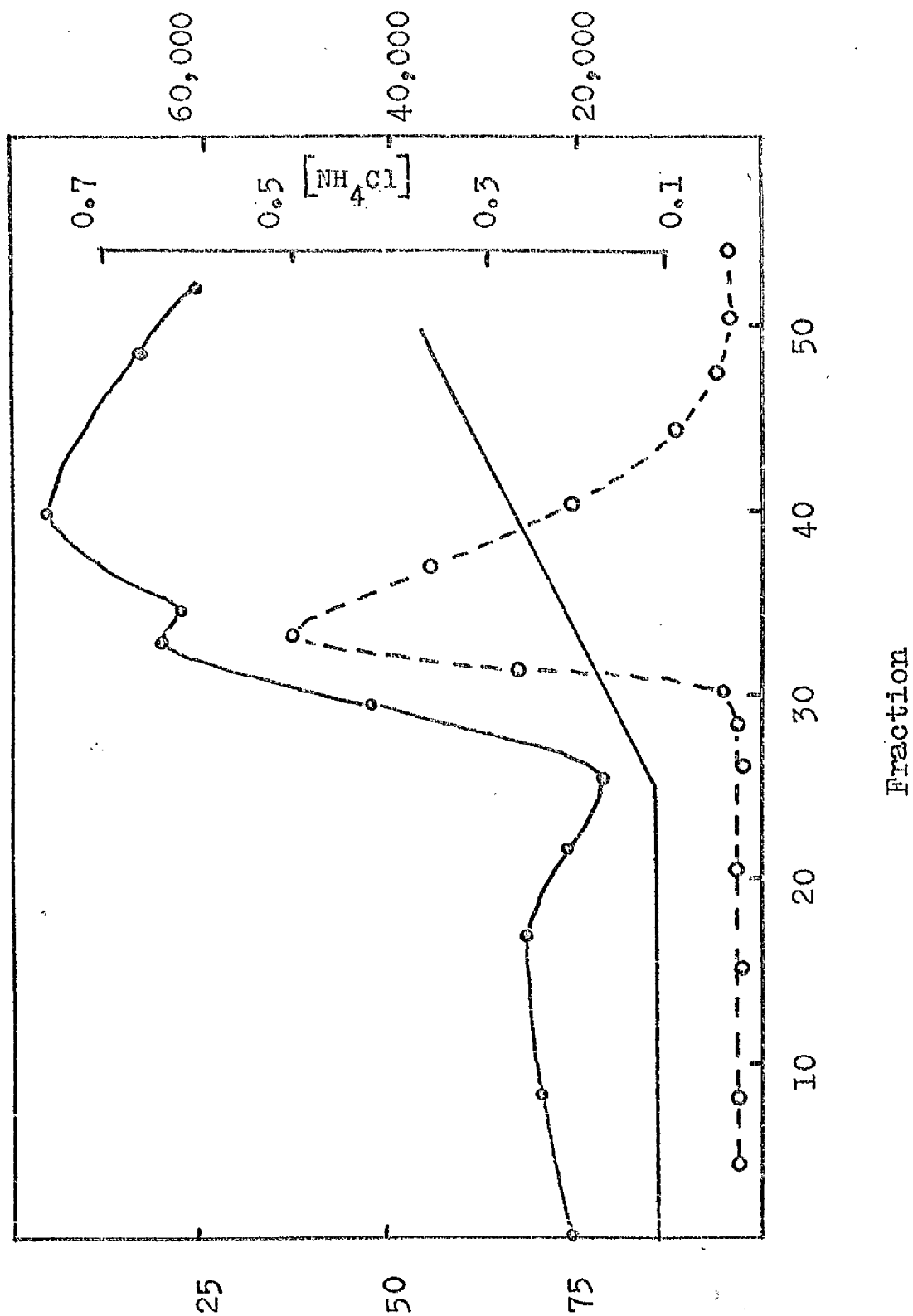
Fraction 3 RNA polymerase, already bound to the DEAE-cellulose and washed three times with prewash buffer (0.05M-tris acetate pH 7.5, 0.11M-NH₄Cl, 0.01M-magnesium acetate, 0.001M-2-mercaptoethanol) was eluted from the 5 X 60cm DEAE-cellulose column with a linear gradient of 0.11M to 0.70M-NH₄Cl in TMA buffer. Each of the closed mixing vessels contained 600ml of buffer. The column flow rate was 90ml/h, 15ml fractions were collected.

o—o Transmission at 254nm in the 3mm flow cell of an LKB Uvicord II.

o - - o RNA polymerase activity measured as the incorporation of ¹⁴C-ATP (as dpm) into acid-insoluble RNA by the procedure outlined in section 2.2.2.

—— NH₄Cl concentration (M).

^{14}C -ATP incorporated into RNA (dpm/ml)



% Transmission (254nm)

FIG. 2.3

FIGURE 2.3. PREPARATIVE SUCROSE GRADIENT PROFILE

2ml of fraction 5 RNA polymerase was applied to a 56ml, 10 to 30% (w/v) sucrose gradient in a Spinco SW 25.2 tube and centrifuged at 25,000rev/min for 20h at 4°C. After centrifugation the gradient was removed from the top by pumping a 35% (w/v) sucrose solution in TMA buffer into the bottom of the tube at 90ml/h and 2.2ml fractions were collected. Each fraction was assayed for both protein (by the method of Lowry et al. (1951)) and for RNA polymerase activity by the standard RNA polymerase assay (2.2.2.).

o—o—o RNA polymerase activity measured as the incorporation of ^{14}C -ATP into acid-insoluble RNA by 1ml of each fraction.

●——● Protein concentration determined by the Lowry (1951) procedure.

^{14}C -ATP Incorporated into RNA (dpm/ml)

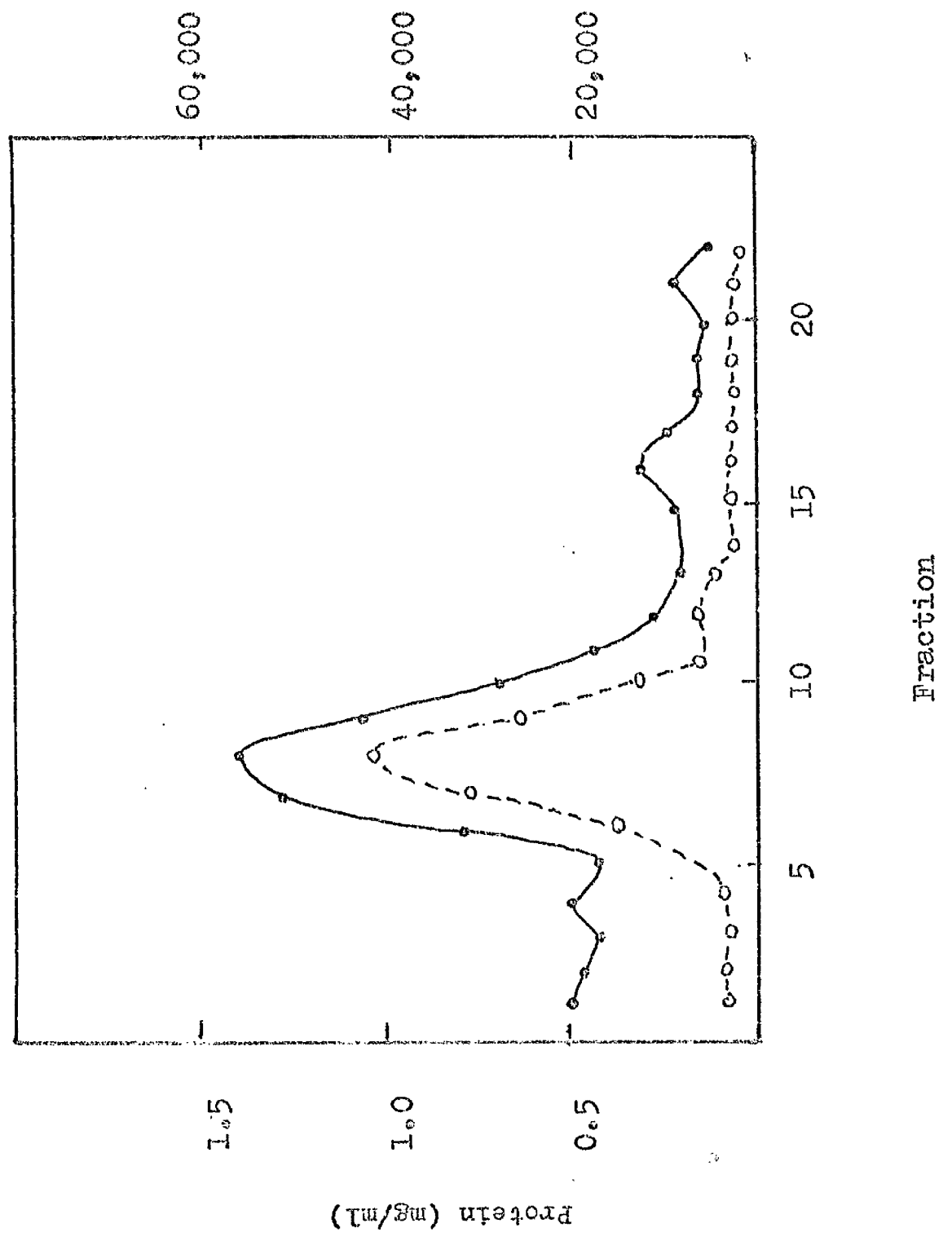


FIG. 2.4

FIGURE 2.4. PREPARATIVE GLYCEROL GRADIENT PROFILE

2ml of fraction 5 RNA polymerase was applied to a 16
to 50% (v/v) linear glycerol gradient in TMA buffer
and centrifuged at 25,000rev/min for 20h at 4°C.
The gradient was then removed from the top of the
tube by pumping a 55% (v/v) glycerol solution in
TMA buffer in through the bottom at 90ml/h. 3.6ml
fractions were collected and assayed for both
protein and RNA polymerase activity.

o - - o RNA polymerase activity measured as the
incorporation of ^{14}C -ATP (as dpm) into
acid-insoluble RNA by the procedure
outlined in section 2.2.2.

o — o Protein concentration determined by the
method of Lowry et al. (1951).

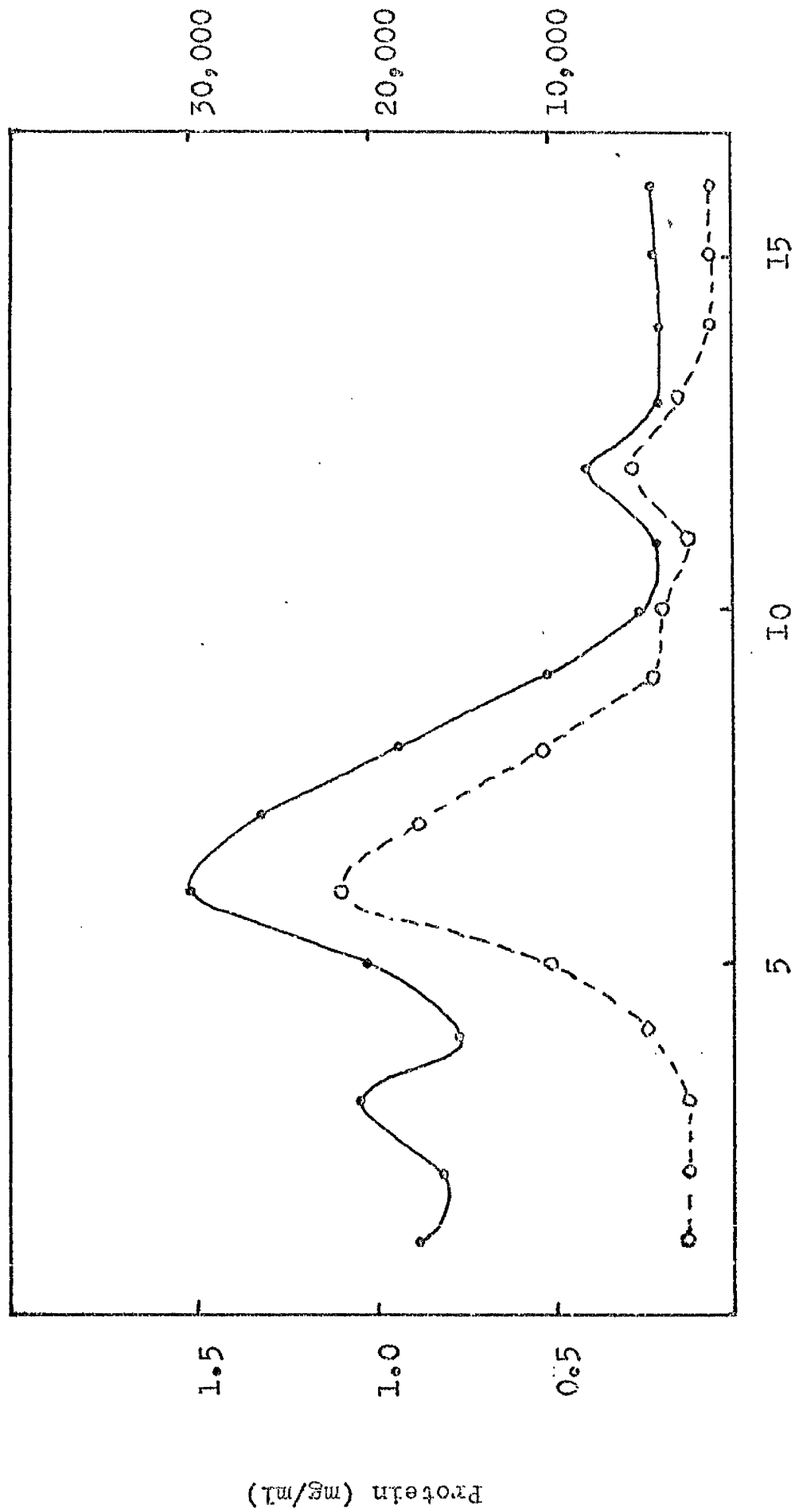


FIG. 2.5

FIGURE 2.5. PREPARATIVE DEAE-CELLULOSE COLUMN PROFILE

500 to 600ml of fraction 3 protein in buffer A were applied to a 2.5 X 20cm column of DEAE-cellulose equilibrated with buffer A at a rate of 54ml/h. 15ml fractions were collected. Tubes 1 to 43 contained the flow-through material. The bound protein and enzyme were eluted from the column at 90ml/h. Tubes 44 to 60 contained the buffer A+0.13M-KCl peak. Tubes 61 to 80, the buffer C+0.23M KCl peak, contained the RNA polymerase activity.

●——● Absorbance at 280nm in the 3mm flow cell of an LKB Uvicord II.

○——○ Enzyme units/ml.

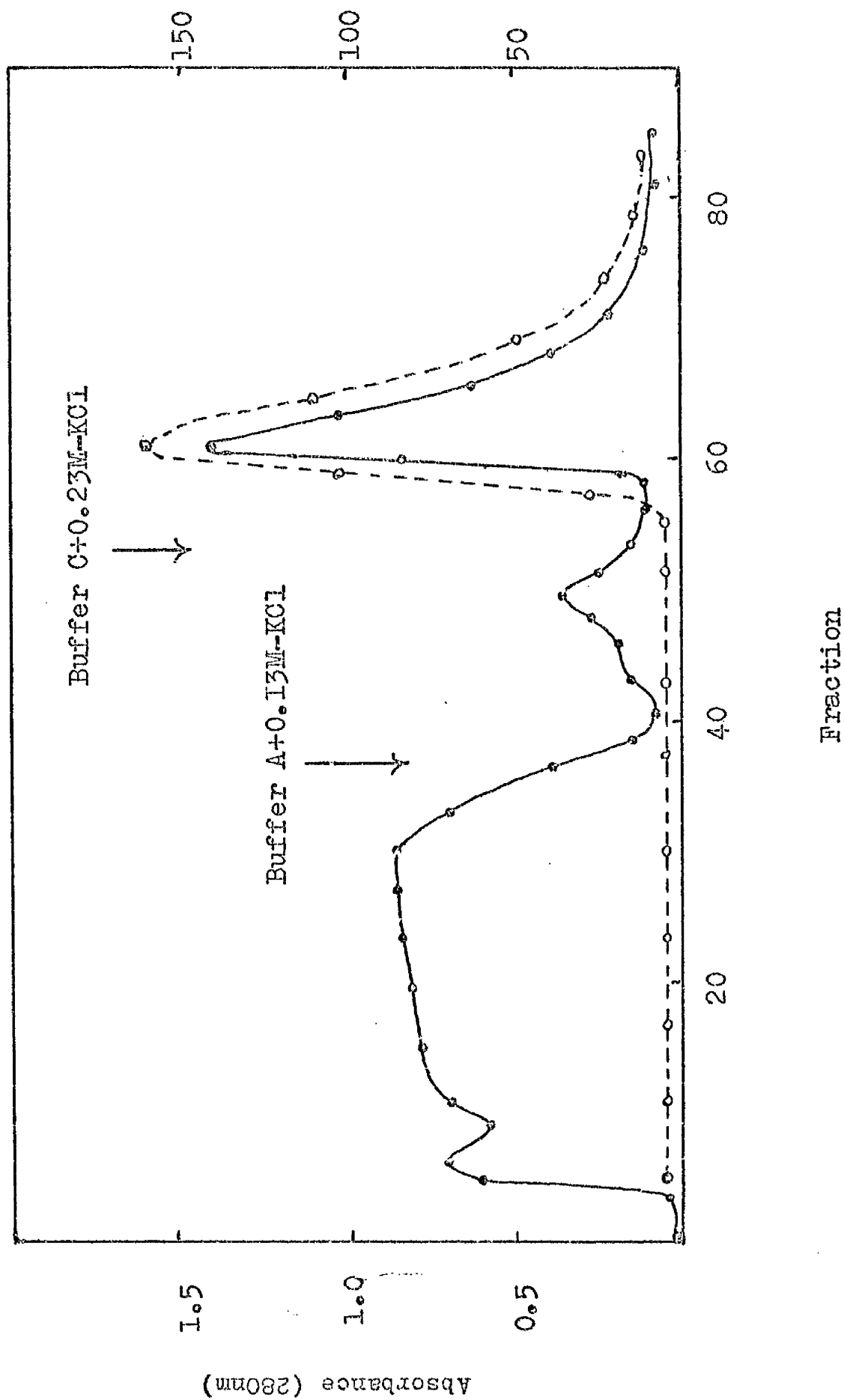


FIG. 2.6

FIGURE 2.6. BIOGEL A-5M AGAROSE GEL PROFILE

20ml of fraction 4 protein, dialysed against buffer C were applied to a 5 X 95cm column of Biogel A-5M equilibrated with buffer C and eluted at 60ml/h. 6ml fractions were collected. Tubes 89 to 116, containing the bulk of the RNA polymerase activity, were pooled.

o——o Absorbance at 280nm in the 3mm flow cell of an LKB Uvicord II.

o — — o Enzyme units/ml.

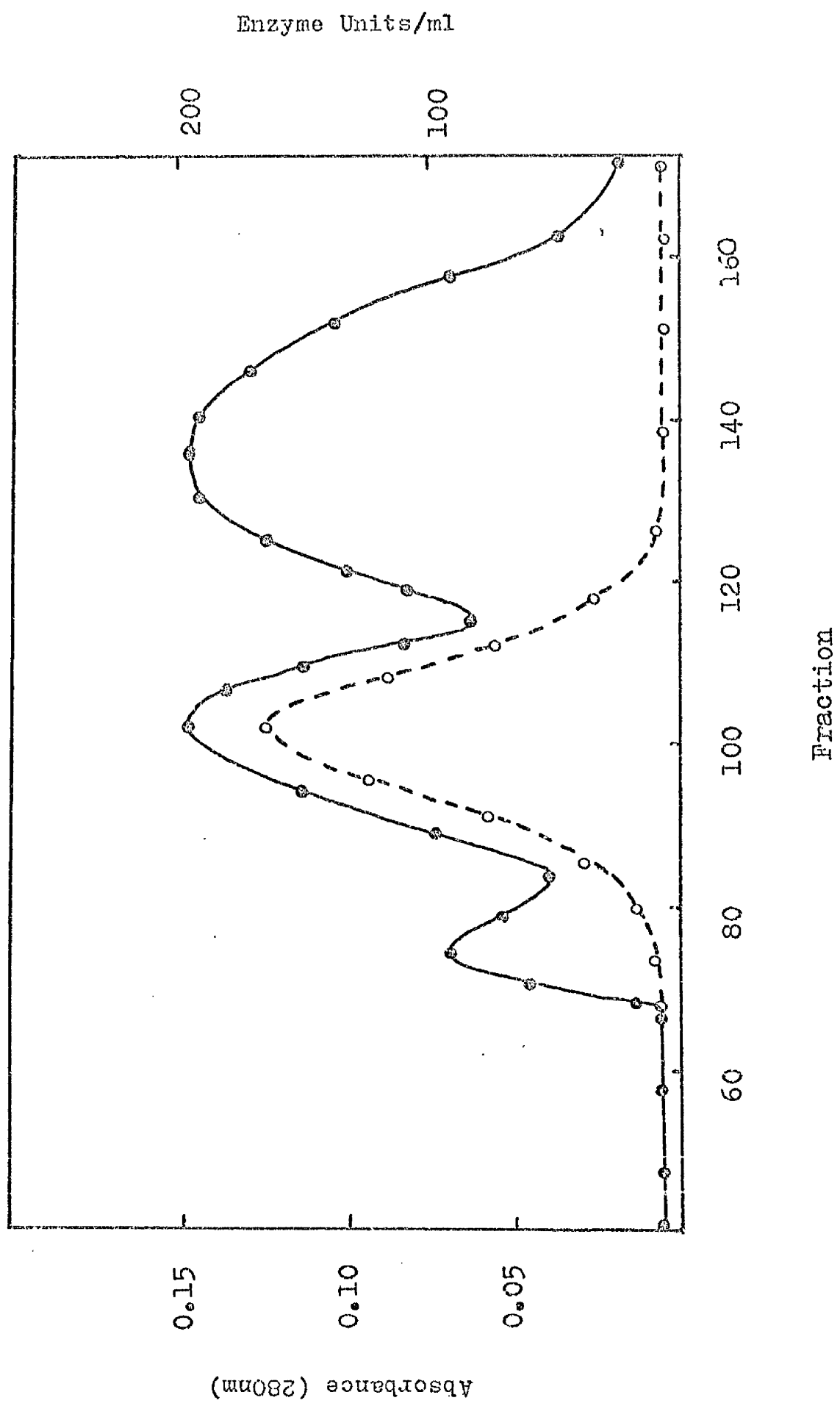


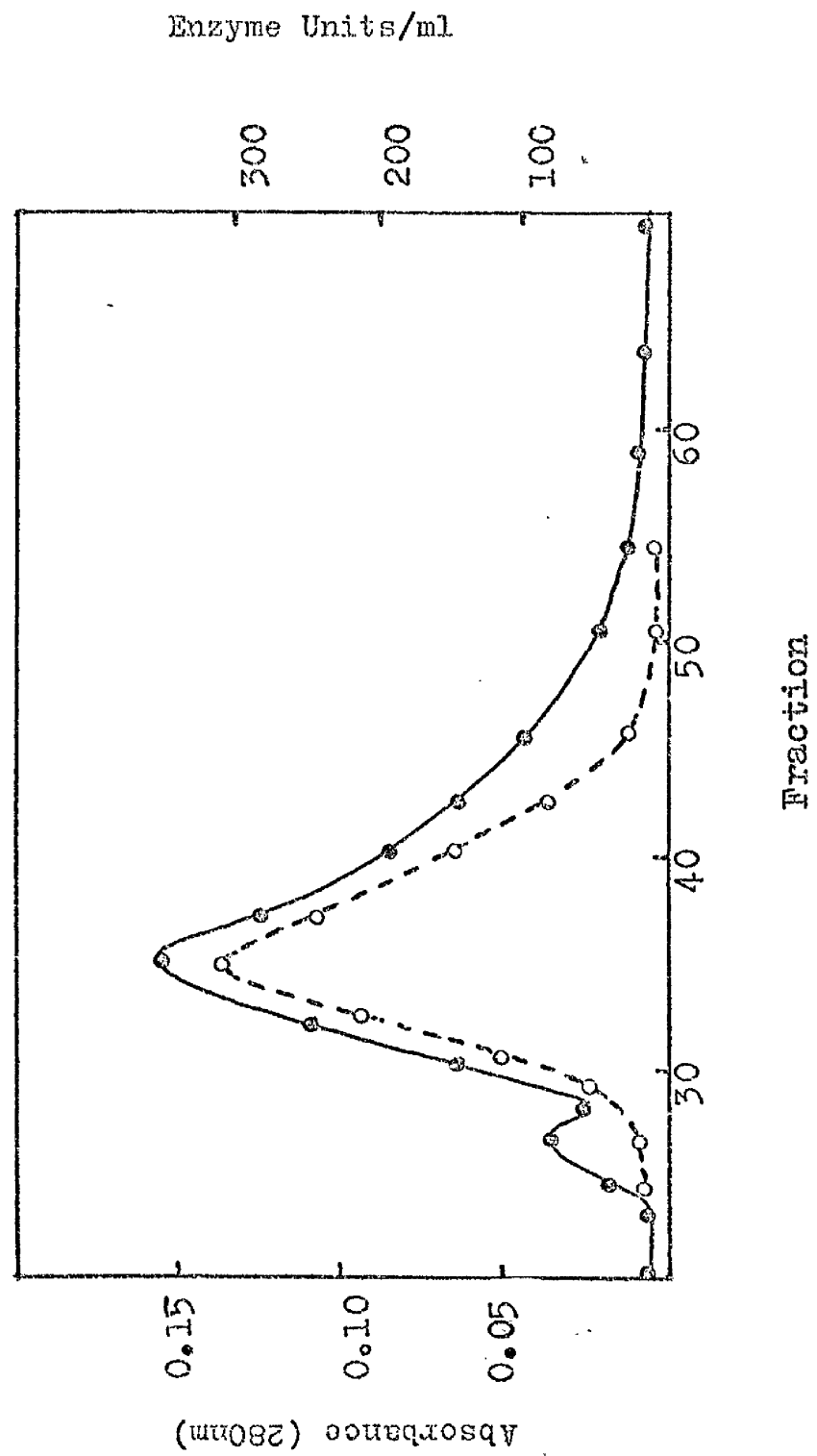
FIG. 2.7

FIGURE 2.7. BIOGEL A-1.5M AGAROSE GEL PROFILE

8ml of fraction 5 protein in buffer A +1.0M-KCl were applied to a 2.5 X 95cm column of Biogel A-1.5M equilibrated with buffer A +1.0M-KCl and eluted at 24ml/h. 6ml fractions were collected. The enzyme containing tubes, of constant specific activity, 29 to 46 were pooled.

—○— Absorbance at 280nm in the 3mm flow cell of an LKB Uvicord II.

○—○ Enzyme units per ml.



SECTION 3 RESULTS

3.1.

PURIFICATION OF RNA POLYMERASE FROM E. COLI MRE 600

3.1.1.

INTRODUCTION

Over the past 25 years there has been an increasing polarisation of effort toward work with the common colon bacterium *Escherichia coli*. Because of its small size, normal lack of pathogenicity and ease of growth under laboratory conditions, *E. coli* is now the most intensively studied organism except for man. The tendency to concentrate work on *E. coli* is increasing because parallel with the chemical studies, extensive genetic analysis is being carried out. Our knowledge of the genetics of *E. coli* is much more complete than our knowledge of any other bacterium or plant and it is now the best understood living organism at the molecular level. To study the physical and chemical properties of DNA-dependent RNA polymerase it would seem preferable to concentrate on the *E. coli* enzyme although it has now been prepared from several other bacteria.

3.1.2.

GROWTH OF E. COLI

For the purposes of purifying DNA-dependent RNA polymerase from *E. coli* it was intended to grow the bacteria under strictly controlled conditions of growth and harvesting procedure as it has been reported (Abraham, 1970) that RNA polymerase isolated from cells harvested at different stages of growth exhibits different properties with respect to

activity on different DNA templates. Several batches of *E. coli* 15224 (a strain available in this department) were grown under strictly controlled conditions as described in section 2.2.1. Although initial studies showed that there was a high RNA polymerase activity in these cells, it did not prove feasible to prepare the very large quantities of bacteria that would be required for the large-scale production of RNA polymerase. Fortunately, large quantities (Kilogrammes) of *E. coli* MRE 600 were made available by the Microbiological Research Establishment Porton, and the preparation of *E. coli* 15224 was discontinued.

E. coli MRE 600 is a strain of *E. coli* C6 and is deficient in the enzyme RNase 1 (Cammack and Wade, 1965; Wade and Robinson, 1966). *E. coli* MRE 600 were grown under conditions of continuous growth and carbon limitation at 37°C and pH 8 as described by Elsworth, Miller, Whitaker, Kitching and Sayer (1968).

3.1.3.

PURIFICATION OF RNA POLYMERASE BY THE ZILLIG PROCEDURE

3.1.3.1.

INTRODUCTION

At the time when this present study of the physical and chemical properties of DNA-dependent RNA polymerase from *E. coli* MRE 600 was commenced (1968) only a limited number of purification procedures, yielding reasonably pure enzyme, as judged by the simple criteria of specific activity and sedimentation homogeneity, were available for the preparation of RNA polymerase. Most of the procedures were inconvenient or even unsuitable for the large-scale preparation of sufficiently pure enzyme for physical and chemical studies. This was mainly due to low enzyme yields. The

procedure of Chamberlin and Berg (1962) was the method of choice of most people requiring RNA polymerase for enzymic studies. The enzyme produced by this procedure is highly active but cannot be used for protein studies without further purification. Richardson (1966b) published a modification of the method which produced such purification by chromatography on hydroxyapatite. However the basic Chamberlin and Berg procedure and modifications all employed precipitation with streptomycin and protamine sulphate in the initial steps to separate the RNA polymerase from the DNA. This is a critical step in the purification procedure and often leads to large losses of activity (Zillig, Zechel and Halbwachs, 1970). These steps have been avoided in a high yield method described by Babinet (1967) which involves the use of a phase partition procedure to separate the RNA polymerase from nucleic acids. The phase partition method however, requires the centrifugation and dialysis of very large volumes if the enzyme purification is performed on a large scale. Of all the procedures available at the time, that of Zillig, Fuchs and Millette (1966) appeared to offer the largest yield of RNA polymerase and it also appeared to avoid the critical protamine sulphate step of the Chamberlin and Berg (1962) procedure. The Zillig purification schemes utilises DEAE-cellulose chromatography to separate RNA polymerase from nucleic acids.

3.1.3.2.

RNA POLYMERASE PREPARATION

The procedure used was modified only slightly in the early steps from that described by Zillig et al. (1966). In the later stages the

principal modification from the published procedure was the substitution of glycerol gradient ultracentrifugation for sucrose gradient centrifugation which led to improved $A_{280} : A_{260}$ ratios for the enzyme.

Cell disruption was performed using the Zillig cell-mill (Zillig and Holzel, 1958). This disintegrates the bacteria by shaking them with fine glass beads at 50Hz. Homogenisation for 2 min leads to the almost complete disruption of the bacteria, homogenisation for longer times does not greatly increase the yield of enzyme (figure 3.1). Zillig et al. (1966) separate the bacterial extract from the glass beads by gentle suction on a sintered glass funnel, but this led to excessive foaming with subsequent protein denaturation. To avoid this the bacterial extract was removed from the glass beads by squeezing by hand through muslin, a difficult task due to the viscosity of the solution. The bacterial debris was removed by centrifugation to yield fraction 2 enzyme.

DNA-RNA polymerase fraction

This involved centrifuging the crude extract for 20h at 78,000g to sediment the ribosomes and the DNA-RNA polymerase complex. Much care was required to successfully remove the DNA-RNA polymerase pellet from the ribosome pellet without contamination with some of the ribosome fraction. This DNA-RNA polymerase pellet was fraction 3 enzyme.

DEAE-cellulose chromatography

This proved to be the most critical step in the entire Zillig purification procedure. The position of RNA polymerase in the elution from the DEAE-cellulose had always to be determined by enzyme assay. The DEAE-

cellulose chromatography was unable to completely separate the RNA polymerase from all nucleic acid contamination as the peak of enzyme activity usually overlapped with the DNA peak. DEAE-cellulose fractionation could cause the enzyme yield to vary over a wide range (20 to 70% of the theoretical maximum).

Ammonium sulphate fractionation

Fraction 4 enzyme, obtained from the DEAE-cellulose step was subject to ammonium sulphate fractionation and collected by ultracentrifugation at 151,000g for 5h. The enzyme pellet collected proved difficult to suspend in TMA buffer, and to avoid agitation, was usually allowed to stand overnight (8h) to bring it into solution. The ammonium sulphate fractionation led to the removal of some of the contaminating nucleic acid material.

Density gradient centrifugation

Fraction 5 RNA polymerase obtained from the ammonium sulphate fractionation was originally subjected to sucrose gradient centrifugation as described by Zillig et al. (1966). This was replaced by glycerol gradient centrifugation which was found to yield an enzyme preparation with decreased nucleic acid contamination. The presence of glycerol is known to stabilise the enzyme and it also meant that the RNA polymerase peak from the glycerol gradient could be stored directly at -10°C without prior precipitation and resuspension in a glycerol storage buffer. The main disadvantages of glycerol or sucrose gradient centrifugation are that time-consuming analyses have to be performed on each of the gradients and more importantly, from a preparative point of view, only relatively small quantities of protein can be applied to each gradient.

TABLE 3.1. SUMMARY OF RNA POLYMERASE PURIFICATION BY THE ZILLIG PROCEDURE

Fraction	Stage	Total enzyme units	Total Protein (mg)	Specific Activity	A ₂₈₀ :A ₂₆₀
1	Homogenate	-	-	-	-
2	Crude extract	240,000	20,000	12	-
3	DNA-protein	100,000	5,000	20	-
4	DEAE-cellulose	27,000	300	90	1.0
5	Ammonium sulphate precipitate	4,740	30	158	1.0
6	Sucrose gradient peak	2,430	15	182	1.3(1.49)*

* obtained with glycerol gradient centrifugation

As can be seen from the purification table (table 3.1) based on eight preparations of RNA polymerase by the Zillig procedure, very low yields of enzyme with only reasonable specific activity were obtained. From the published procedure (Zillig et al., 1966) much higher yields of more active enzyme should have been expected. Probably the major reason for the low yields obtained lies in the incomplete separation of RNA polymerase from nucleic acids in the DEAE-cellulose step.

Zillig et al. (1970) have now published a much improved purification procedure which avoids this critical DEAE-cellulose step.

3.1.4.

PURIFICATION OF RNA POLYMERASE BY THE BURGESS PROCEDURE

3.1.4.1.

INTRODUCTION

This purification procedure was outlined by Burgess et al. (1969) before publication in detail (Burgess, 1969a). As the purity of most RNA polymerase preparations had not been convincingly demonstrated he designed a new procedure which overcame most of the shortcomings of the other methods. It achieves an improved yield and purity of the enzyme avoiding the critical and unreliable steps of other procedures. The amount of high-speed centrifugation of the enzyme was greatly reduced and treatment of the crude extract with DNase at an early stage in the enzyme preparation disrupts the DNA-RNA polymerase complex avoiding streptomycin and protamine sulphate fractionation and also reduces the viscosity of the crude extract. The addition of 5% (v/v) glycerol to every buffer employed in the purification improves the stability of the enzyme. We have found that the Burgess RNA polymerase purification pro-

cedure is so reproducible that few enzyme assays are required to monitor the purification. Complete purification of RNA polymerase from *E. coli* MRE 600 can be achieved in under four days.

3.1.4.2.

RNA POLYMERASE PREPARATION

The procedure was partly modified from that of Burgess (1969a) and uses agarose gel column chromatography in place of glycerol gradient centrifugation. The object of the purification scheme was to prepare RNA polymerase holoenzyme.

Homogenisation and DNase Treatment

Homogenisation of the *E. coli* was performed as described in section 2.3. During RNA synthesis RNA polymerase is part of a DNA-RNA-RNA polymerase complex. If the rapidly sedimenting complex remained intact after cell disintegration and was pelleted during the removal of ribosomes by high-speed centrifugation then some loss of enzyme might be expected to occur. Treatment of the cell homogenate with DNase results in DNA degradation in the complex thus preventing early enzyme losses. DNase solutions were freshly prepared for each preparation of the enzyme as they were found to rapidly lose their activity at pH 7.5. The amount of DNase added to the crude extract was 2.5mg per crude extract of 200g of *E. coli*. This is 25% greater than that used by Burgess (1969a) but was found to be required for DNA degradation in *E. coli* MRE 600 homogenates. Most of the oligonucleotides formed by the DNase treatment are soluble in the ammonium sulphate solutions and are removed during the ammonium sulphate fractionation step. The addition of large amounts of DNase to the crude

extract can lead to the inactivation of RNA polymerase due to fragments of RNA, produced by RNase 1, binding to the now free enzyme (Alberts et al., 1968). Fortunately E. coli MRE 600 is an RNase 1-free strain and this complication does not occur. DNase treatment also decreases the viscosity of the crude extract (figure 3.2) and makes the removal of the glass beads much easier. The presence of 0.01M-MgCl₂ in the homogenising buffer maintains the ribosomes mainly in the 70S form and facilitates their removal by the high-speed centrifugation step.

High-speed Centrifugation

In order to obtain a packed pellet of ribosomes and cellular debris it was necessary to centrifuge for 4h at 78,000g, two hours longer than suggested in the published purification procedure (Burgess, 1969a). The supernate is fraction 2 enzyme.

Ammonium Sulphate Fractionation

This leads to very little loss of enzyme if properly carried out, care must be taken to solidly pack the protein pellet at each stage in the fractionation. This yields fraction 3 RNA polymerase.

DEAE-cellulose chromatography

A column with a small length to diameter ratio was used to give the maximum flow rate. Fraction 3 enzyme was pumped onto the column overnight (10h) at a slow rate (50ml/h) to ensure total binding of the RNA polymerase to the DEAE-cellulose. The column turned a brownish colour throughout its entire length. During the elution of the protein peaks the top of the column was stirred to reduce the tailing of the peaks and to maintain a fast flow rate (90ml/h).

Agarose gel filtration chromatography

RNA polymerase is known to exist as a monomer in high ionic strength buffer and as a dimer in low ionic strength buffer (Richardson, 1966a). Agarose gel filtration, rather than glycerol gradient ultracentrifugation, was used as the preferred purification steps as much larger quantities of material could be handled.

(1) Biogel A5M (low salt buffer). A small peak of high molecular weight RNA comes through in the void volume then RNA polymerase elutes from the column well ahead of the bulk of the contaminating lower molecular weight protein. The approximate molecular weight of RNA polymerase dimer can be determined by its elution volume from the agarose column and this was found to be $880,000 \pm 130,000$ daltons. RNA polymerase is now at least 80% pure as judged by SDS-polyacrylamide gel electrophoresis. In some preparations of RNA polymerase the Biogel A5M column was replaced by a similar column of Sepharose 6B, but this led to poorer resolution of the RNA polymerase peak from the contaminating protein peak, leading to less pure fraction 5 RNA polymerase.

(2) Biogel A1.5M (high salt buffer). The ionic strength of the buffer in this agarose column was 1.0. Any high molecular protein contaminant of RNA polymerase was removed by means of this agarose column. RNA polymerase elutes from the column as a monomer with an approximate molecular weight, as determined from its elution volume, of $440,000 \pm 60,000$ daltons. This is fraction 6 RNA polymerase and is usually at least 95% pure (see section 3.2), has an A_{280}/A_{260} of 1.6 and a specific activity of 600 using calf thymus DNA as template in the enzymatic assay (specific activities of up to 1,000 have been obtained from some

enzyme preparations). Table 3.2 is a purification table compiled from several RNA polymerase purifications. Table 3.3 shows the specific activity of a typical RNA polymerase preparation on different DNA templates. This demonstrates the presence of σ subunit in the RNA polymerase holoenzyme preparation.

3.1.5.

STABILITY OF RNA POLYMERASE

Some preparations of E. coli MRE 600 have been stored for up to 18 months at -10°C with a 30% loss of activity as measured with calf thymus DNA template. Stored samples of enzyme sometimes formed crystals in their glass storage bottles but when assayed these had no significant RNA polymerase activity.

TABLE 3.2. SUMMARY OF RNA POLYMERASE PURIFICATION BY THE BURGESS PROCEDURE

Fraction	Stage	Total Protein (mg)	Total Enzyme Units	Specific activity	A ₂₈₀ : A ₂₆₀
1	Homogenate	26,000	-	-	-
2	Crude extract	12,000	30,000	2.5	-
3	Ammonium sulphate precipitate	2,200	28,000	13	-
4	DEAE-cellulose	430	30,000	70	1.0
5	Biogel A1.5M	40	20,000	500	1.30
6	Biogel A 5M	32	19,000	600	1.62

This purification table was compiled from sixteen preparations of RNA polymerase by this procedure.

TABLE 3.3. SPECIFIC ACTIVITIES OF RNA POLYMERASE ON DIFFERENT DNA TEMPLATES

DNA	calf thymus	T7	E. coli	Ascites
specific activity	600 (1015)	1294 (2691)	196	296

The RNA polymerase was assayed on the different DNA templates in the standard radioactive assay described in section 2.2.3. DNA was in excess in all the reaction mixtures. The concentration of protein was measured by the method of Lowry et al. (1951). The figures in parentheses are those of a preparation with the highest specific activity obtained in this work.

3.2.

PURITY OF RNA POLYMERASE

3.2.1.

ENZYMIC PURITY

The criteria of purity for RNA polymerase have already been described in section 1. The specific activity of RNA polymerase, measured with calf thymus DNA as template, isolated from E. coli MRE 600 by the Burgess procedure, is comparable to that of published highly purified enzyme preparations (Zillig et al., 1966; Burgess, 1969a). The specific activity determined with T7 bacteriophage DNA as template is more than twice that with calf thymus DNA as template indicating that there is sigma subunit present in the enzyme (table 3.3). RNA polymerase prepared by all other published procedures (see Richardson, 1969) has been shown to be essentially free of both RNase and DNase activity. Martelo et al. (1970) have suggested that the specific activity of RNA polymerase may be affected by the degree of phosphorylation of one of its subunits. An assay was performed to test for the presence of alkaline phosphatase in the RNA polymerase preparation.

Ribonuclease - The RNA solubilisation assay for exonuclease and large amounts of endonuclease was performed by the method of Zillig et al. (1966) using fraction 6 RNA polymerase. After a 10min RNA synthesis period the reaction was stopped by the addition of actinomycin D (an RNA polymerase inhibitor) and the fate of the synthesised radioactive RNA was followed by precipitation with TCA after various times of further incubation. There was no detectable RNase activity (figure 3.3).

Deoxyribonuclease - The conditions of the assay for endonuclease activity were those of Lehman, Roussos and Pratt (1962). The assay was performed using a low-shear Zimm viscometer. The relative viscosity of a λ bacteriophage DNA solution was measured at various time intervals after the addition of fraction 6 RNA polymerase. As can be seen from figure 3.4 there was no detectable DNase activity. This shows that not only the DNase added in the first stage of the enzyme purification has been effectively removed but also any indigenous DNase.

Alkaline phosphatase - This assay was performed as described by Richardson (1966a). No detectable alkaline phosphatase activity was present (figure 3.5).

3.2.2.

PHYSICAL PURITY OF RNA POLYMERASE

The physical purity of an enzyme is generally assessed by its sedimentation and electrophoretic homogeneity.

3.2.2.1.

SEDIMENTATION HOMOGENEITY

Like many other proteins, RNA polymerase has a tendency to aggregate and can exist as either a monomer or dimer. This aggregation is strongly dependent on the ionic strength of the solution. A very pure preparation of RNA polymerase, as judged by only one peak in a Schlieren photograph obtained by sedimentation velocity analytical ultracentrifugation is at least 95% homogeneous (Richardson, 1969). Fraction 6 RNA polymerase purified by both the Zillig and Burgess procedures

(sections 2.2.2 and 2.2.3) was essentially homogeneous as can be seen from an analysis of their sedimentation velocity patterns (figures 3.6 and 3.7) at both high and low ionic strengths which both show only one sedimenting species present.

3.2.2.2.

ELECTROPHORETIC HOMOGENEITY

3.2.2.2.1.

QUALITATIVE ELECTROPHORESIS

RNA polymerase was first analysed by subjecting it to electrophoresis in the standard pH 8.7 polyacrylamide gels in the absence of any dissociating agents by the general method of Ornstein (1964) and Davis (1964) as outlined by Brewer and Ashworth (1969). The analysis of several RNA polymerase preparations showed only one major slow-moving protein species and two smaller faster-moving species of unknown composition (figure 3.8). The low ionic strength ($\mu = 0.01$) and high pH (pH = 9.5) conditions in the Ornstein and Davis polyacrylamide gel analytical procedure are known to lead to aggregation and denaturation of RNA polymerase with partial breakdown of the native enzyme into subunits likely to occur (Eurgess, 1969a). Analysis of the electrophoretic purity of the enzyme was therefore performed in the presence of known dissociating agents (SDS, urea). Now that the subunit structure of RNA polymerase is known, this would appear to be the best method of displaying enzyme purity.

The two other polyacrylamide gel procedures used to analyse RNA polymerase preparations were those of Jovin, Chrambach and Naughton

(1964) where 8M-urea is the dissociating agent and that of Shapiro, Vinuela and Maizel (1967) where 1% (w/v) SDS is used to dissociate RNA polymerase into its various subunits. Electrophoresis in polyacrylamide gels in the presence of the anionic detergent SDS has proved to be a very useful tool in separation and identification of polypeptide chains.

Shapiro et al. (1967) have shown that the separation of proteins on SDS-polyacrylamide gels is dependent on the molecular weights of their polypeptide chains and the technique has now been widely applied to the rapid and simple estimation of molecular weights. The validity of this method of determining molecular weights has been further verified by Weber and Osborn (1969). Although electrophoresis on acrylamide gels in the presence of SDS has now been widely acclaimed as a convenient and accurate method for determining molecular weights of polypeptide chains, Tung and Knight (1971) have reported that substantial errors could result in interpreting the molecular weight of unknown proteins as they find that charge effects, normally assumed to be swamped out by the protein-bound SDS anions so that proteins migrate in gels almost entirely according to their molecular size, may not be entirely eliminated by the SDS.

SDS-polyacrylamide gel electrophoresis has been applied by several authors (table 1.4) to the subunit structure of RNA polymerase purified from several different strains of *E. coli* as well as from other bacteria and estimates of the molecular weights of the component polypeptide chains have been obtained.

The technique for the analysis of *E. coli* MRE 600 RNA polymerase was performed as described in section 2.2.6.3. Figure 3.9 shows an analysis of fraction 6 RNA polymerase (holoenzyme) and core enzyme by electrophoresis on 5% polyacrylamide gels in the presence of SDS. Figure 3.10 shows both forms of the active enzyme after dissociation of the subunits in 8M-urea and electrophoresis as described in section 2.2.6.2. The subunit polypeptide chains β' and β (only clearly resolved by prolonged electrophoresis), σ and α , first described by Burgess et al. (1969) are present. As well as these four subunits, there is present another major subunit which we have designated θ , the molecular weight of this protein species (58,000) lies between that of σ and α . In all preparations of *E. coli* MRE 600 RNA polymerase in our laboratory, θ is present in at least the same concentration if not sometimes greater, than that of σ as determined by the intensity of staining with both amido black and coomassie blue dyes. Two other protein species of mobility greater than that of α can also be detected, one of these is probably ω (Burgess et al., 1969). In all preparations of core enzyme, only the β' , β and α subunits are present. The trace impurities found in some preparations of RNA polymerase include τ (Burgess et al., 1969). Several polypeptide species with an electrophoretic mobility less than that of β' and β were sometimes present in holoenzyme preparations, the molecular weight of such species was around 200,000 daltons and were probably incompletely dissociated enzyme subunits.

It would appear therefore from electrophoretic analysis that RNA polymerase both the core and the holoenzyme are at least 95% pure, assuming that θ subunit is a true subunit or component of a subunit of the native enzyme. The relationship of θ to the native enzyme was investigated (see section 3.3).

3.2.2.2.2.

MOLECULAR WEIGHT OF RNA POLYMERASE SUBUNITS

The molecular weight of each of the subunits of RNA polymerase prepared from *E. coli* MRE 600 was determined by the procedure of Shapiro et al. (1967). A standard curve was prepared with proteins of known molecular weight (table 3.4) using both 5% and 10% polyacrylamide gels. The mobility of each protein was plotted against the logarithm of the molecular weight and for both 5% and 10% polyacrylamide gels straight lines were obtained. For the 5% polyacrylamide gels the relationship of the logarithm of the molecular weight to mobility was a linear one over the wavelength range 23,000 to 147,000 daltons (figure 3.11), this line satisfied the equation

$$\text{Molecular weight} = 3.10^5 \cdot (10^{-1.115 \text{ Mobility}})$$

or $\log_{10}(\text{Mol. wt}) = 5.48 - 1.115 (\text{Mobility}).$

This is almost identical to the line obtained by Burgess (1969a) and is also very similar to that of Shapiro et al. (1967). The standard curve obtained with the 10% polyacrylamide gels was linear over the wavelength range 13,000 to 68,000 daltons and satisfied the equation

TABLE 3.4.

MOLECULAR WEIGHTS OF STANDARD
PROTEINS

PROTEIN	MOLECULAR WEIGHT	REFERENCE
γ -globulin (sheep)	147,000	All molecular weights were taken from Weber and Osborn (1969).
bovine serum albumin	68,000	
γ -globulin (H-chain)	50,000	
ovalbumin	43,000	
pepsin	35,000	
γ -globulin (L-chain)	23,500	
trypsin	23,300	
Lysozyme	14,300	

$$\log_{10}(\text{Mol. wt.}) = 2.18 - 0.95(\text{Mobility})$$

The molecular weights of the E. coli MRE 600 RNA polymerase subunits are shown in table 3.5 and are similar, in general, to those published by other authors (table 1.). The θ component which is found in our preparations of RNA polymerase has a molecular weight of $58,000 \pm 1,000$. σ subunit has a molecular weight of $78,000 \pm 1,200$ daltons, this is more than 10% lighter than that originally obtained by Burgess et al. (1969) for the E. coli K12 σ subunit (95,000), but is approximately the same as that obtained by Seifert et al. (1969) (80,000). Of all the molecular weights of E. coli RNA polymerase subunits (tables 1.4 and 3.5) it can be seen that the greatest reported variation in the molecular weight of any of the subunits is in that of the σ subunit, and since the SDS-polyacrylamide gel electrophoresis technique for obtaining molecular weights is fairly reproducible, this would appear to suggest that σ subunits vary, in at least their molecular weights, in the different strains of E. coli. The greatest error in the estimation of the molecular weight occurs with the β and β' subunits due to the fact that very few pure high molecular weight markers are available for the protein standard curve, and also that the scale is a logarithmic one, nevertheless the average molecular weight of the combined $\beta' + \beta$ band is very similar to that published.

3.2.2.2.3.

SUBUNIT COMPOSITION OF RNA POLYMERASE

It has been shown for coomassie brilliant blue by Fazekas de St. Groth, Webster and Datyner (1963) that the amount of dye bound to a protein is

proportional to the amount of protein present with a variation of less than 10% among the different proteins tested. A similar result is shown in figure 3.12, where the area under the peak on a densitometer trace of the polyacrylamide gel is proportional to the amount of protein applied to the gel. However preliminary experiments in our laboratory with widely different types of protein have been performed and suggest that different proteins have quite different staining intensities with both amido black and coomassie blue.

Making the assumption that the intensity of staining with either amido black or coomassie blue is proportional to the amount of protein present in each band of E. coli RNA polymerase subunits in the polyacrylamide gels, the core enzyme appears to have the subunit composition $\beta'\beta\alpha_2$ (Burgess et al., 1969). The holoenzyme probably consists of two species as purified from E. coli MRE 600, one with the subunit composition $\beta'\beta\alpha_2\sigma$ and the other with the subunit composition $\beta'\beta\alpha_2\theta(v)$. The proportion of $\beta'\beta\alpha_2\theta(v)$ is probably slightly greater than that of $\beta'\beta\alpha_2\sigma$.

TABLE 3.5. MOLECULAR WEIGHTS OF THE SUBUNITS OF E. COLI MRE 600 DNA-DEPENDENT RNA POLYMERASE DETERMINED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SUBUNIT	$\beta' + \beta$ (mean)	σ	θ	α	ν	ω
MOLECULAR WEIGHT	145,000 \pm 7,000	78,000 \pm 1200	58,000 \pm 1,000	37,000 \pm 1000	17,000 \pm 700	-

These are the mean of five preparations of RNA polymerase stained with either amido black or coomassie blue.

3.3.

PROPERTIES OF E. COLI MRE 600 RNA POLYMERASE

3.3.1.

INTRODUCTION

The subunit structure of E. coli MRE 600 RNA polymerase is similar to that reported by other workers for other E. coli RNA polymerases (section 3.2.2.2) except for the presence of the θ component of molecular weight 58,000. RNA polymerase purified from E. coli MRE 600 by Martelo et al. (1970) may also show this component (SDS-polyacrylamide gels not published). SDS-polyacrylamide gel electrophoresis of RNA polymerase purified from E. coli A19, also an RNase 1⁻ strain of E. coli like MRE 600, shows a protein subunit of similar molecular weight to θ (Hirschbein et al., 1969). Chelala et al. (1971) have also reported the presence of another subunit when they purify RNA polymerase by the Babinet (1967) procedure from E. coli A19. Zillig et al. (1970a) have detected a similar protein which they term μ of molecular weight 60,000 when the σ component of RNA polymerase is subject to denaturation in 6M-urea, pH 11, and detected by polyacrylamide gel electrophoresis, thus μ appears to be a breakdown product of σ . A small protein of molecular weight 15,000 (ν) was also detected on the gels and Zillig et al. (1970a) have suggested that σ is a protein with the subunit composition $\mu\nu_2$.

An investigation into the chromatographic properties of E. coli MRE 600 RNA polymerase was carried out in an attempt to elucidate the relationship of θ to core enzyme and σ and if possible to elucidate the role, if any, of θ in the transcription process.

3.3.2.

DENATURATION STUDIES

The observation by Zillig et al. (1970a) that σ can be partially dissociated or broken down into two protein species of molecular weights 60,000 and 15,000 suggested the possibility that θ was a breakdown product of the E. coli MRE 600 σ subunit. Similar degradative studies were performed on RNA polymerase holoenzyme and on peak B protein ($\alpha\beta\sigma$) from the stepwise elution of RNA polymerase from the phosphocellulose column (see section 3.3.3).

Several RNA polymerase and $\alpha\beta\sigma$ samples were dialysed for 24h at 20°C against denaturing solutions - 8M-urea, 6M-guanidium chloride (see also section 3.8) and 0.01M-NaOH. Although the RNA polymerase sample in the 8M-urea could be electrophoresed directly without prior suspension in the electrophoresis buffer, all the samples were further dialysed against 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.1M-sodium phosphate buffer, pH 7.1, for 16h, with several buffer changes, before electrophoresis on 10% polyacrylamide gels as described in section 2.2.6.3. A sample of the same preparation of RNA polymerase which had received no prior pre-treatment before dialysis against the dialysis buffer was used as a control. The results (figure 3.13) show that no further breakdown or dissociation of σ after denaturation with either urea, guanidinium chloride or alkali solutions has occurred.

There is the observation by Martelo et al. (1970) that the degree of phosphorylation of σ may play an important role in controlling the activity of RNA polymerase. This could be done if σ (active) was able

to be broken down to θ (inactive) by a phosphatase. To test this hypothesis both RNA polymerase holoenzyme and peak B material from the stepwise elution of the holoenzyme from a phosphocellulose column (section 3.3.3) were incubated with alkaline phosphatase at 37°C for 30min before dialysis against the usual dialysis buffer (section 2.2.6.3) followed by SDS-polyacrylamide gel electrophoresis. The results were similar to those obtained above - no breakdown or dissociation of σ to θ had occurred.

From the above results it would appear that the σ subunit of RNA polymerase from *E. coli* MRE 600 is unable to be further dissociated by common denaturing treatments or a phosphatase enzyme into smaller subunits.

3.3.3.

PHOSPHOCELLULOSE CHROMATOGRAPHY

Burgess et al. (1969) first reported that *E. coli* RNA polymerase could be separated into two main components by chromatography on a column of phosphocellulose. One component is the stimulating factor (σ) and the other is the core enzyme ($\beta'\beta\alpha_2$). Phosphocellulose chromatography has also been used to remove σ factor from *B. subtilis*, *A. vinelandii* and *Ps. putida* RNA polymerases (Avila et al., 1970; Krakow et al., 1969; Johnson et al., 1971).

Chromatography of purified *E. coli* MRE 600 RNA polymerase (fraction 6 enzyme) on a column of Whatman PC11 phosphocellulose was performed as described in section 2.2.4.1., initially by the gradient elution method of Burgess et al. (1969) and latterly by the stepwise elution procedure

of Bautz et al. (1970).

Gradient elution of the phosphocellulose column to which RNA polymerase had been applied resulted in only two protein peaks (figure 3.14), not three peaks as found by Burgess et al., (1969). Peak A was the flowthrough material (buffer C + 0.05M-KCl) which did not bind to the phosphocellulose. This contained the stimulating activity (σ) and also some core enzyme as can be seen from table 3.6. Peak B, a very dilute diffuse peak, eluted over a wide range of KCl concentration (0.15M to 0.35M-KCl in buffer C) with a maximum at 0.25M-KCl. This appeared to contain only core enzyme as judged by the specific activity of the peak on both calf thymus and T7 DNAs (table 3.6). SDS-polyacrylamide gel analysis of the two peaks showed that peak A contained mainly σ and θ with trace β' , β and α whilst peak B contained only the β' , β and α subunits (figure 3.16). Similar results have been obtained by Hirschbein et al. (1969) using E. coli A19 RNA polymerase. They too obtained only two protein peaks on gradient elution from phosphocellulose and the SDS-polyacrylamide gel patterns obtained are similar to those in figure 3.16.

Stepwise elution of the phosphocellulose column to which fraction 6 RNA polymerase holoenzyme had been applied was found to be a more reproducible procedure and gave an elution profile of three protein peaks (figure 3.15). Peak A was again the flow-through material (0.05M-KCl in buffer C), peak B eluted at 0.05M to 0.25M-KCl in buffer C, and peak C was the 0.25M to 0.40M-KCl in buffer C material. Peaks A and B both contained stimulating protein when added to peak C. Peak A was active on both T7 and calf thymus DNA as template, peak B showed some activity on calf thymus and T7 DNAs (table 3.7).

TABLE 3.6. SPECIFIC ACTIVITIES OF PROTEIN PEAKS
FROM GRADIENT ELUTION OF RNA POLYMERASE
FROM PHOSPHOCELLULOSE

Template DNA	specific activity		
	A	B	B+A
calf thymus	62	600	-
T7	124	0	286

The assay mixture was that described in section 2.2.3. DNA was added in excess (50 μ g). 20 μ g RNA polymerase was added to each assay. 10 μ g of peak A material was added to peak B and stimulation of RNA synthesis on T7 DNA was obtained. Adding more of peak A protein to peak B (core enzyme) increases the specific activity of peak B protein on T7 DNA relative to calf thymus DNA.

Fractions from each of the peaks were analysed by SDS-polyacrylamide gel electrophoresis to determine the subunit species present in each peak (figure 3.16). Peak A contained mainly σ and θ (the ratios of these two species present could vary depending on several factors which are discussed below) as well as trace β' , β and α ; peak B contained α , β and σ , with σ in excess stoichiometrically as judged by the intensity of the staining of the protein species in the gel with amido black dye; peak C was the core enzyme $\beta'\beta\alpha_2$. These results are similar to those obtained by Bautz et al. (1970) with E. coli B RNA polymerase except that in peak A only σ subunit was present. Their peak B (0.05 to 0.25M-KCl) also showed the $\alpha\beta\sigma$ species.

The amount of sample applied to the phosphocellulose column and also the rate of application of the RNA polymerase affected the subsequent protein elution profile by varying the amount of protein in each of the peaks and also the relative proportions of the subunits in peak A. Burgess et al. (1969) suggest that the RNA polymerase sample be applied to the column at about one column volume per hour to allow the enzyme time to bind to the phosphocellulose. In practice it was found that a much slower application rate was required, about one half a column volume per hour was satisfactory. If the sample of RNA polymerase is pumped onto the phosphocellulose column too rapidly, much more core enzyme appears in peak A, the flow-through material, with subsequent diminution of peak C, i.e. the yield of pure core enzyme is decreased.

The application to the column of a relatively small amount of RNA polymerase protein relative to the amount of phosphocellulose (1mg RNA polymerase to 10ml packed volume of phosphocellulose, compared with

TABLE 3.7. SPECIFIC ACTIVITIES OF PROTEIN PEAKS
FROM STEPWISE ELUTION OF RNA POLYMERASE
FROM PHOSPHOCELLULOSE

template DNA	specific activity				
	A	B	C	C+A	B+C
calf thymus	402	125	512	-	-
T7	128	-	300	1137 (1)	988 (2)

All assay mixtures contained 30 μ g of protein. Both peaks A and B contain stimulating factor (σ). Protein concentration was measured by the method of Lowry et al. (1951).

(1) 18 μ g of peak A added to 30 μ g of peak C

(2) 38 μ g of peak B added to 30 μ g of peak C

10mg/10ml phosphocellulose as was usually used leads to peak A containing a much higher proportion of θ to σ , with most of σ eluting in peak B along with α and β subunits.

As judged by SDS-polyacrylamide gel electrophoresis, peak A, as well as containing θ , σ and trace core enzyme also contains all the trace impurities, if any, present in any particular enzyme preparation; peak B and peak C contain only RNA polymerase subunits i.e. they are probably at least 95% pure.

Phosphocellulose chromatography was unable to completely separate θ subunit from RNA polymerase holoenzyme. The stepwise elution procedure of Bautz et al. (1970) was used to prepare the core enzyme for the physical studies on RNA polymerase core enzyme.

3.3.4.

HYDROXYAPATITE CHROMATOGRAPHY

Pettijohn and Kamiya (1967) first reported that purified E. coli MRE 600 RNA polymerase could be separated into two peaks on a column of hydroxyapatite. Although both peaks had some RNA polymerase activity there were slight differences in their other properties. The subunit composition of the enzyme in each of the peaks was unknown. Both Richardson (1966b) and Nicholson (1971) have used hydroxyapatite as a final purification step in the preparation of E. coli RNA polymerase.

A column of hydroxyapatite was prepared as described in section 2.2.4.3. Fraction 6 RNA polymerase holoenzyme was applied to the column in 0.04M-sodium phosphate, pH 7.0, containing 5% (v/v) glycerol and 1mM-dithiothreitol. The enzyme protein was eluted from the column with a

0.04M to 0.40M-sodium phosphate, pH 7.0, gradient. Two main protein peaks were obtained (figure 3.17). The first peak, HA-1, eluted at 0.05M-sodium phosphate, and the second peak HA-2, eluted at 0.11M-sodium phosphate. The protein in each peak was precipitated by the addition of solid ammonium sulphate to give 60% ammonium sulphate saturation. The precipitate was collected by centrifugation and suspended and stored in buffers at -10°C . Both peaks were analysed by SDS-polyacrylamide gel electrophoresis. HA-1 contained θ and σ subunits with trace core enzyme present whilst HA-2 was mainly the core enzyme ($\beta'\beta\alpha_2$) with trace θ and σ subunits present (figure 3.18). Therefore hydroxyapatite may also be used as an alternative procedure to phosphocellulose in the preparation of RNA polymerase core enzyme. The results obtained appear to be almost identical to those of gradient elution of RNA polymerase from a phosphocellulose column (section 3.3.3).

Hydroxyapatite chromatography was unable to separate the θ component from σ subunit of RNA polymerase.

3.3.5.

ANALYTICAL DEAE-CELLULOSE CHROMATOGRAPHY

Abraham (1970) has reported that RNA polymerase from *E. coli* MRE 600 can be separated into two distinct species by stepwise elution from a column of DEAE-cellulose, one of the species being inactive with T7 DNA as template. He finds that RNA polymerase lacking σ elutes from the column with a buffer containing 0.16M-KCl, whilst enzyme molecules containing σ are released from the column by a buffer containing 0.22M-KCl. This

suggested that RNA polymerase with $\theta(\beta'\beta\alpha_2\theta)$ could perhaps be separated from RNA polymerase with $\sigma(\beta'\beta\alpha_2\sigma)$.

Analytical DEAE-cellulose chromatography was performed as described in section 2.2.4.2. RNA polymerase in buffer A was applied to the column which was then eluted with buffer A + 0.16M-KCl and buffer A + 0.22M-KCl. Two protein peaks were obtained (figure 3.19) and representative tubes of each were analysed by SDS-polyacrylamide gel electrophoresis (figure 3.20). Peak A contained the core enzyme with θ and σ , peak B contained only β', β, α and θ subunits. Peak B protein had a very low activity using T7 DNA as template (table 3.8) compared to calf thymus DNA as template. This result shows that θ does not have any stimulating activity like σ with respect to its ability to initiate RNA synthesis on T7 DNA. It was decided to investigate the properties of the peak B material ($\beta'\beta\alpha_2\theta$) further.

If θ was a sigma-like subunit perhaps the presence of a different divalent cation in the enzyme assay would have some affect on its property to synthesise RNA on a T7 DNA template Peak B material from the analytical DEAE-cellulose column was assayed with either Mg^{2+} or Mn^{2+} on E. coli, calf thymus and T7 DNA templates in the standard RNA polymerase assay (section 2.2.3). The manganese concentration in the RNA polymerase assay was that of Maitra and Hurwitz (1967). The results are shown in table 3.8. It can be seen that having either Mg^{2+} or Mn^{2+} in the assay leads to no significant differences in the activity of the enzyme on the different DNA templates. The peak B

TABLE 3.8. PROPERTIES OF PEAK B PROTEIN FROM ANALYTICAL
DEAE-CELLULOSE CHROMATOGRAPHY OF RNA POLYMERASE

Divalent cation in RNA polymerase assay	Cts/min incorporated into RNA		
	E. coli DNA	calf thymus DNA	T7 DNA
Mg ²⁺	863	8431	4997
Mn ²⁺	1248	8165	5312

The standard RNA polymerase assay as described in 2.2.3 was used. The magnesium concentration was 0.01M (Burgess, 1969a), the manganese concentration was 0.001M.

The radioactive assay contained ³H-GTP. 20µg of peak B material was added to each of the reaction mixtures.

enzyme ($\beta'\beta\alpha_2\theta$) was most active on calf thymus DNA and only about 50% as active, compared with E. coli DNA, on the T7 DNA (the activity on T7 DNA was probably due to trace σ present). There was very little activity on E. coli DNA as template.

Some physical studies (analytical ultracentrifugation) were performed on the peak B material ($\beta'\beta\alpha_2\theta$) and anomalous sedimentation results were obtained (see section 3.7).

3.3.6.

THE ROLE OF θ IN TRANSCRIPTION

Sigma subunit of RNA polymerase is known to be released from the core enzyme sometime after the initiation of RNA synthesis (Chamberlin, 1970). This led to an investigation into what was the possible implication of the θ subunit of E. coli MRE 600 RNA polymerase in transcription; was it released like σ by the process of transcription or did it remain bound to the core enzyme.

A standard RNA polymerase assay, four times the usual volume (see section 2.22) was prepared, containing in 2ml - 300 μ g of calf thymus DNA, 150 μ g of fraction 6 RNA polymerase and all four ribonucleoside triphosphates (non-radioactive) in 1.5mM concentration. The control tube contained no ribonucleoside triphosphates. The reaction was started by the addition of the triphosphates. After 5min incubation at 37°C, the reaction was stopped by the addition of 0.1ml of a 5% (w/v) ethidium bromide solution. Both reaction mixture and control were centrifuged at 40,000 rev/min (100,000g) for 1.5h to sediment the RNA-DNA-RNA polymerase complex in the reaction tube and the DNA-RNA polymerase in

the control tube but not the relatively low molecular RNA polymerase subunits which may have been released from RNA polymerase during RNA synthesis. Samples of both supernates and resuspended precipitates were prepared and analysed by SDS-polyacrylamide gel electrophoresis. Although not all the RNA polymerase appeared to bind to the DNA or else there were free dissociated subunits α, β and β' present in the enzyme preparation (this has been observed by Sethi and Zillig (1970)), it can be seen from the results shown in figure 3.21 that both σ and θ are released when there is active RNA synthesis and that their ratios are the same as those found in the particular RNA polymerase preparation which was used for this particular experiment. Thus it would appear that θ is released as well as σ by the process of transcription suggesting that its physical properties may well be similar to σ and that it could be related to σ .

3.4.

A FACTOR INHIBITING RNA SYNTHESIS

3.4.1.

INTRODUCTION

With one of the current interests in *E. coli* RNA polymerase being centred on the effect of various protein factors on the activity of the enzyme and on the selection of its transcripts, it was decided to investigate whether any protein factors which could influence the activity of *E. coli* MRE 600 RNA polymerase were being lost during the purification of the enzyme. This investigation was also performed in an attempt to perhaps identify protein factor which could have a bearing on the θ enigma of *E. coli* MRE 600 RNA polymerase.

For instance it is known that the purification procedure for Q β replicase (Kamen, 1970) parallels the published Burgess (1969a) RNA polymerase purification procedure and that two components of Q β replicase are factors which are known to influence the synthesis of RNA by *E. coli* RNA polymerase (Travers et al., 1970). As all steps in the purification of RNA polymerase by the procedure described in section 2.2.3. are batchwise e.g. ammonium sulphate fractionation and stepwise elution from DEAE-cellulose, and not selective until the first agarose gel filtration step (Biogel A5M) it is here, where the elution from the Biogel A5M chromatographic column is molecular weight and size dependent, that a search for protein factors which partially copurify with RNA polymerase and may have some effect on its activity, was undertaken. RNA polymerase elutes as a dimer, estimated molecular weight 880,000, from this column and usually the large peak of RNA polymerase lower molecular protein is discarded. One could conceivably expect to find in this large peak (see figure 2.6) several

protein factors known to affect the activity of RNA polymerase e.g. ψ factors (molecular weight 40,000 to 50,000 daltons), ρ factor (200,000) and perhaps free σ factor (80,000) as well as other protein factors as yet unknown.

3.4.2.

ANALYSIS OF BIOGEL A5M COLUMN ELUATE

During the preparation of a batch of RNA polymerase from *E. coli* MRE 600 fractions from the large peak of relatively low molecular weight protein (less than 500,000 daltons) were assayed in the standard radioactive assay (section 2.2.3) along with RNA polymerase holoenzyme in order to detect any stimulating activity. *E. coli* DNA was used as the template DNA for the reason that ψ factors are known to give increased activity of RNA polymerase on *E. coli* DNA templates (Travers et al., 1970). Aliquots of fractions across the peak were added to the assay mixture before the reaction was started by the addition of the ribonucleoside triphosphates. The results can be seen in figures 3.22. The addition of these fractions from the large diffuse non-RNA polymerase protein peak led to the complete inhibition of RNA synthesis by RNA polymerase, peak of the inhibition coinciding with the protein peak. Peak fractions of inhibitory protein, as it was termed, were collected and stored frozen at -10°C for further investigation.

3.4.3.

PROPERTIES OF PROTEIN INHIBITOR

From the method of preparation of the inhibitory protein (ammonium

sulphate precipitation and chromatography on DEAE-cellulose) it seemed likely that the inhibitor was a protein. Consistent with this suggestion is the observation that the inhibitory activity is thermolabile, being totally inactivated by heating to 100°C for one minute, and also has an $A_{280}:A_{260}$ of 1.4 indicating a nucleic acid content of less than 1%. The inhibitory factor elutes from the Biogel A5M column at a position corresponding to a molecular weight, assuming that it is a globular protein, of $320,000 \pm 50,000$ daltons. A sample of the inhibitory protein peak was analysed by SDS-polyacrylamide gel electrophoresis and was found to contain multiple protein species (figure 3.23); one had a molecular weight of approximately 100,000, and there were four other proteins in the molecular weight range 35,000 to 55,000. On this analysis it would appear that the protein inhibitor peak of RNA polymerase is not a pure protein species and the factor is probably only an aggregate of one of the proteins detected.

Preliminary experiments with RNA polymerase assay to which inhibitor protein had been added, using *E. coli*, calf thymus and T7 DNA templates, showed that, although there was an overall depression of RNA synthesis as judged by the incorporation of labelled ribonucleoside triphosphate into RNA, the inhibition was most pronounced with T7 DNA as template (table 3.9). These results obtained with freshly prepared inhibitor protein appeared to suggest that the effect of the inhibition was twofold

- (1) there was a general depression of RNA synthesis dependent on

the amount of impure inhibitor protein added,

(ii) the effect of inhibitor protein was most marked on the initiation of the transcription mechanism as judged by the greater degree of inhibition of RNA synthesis on T7 DNA compared with calf thymus and *E. coli* DNAs as templates.

There is a distinct possibility that this dual effect is due to the presence of more than one protein factor as SDS-polyacrylamide gels showed at least five distinct protein species.

The hypothesis was suggested that perhaps the effect of the factor inhibiting or depressing RNA synthesis and inhibiting initiation was due to the action of a specific or even non-specific enzyme which was responsible for the dissociation or breakdown of σ to θ or even lower molecular weight protein species. To test this hypothesis preliminary experiments were performed by incubating RNA polymerase holoenzyme i.e. with σ , with the inhibitory factor for one hour at 37°C and subjecting the incubation mixture to SDS-polyacrylamide gel electrophoresis. The results showed that under the conditions employed, there was no specific breakdown of σ to θ but that some proteolytic action had occurred and both σ and θ subunits of *E. coli* MRE 600 RNA polymerase appeared to have undergone some proteolytic digestion due to the presence of new trace protein bands as well as the partial disappearance of θ and σ compared to the control experiment. The conclusion which could be reached was that the inhibition fraction had proteolytic activity of some form. The implications of this discovery will be discussed in

TABLE 3.9. ACTIVITY OF RNA POLYMERASE WITH INHIBITOR
 PROTEIN ON DIFFERENT DNA
 TEMPLATES

μmoles GMP incorporated into RNA	DNA Template		
	E. coli	calf thymus	T7
- inhibitor protein (control)	1383	7624	3967
+ inhibitor protein	510	1570	94
% original activity	37	21	2.4

The standard RNA polymerase assay (2.2.3) with H-GTP was used. The reaction mixtures containing inhibitor protein were preincubated for 10min with RNA polymerase (20μg) before the reaction was started by the addition of the ribonucleoside triphosphates. The particular preparation of RNA polymerase (holoenzyme) used in this experiment contained less than the usual amount of o.

section 4.2.

3.4.4.

DEAE-CELLULOSE CHROMATOGRAPHY OF THE PROTEIN INHIBITOR FRACTION

An attempt was made to further purify the large pooled peak of the RNA synthesis inhibition fraction from the Biogel A5M agarose column by chromatography on DEAE-cellulose. The protein was precipitated by the addition of ammonium sulphate to give 67% saturation, collected by centrifugation, suspended in 5ml of buffer A and dialysed overnight (18h) at 4°C. The sample was then applied to a column of DEAE-cellulose equilibrated with buffer A (section 2.2.3). The protein was eluted with a 0 to 0.4M-KCl gradient in buffer A. Only one protein peak was obtained which coincided with the maximum depression of RNA synthesis (figure 3.24). The peak was pooled and stored in buffer S. T7 DNA was used as the template DNA in the inhibition assays and it was found that the almost complete inhibition of initiation was no longer present although the property to depress RNA synthesis remained i.e. the degree of inhibition on both calf thymus and T7 DNA was the same. The properties of the inhibitory protein are now being further investigated in this laboratory.

3.5.

AMINO ACID ANALYSIS OF E. COLI MRE 600 RNA POLYMERASE

3.5.1.

INTRODUCTION

The amino acid composition of RNA polymerase purified from E. coli (Maitra and Hurwitz, 1967), E. coli K-12 (Priess and Zillig, 1967; Burgess, 1969b) and E. coli B (Nicholson, 1971) have been published. Only two of the amino acid analyses were performed on enzyme of defined subunit composition (Burgess, 1969b; Nicholson, 1971). The amino acid composition of the subunits of RNA polymerase has now been determined (Zillig et al., 1970b; Burgess, 1969b).

In the present work the amino acid composition was determined for several reasons -

- (i) to chemically characterise the E. coli MRE 600 RNA polymerase,
- (ii) to obtain a value for the mean residue weight for use in ORD studies of the enzyme,
- (iii) to estimate the helix content of the enzyme by the method of Goldsack (1969),
- (iv) to determine a value for the partial specific volume (\bar{v}) for the calculation of analytical ultracentrifugation equilibrium molecular weight,
- (v) to calculate an extinction coefficient for the enzyme by the method of Beaven and Holiday (1952).

3.5.2.

AMINO ACID ANALYSES

The analysis was performed on both RNA polymerase holoenzyme and on core enzyme purified from *E. coli* MRE 600 by the Burgess procedure (section 2.2.5). The core enzyme was prepared by column chromatography of fraction 6 RNA polymerase on phosphocellulose (section 3.3.3). The preparation of samples and the analyses were performed as described in section 2.2.5. The results of the analyses are shown in Table 3.10. These are an average of three determinations of both 24h and 72h hydrolyses except for serine, threonine, methionine and cysteine whose values were corrected for destruction by a linear extrapolation of the 24h and 72h values. The value for tryptophan, for the core enzyme, was calculated from the ultraviolet spectrum in 6M-guanidinium chloride, 0.02M-sodium phosphate, pH 6.5, by the method of Edelhoch (1967). No value for tryptophan was obtained for the holoenzyme due to the possible presence of nucleic acid ($A_{280}/A_{260} = 1.60$) as a contaminant which would interfere with the spectrophotometric tryptophan determination.

3.5.3.

RESULTS

3.5.3.1.

AMINO ACID COMPOSITION

The amino acid composition of both the holoenzyme and core enzyme are quite similar, the only outstanding differences being in the values for glutamic acid where the holoenzyme has considerably greater and for

cysteine where the holoenzyme has considerably less than the core enzyme. Both protein species have 33% hydrophobic amino acid (alanine, leucine, isoleucine, methionine, phenylalanine, tryosine and tryptophan) residues.

The amino acid composition of *E. coli* MRE 600 RNA polymerase is similar to that reported by Priess and Zillig (1967) for the *E. coli* K12 holoenzyme.

The amino acid composition of *E. coli* MRE 600 core enzyme, shown by SDS-polyacrylamide gel electrophoresis to be at least 95% pure (figure 3.9) is quite significantly different from the *E. coli* K12 and *E. coli* B core enzymes (table 3.11). Comparing MRE 600 core enzyme to K12 core enzyme, the value for histidine is over 20% greater and for glutamic acid it is more than 20% less. Comparing *E. coli* MRE 600 core polymerase with that from *E. coli* B, the differences are even more pronounced with the values for lysine, histidine, proline, valine and isoleucine more than 20% greater, and that for glutamic acid more than 20% less, than that published by Nicholson (1971). From the above results it would appear that the core enzyme isolated from *E. coli* B, K12 and MRE 600 shows a different amino acid composition for each strain as the differences appear to be too great for experimental error.

3.5.3.2.

PARTIAL SPECIFIC VOLUME

The partial specific volume (\bar{v}) of both *E. coli* MRE 600 core enzyme and holoenzyme was calculated from the partial specific volumes of the component amino acids as published by Cohn and Edsall (1943). The values

obtained $\bar{v} = 0.744$ for both the core enzyme and holoenzyme are higher than those determined by other authors (table 3.12).

3.5.3.3.

MEAN RESIDUE WEIGHT

The values for the mean residue weight (MRW) of 112 for the E. coli MRE 600 RNA polymerase core enzyme and 108 for the holoenzyme are similar to those already published whose values range from 109 to 111. (table 3.12)

3.5.3.4.

DETERMINATION OF HELIX CONTENT FROM THE AMINO ACID COMPOSITION

Goldsack (1969) has validated the hypothesis that there is a linear relationship between the amino acid composition of a protein and the b_0 parameter determined from the ORD. The amino acid compositions of over one hundred different proteins have been analysed with respect to their content of helix-forming (alanine, arginine, aspartic acid, cysteine, glutamic acid, leucine, lysine) and non helix-forming amino acids (glycine, phenylalanine, proline, serine threonine, tryptophan, tyrosine), and these have been correlated with the Moffitt-Yang b_0 which is a direct measure of helix content of a protein (Urnes and Doty, 1961). The results obtained from an analysis of E. coli MRE 600 RNA polymerase as well as those from the published amino acid compositions of all the other E. coli are shown in table 3.12.

3.5.3.5.

THE EXTINCTION COEFFICIENT

The extinction coefficient (ϵ) of a protein determined at its absorption

maximum around 280nm is the sum of the extinction coefficients of only three of its constituent amino acids, tyrosine ($\epsilon_{\text{max}} = 1340$ at 274.5nm), tryptophan ($\epsilon_{\text{max}} = 5550$ at 278nm) and cysteine ($\epsilon_{\text{max}} = 150$ at 280nm) (Beaven and Holiday, 1952). Determining the extinction coefficient of a protein using the value for the amount of tryptophan in the protein already determined spectrophotometrically produces a circular argument. Wetlaufer (1962) has examined the extinction coefficients of several proteins and compared them with those calculated from their amino acid compositions. The results show that the calculation has a real value, all the calculated extinction coefficients are within 10% of the observed. The results for E. coli MRE 600 core enzyme and those calculated from the data of Burgess (1969b) and Nicholson (1971) are shown in table 3.12.

TABLE 3.10

AMINO ACID COMPOSITION

OF

E. COLI MRE 600 RNA POLYMERASE

AMINO ACID	MOLE %	
	HOLOENZYME	CORE ENZYME
LYSINE	6.55	6.42
HISTIDINE	1.91	2.26
ARGININE	6.48	6.77
ASPARTIC ACID	9.98	9.41
THREONINE	5.69	5.77
SERINE	4.70	5.61
GLUTAMIC ACID	11.12	9.05
PROLINE	4.33	4.43
GLYCINE	7.57	7.85
ALANINE	8.36	7.30
CYSTEINE	1.02	1.40
VALINE	8.49	9.21
METHIONINE	3.08	2.49
ISOLEUCINE	6.50	7.18
LEUCINE	9.62	10.14
TYROSINE	2.48	2.22
PHENYLALANINE	2.63	2.72
TRYPTOPHAN	-	0.55

TABLE 3.11. COMPARISON OF THE AMINO ACID COMPOSITIONS
OF RNA POLYMERASE CORE ENZYME FROM
DIFFERENT E. COLI.

AMINO ACID	E. coli		
	K 12 (1)	B (2)	MRE 600
LYSINE	5.70	5.4	6.42
HISTIDINE	1.52	1.6	2.26
ARGININE	6.72	5.8	6.77
ASPARTIC ACID	9.67	10.2	9.41
THREONINE	4.91	5.3	5.77
SERINE	5.04	6.7	5.61
GLUTAMIC ACID	13.85	13.6	9.05
PROLINE	4.35	3.4	4.43
GLYCINE	7.65	7.4	7.85
ALANINE	7.38	8.8	7.30
HALF-CYSTINE	0.87	-	1.40
VALINE	8.08	6.7	9.21
METHIONINE	2.29	2.9	2.49
ISOLEUCINE	6.37	5.6	7.18
LEUCINE	10.21	10.9	10.14
TYROSINE	2.35	2.6	2.22
PHENYLALANINE	2.46	2.8	2.72
TRYPTOPHAN	0.55	0.57	0.55

(1) Burgess (1969b)

(2) Nicholson (1971)

3.12 PHYSICAL PARAMETERS OF E. COLI RNA POLYMERASES CALCULATED FROM THE AMINO ACID COMPOSITIONS

		Core enzyme			Holoenzyme		
Reference		(1)	(2)	(3)	(4)	(5)	(3)
E. coli		K12	B	B	MRE 600		B
MFW		110	111	-	112	109	-
\bar{v}		0.738	0.738	0.737	0.744	-	0.744
b_0	helix	-240	-100	-	-45	-240	-160
	non-helix	-270	-250	-	-250	-320	-300
% Helix (5)		38,43	16,40	-	7,40	38,51	25,48
$E_{1\text{cm}}^{1\%}$		5.8	5.41	-	6.2	-	-

- (1) Burgess (1969b) (2) Nicholson (1971) (3) Berg and Chamberlin (1970)
 (4) Priess and Zillig (1967) (5) Maitra and Hurwitz (1967) (6) Urnes and Doty (1961)

3.6.

ULTRA-VIOLET SPECTROSCOPY OF E. COLI MRE 600 RNA POLYMERASE

3.6.1.

INTRODUCTION

The ultra-violet spectrum of RNA polymerase from several different strains of E. coli has been published by several authors (Neuhoff et al., 1969; Anderson and Abraham, 1970; Nicholson, 1971). Only Nicholson (1971) defines the subunit composition of his enzyme preparation (core enzyme). This UV spectrum of RNA polymerase shows an absorption maximum at 278nm and a minimum around 251nm, which are characteristic of most proteins.

One of the criteria of purity of a preparation of RNA polymerase is the ratio of the absorbance at 280nm to that at 260nm. Enzyme preparations with a ratio below 1.65 (Anderson and Abraham, 1970) are assumed to contain some contaminating nucleic acids or their derivatives. All of the A_{280}/A_{260} ratios of RNA polymerase preparations from E. coli published are different and range from 1.25 (Zillig et al., 1966) to 1.9 (Burgess, 1969a).

Richardson (1966a) has determined the specific absorbance of E. coli B RNA polymerase ($A_{280}/A_{260} = 1.8$) by three different methods based on a comparison with bovine serum albumin. By the Biuret and Lowry (1951) procedures, RNA polymerase has a specific absorbance of 0.59/mg/ml at 280nm. Using the synthetic boundary cell in the Spinco model E analytical ultracentrifuge, and assuming that RNA polymerase and bovine serum albumin have identical refraction increments a value of 0.67/mg/ml was obtained for the specific absorbance at 280nm. Richardson (1966a) assumes a

compromise value of 0.65/mg/ml at 280nm for all his physical studies (the uncertainty of this value is about 10%). Since 1966, the value for the specific absorbance of 0.65/mg/ml for RNA polymerase has been generally accepted. Recently (1971), Nicholson has published a value for the specific absorbance of E. coli B core enzyme of 0.541/mg/ml.

Several things are important when determining both the specific absorbance and the A_{280}/A_{260} of RNA polymerase. Firstly, the observation that each strain of E. coli so far studied appears to have a different amino acid composition; as the absorption around 280nm is due to the absorption of only three of the amino acids (tryosine, tryptophan and cysteine) in the protein, their relative concentrations in RNA polymerase will greatly affect the value of the specific absorbance. Secondly, the discovery (Chelala et al 1971) that RNA polymerase can undergo adenylation in the presence of ATP and magnesium must interfere with the determination of both specific absorbance and the A_{280}/A_{260} . Finally the state of aggregation of the enzyme itself is important. RNA polymerase is a very large protein molecule (holoenzyme molecular weight ~480,000 daltons) and can exist as a dimer (960,000 daltons) and even higher aggregates (core polymerase can form a hexamer (Berg and Chamberlin, (1971)) in buffers of low ionic strength. This can lead to a high degree of light scattering which interferes very significantly with both the A_{280}/A_{260} and the true value for the extinction coefficient at 280nm. To avoid these complications the extinction should be measured on millipore filtered protein solutions in buffers of high ionic strength ($\mu = 0.5$) to diminish the contribution of scattering from large aggregates.

3.6.2.

ULTRA-VIOLET SPECTROSCOPY OF RNA POLYMERASE

All the ultra-violet spectra of *E. coli* MRE 600 RNA polymerase (core enzyme and holoenzyme) were measured on a Cary 15 double-beam recording spectrophotometer with automatic slit width control. The calibration of the instrument was routinely checked using a 0.004% (w/v) solution of K_2CrO_4 in 0.05M-NaOH which gives an absorbance of 1.00 at 373nm in a 1cm path length cell (Yang and Samejima, 1963). Most RNA polymerase spectra were recorded at room temperature (20°C). All protein solutions were filtered through 200nm pore size Sartorius membrane filters. The solvent base line was set by using the multipot system and the solvent and sample quartz cells were matched before use.

The ultra-violet spectra of both RNA polymerase holoenzyme and core enzyme (prepared by phosphocellulose chromatography) from *E. coli* MRE 600 are shown in figures 3.25 and 3.26. Both show an absorption maximum at 275.5nm and a minimum at 251nm. In both forms of the enzyme the presence phenylalanine can be detected by the inflexions in the ultra-violet spectra at 269, 265, 259 and 254nm. The difference spectrum between the two forms of the enzyme shows the presence of more exposed tyrosyl and tryptophan residues in the core enzyme.

The specific absorbance of both holoenzyme and core enzyme was measured by the Lowry (1951) procedure based on crystalline bovine serum albumin, $E_{280nm}^{0.1\%} = 0.66$ (Tanford and Roberts, 1952). The values obtained were 0.63 for the core enzyme (this compares favourably with the value (6.2) determined from the amino acid composition, table 3.12) and 0.62 for

the holoenzyme. In all spectrophotometric estimations of RNA polymerase a specific absorbance of 0.65/mg/ml (Richardson, 1966a), within 5% of that determined above, was assumed for all other work involving concentrations of RNA polymerase.

The Warburg and Christian (1941) data relating the amount of nucleic acid contamination of a protein to the A_{280}/A_{260} has been used for many years as a measure of protein purity. Warburg and Christian (1941) based their data on crystalline yeast enolase ($A_{280}/A_{260} = 1.75$) and yeast nucleic acid. Kalckar (1947) has applied the Warburg and Christian data and formulated an equation which allows the concentration of a protein, in the presence of nucleic acid, to be determined from the ultra-violet spectrum.

Protein concentration (mg/ml) = $1.55 A_{280} - 0.76 A_{260}$
(Layne, 1957). Strictly this equation only holds for yeast enolase and yeast nucleic acid but in practice it has been widely applied in the spectrophotometric estimation of protein concentrations.

A revised form of this equation was constructed using an $A_{280}/A_{260} = 1.85$ (the highest ratio obtained for active E. coli MRE 600 core polymerase), a specific absorbance of 0.65/mg/ml, and E. coli DNA, in order to determine more accurately both the protein concentration and nucleic acid contamination of RNA polymerase during purification, the following equation was arrived at

$$\text{protein concentration (mg/ml)} = 2.1348A_{280} - 1.1091A_{260} \quad .$$

This provides an accurate spectrophotometric method of determining RNA

polymerase concentrations. Figure 3.27 expresses graphically the relationship of A_{280}/A_{260} to the per cent (w/w) contamination of RNA polymerase with nucleic acids.

3.7.

ANALYTICAL ULTRACENTRIFUGATION STUDIES

3.7.1.

INTRODUCTION

The basic physical properties of RNA polymerase were the subject of considerable confusion and controversy for several years. The confusion was most evident in the various determinations of the sedimentation coefficient and molecular weight of the enzyme (tables 1.2 and 1.3). A significant advance was made in our understanding of the physical properties of the enzyme was made by Richardson (1966a) who demonstrated that the s -value was highly dependent on ionic strength and that the enzyme could undergo reversible aggregation at low ionic strength. The variables now known to affect sedimentation are ionic strength (Stevens et al., 1966; Richardson 1966a); age of the enzyme (Richardson, 1966a) and subunit composition (Berg and Chamberlin, 1970). The most careful recent study which takes these variables into account and adequately demonstrates the subunit composition of the RNA polymerase studied is that of Berg and Chamberlin (1970). They showed that the holoenzyme and core enzyme differ considerably in their response to ionic strength (μ). The holoenzyme monomer has an $s_{20,w}$ of 14.9S ($\mu > 0.1$) and the dimer has an $s_{20,w}$ of 23S ($\mu < 0.1$). Core enzyme monomer has an s -value of 12.55 ($\mu = 0.26$) but aggregates as the ionic strength decreases to give s -value as high as 48S (hexamer). When a mixture of holoenzyme and core polymerase is studied results intermediate to those above are obtained.

The variables affecting the state of aggregation of the enzyme have obviously caused difficulty in determining the true molecular weight as values for the molecular weight of the enzyme have ranged widely from 360,000 to 480,000 (see table 1.3). Recently, however, the molecular weights of both core and holoenzyme have been determined by several independent methods. Careful sedimentation equilibrium analysis by Berg and Chamberlin (1970) provide a value of 383,000 for the core enzyme. Attempts to measure the molecular weight of the holoenzyme resulted in lower values than expected due in part to partial dissociation into core enzyme and sigma during centrifugation (Berg and Chamberlin, 1970). Values of $400,000 \pm 10\%$ and $430,000 \pm 10\%$ have been obtained for core enzyme and holoenzyme respectively by Burgess (1969b) by computation of the molecular weight from the subunit molecular weight and subunit stoichiometry.

3.7.2.

SEDIMENTATION VELOCITY STUDIES

Originally sedimentation velocity studies were performed with fraction 6 polymerase, purified by the Zillig (1966) procedure (section 2.2.2), suspended in TMA buffer, pH 7.5, ($\mu = 0.05$) in a Spinco Model E equipped with Schlieren optics. The subunit composition of the RNA polymerase was not known. Analytical ultracentrifugation (figure 3.6) shows that the enzyme was essentially homogeneous. In TMA buffer the enzyme exists as dimer. The s-value was found to exhibit a slight dependence on the protein concentration. Extrapolation to zero protein concentration and

correction for the solvent gave an $s_{20,w}$ of 23.3.

Fraction 6 RNA polymerase (holoenzyme) was prepared by the Burgess (1969a) procedure (section 2.2.3) and core enzyme by phosphocellulose chromatography (section 3.3.3). The subunit compositions of both RNA polymerase species were determined by SDS-polyacrylamide gel electrophoresis (figure 3.9). The sedimentation coefficients of the enzyme samples dialysed 12h at 4°C against buffer A + 0.5M-KCl ($\mu = 0.51$), were determined at 20°C using the UV scanner in the Spinco Model E analytical ultracentrifuge as described in section 2.2.8.1. The protein concentrations were 300µg/ml. In all cases, plotting $\log_{10} r$ against t yielded a straight line. Holoenzyme and core enzyme had s -values of 12.74 and 12.89 respectively. Correcting for the solvent (the density of buffer A + 0.5M-KCl was determined from data obtained from International Critical Tables vol. 3, p.87), the $s_{20,w}$ values became 13.6 and 13.7 respectively (table 3.13), assuming no preferential hydration of the protein.

Samples of both RNA polymerase species stored for considerable lengths of time were found to undergo partial breakdown into smaller species. On sedimentation there was often up to 30% non-sedimenting UV absorbing material.

Peak B protein from the analytical DEAE-cellulose chromatography of RNA polymerase holoenzyme (section 3.3.3), shown by SDS-polyacrylamide gel electrophoresis to have the subunit composition $\beta'\beta\alpha_2\theta$ (figure 3.9), was dialysed at 4°C for 12h against both buffer A and buffer A + 0.5M-KCl. The sedimentation coefficients of each of the protein in each of the solutions was determined. The $s_{20,s}$ in buffer A ($\mu = 0.01$) was 16.7S

and in buffer A + 0.5M-KCl ($\mu = 0.51$), 13.55. These results appear to show that θ is interfering with the normal aggregation properties of RNA polymerase, the s-value for the peak B protein in high ionic strength being similar to that of the holoenzyme monomer, but in low ionic strength buffer there was no dimer formation. The fact that the s-value has increased to 16.7 indicates that some association (or dimerisation) may have taken place - this may be due to the presence of trace amounts of σ (detectable by SDS-polyacrylamide gel electrophoresis).

3.7.3.

SEDIMENTATION EQUILIBRIUM STUDIES

Since core polymerase behaves as a single sedimenting species at high ionic strengths, it is possible to determine the molecular weight of this species by low-speed sedimentation equilibrium. Accordingly core polymerase was subjected to equilibrium sedimentation in buffer A + 0.5M-KCl. A graph of $\log c$ versus r^2 obtained with core polymerase was linear over the portion of the cell used for calculations. The value obtained for the weight average molecular weight was 364,000. No correction was made for preferential hydration. The partial specific volume (\bar{v}) used in the calculation was 0.744 (obtained from the amino acid composition, section 3.5). This is over 2% higher than that obtained by other authors (see table 3.12), if a partial specific volume of 0.738 is assumed (Burgess, 1969a; Nicholson, 1971), a weight average molecular weight of 355,000 is the result, Berg and Chamberlin (1970) have observed that approximately 12% of the total material breaks down irreversibly in 8S species. This would be expected to lower the molecular weight by 4%, correcting for this 8S material by the correction factor of Berg and

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Chamberlin (1970) the revised molecular weight becomes 378,000.

The sedimentation equilibrium molecular weight is comparable to that calculated from the subunit molecular weights of the core enzyme ($\beta'\beta\alpha_2$) determined by SDS-polyacrylamide gel electrophoresis which gives a value of $364,000 \pm 16,000$.

RNA polymerase holoenzyme was also subjected to low-speed equilibrium sedimentation in buffer A + 0.5M-KCl where the 13.6S species of the enzyme is known to exist. The weight average molecular weight found was 431,000 (uncorrected for preferential hydration). The molecular weight is lower than that for RNA polymerase holoenzyme published by other authors (Richardson, 1966a; Ruet et al. 1970) (see table 1.3). It is close to that predicted from the subunit molecular weights determined by SDS - polyacrylamide gel electrophoresis, if the E. coli MRE 600 RNA polymerase consists of equal amounts of $\beta'\beta\alpha_2\sigma$ and $\beta'\beta\alpha_2\theta$, the latter species may have ν present which would give it the same molecular weight as $\beta'\beta\alpha_2\sigma$ ($442,000 \pm 18,500$). No account has been taken of the molecular weight of ω (9,000) if present. Berg and Chamberlin (1970) have shown that low values of the sedimentation equilibrium molecular weight are due to partial dissociation of the holoenzyme to give core polymerase and σ component during sedimentation.

One attempt was made to determine the weight average molecular weight of RNA polymerase holoenzyme in buffer A (dimcr). A value of $886,000 \pm 10\%$ was obtained.

TABLE 3.13 SEDIMENTATION CONSTANTS AND MOLECULAR WEIGHTS

OF

E. COLI MRE 600 RNA POLYMERASE

enzyme	μ	$S_{20,w}$	M_w	polyacrylamide gels
core enzyme	0.51	13.7	$364,000 \pm 10\%$	$364,000 \pm 15,000$
holoenzyme	0.51	13.6	$431,000 \pm 10\%$	$442,000 \pm 18,500$
	0.01	23.3	$886,000 \pm 10\%$	-

* Uncorrected for subunit dissociation, if corrected by the value obtained by Berg and Chamberlin (1970) the molecular weight would become 378,000.

3.8.

ORD STUDIES ON E. COLI MRE 600 RNA POLYMERASE

3.8.1.

OPTICAL ROTATORY DISPERSION: INTRODUCTION

Until about fifteen years ago, protein chemists were mostly preoccupied with studying the primary structure and the hydrodynamic properties of protein molecules, but now there is great interest in probing the fine conformational structure of protein molecules. The work of Perutz, Kendrew and co-workers on the X-ray diffraction of haemoglobin and myoglobin represents a milestone in the development of protein chemistry. The three-dimensional structures of many other proteins should be elucidated by this isomorphous replacement method in the coming years. Nevertheless, this powerful technique is only applicable to crystals. To investigate protein conformations in solution, not amenable to X-ray diffraction studies, we must use various other physical methods. Among the current techniques used optical rotatory dispersion (ORD) provides a semi-quantitative estimate of secondary structure, especially of the degree of helical conformation of a protein in solution. ORD is a powerful technique in the study of protein-protein interactions and for the detection of conformational changes.

Numerous excellent reviews on the ORD of polypeptides, proteins and nucleic acids have appeared in the last few years. The literature up to 1961 has been reviewed by Urnes and Doty (1961). Other more recent reviews are those of Fasman (1962), Yang (1967), Adler and Fasman (1968) and Tinoco (1970).

3.8.2.

OPTICAL ROTATORY DISPERSION AND HELIX CONTENT

3.8.2.1.

ORD OF POLYPEPTIDES

The optical rotation of a protein consists of two contributions, one attributable to the amino acid residues and the other arising from certain ordered structural elements. Such a structural element is Pauling's right-handed α -helix. Much of the early work on the conformation of polypeptides in solution was performed on the synthetic polypeptide poly- γ -benzyl-L-glutamate (Yang and Doty, 1957). The ORD of both the helical and randomly coiled conformations were found to be different. Similar results were obtained for poly-L-glutamic acid (Doty, Wada, Yang and Blout, 1957). Thus the α -helix is optically active and makes a significant contribution to the optical rotation of a protein in its native state. Thus the ORD of a protein can provide a powerful means of investigating protein conformations.

With the advent of the recording spectropolarimeters ORD measurements were extended into the UV and the peptide Cotton effects could then be characterised for helical and randomly coiled polypeptides and proteins. The helical form has a trough at around 233nm, a peak at 198nm and a crossover around 224nm; the randomly coiled (disordered) conformation has a trough at 205nm, a peak at 190nm and it may have a small trough around 230nm (Yang, 1967).

3.8.2.2.

PHENOMENOLOGICAL TREATMENT OF EXPERIMENTAL DATA

THE MOFFITT EQUATION: While experimental work on polypeptides and proteins was in progress, Fitts and Kirkwood (1956) and Moffitt (1956) independently developed a theory of optical rotation of the α -helix. Moffitt's equation,

$$[\text{m}'] = \frac{a_o \lambda_o^2}{(\lambda^2 - \lambda_o^2)} + \frac{b_o \lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

was an immediate success and it fitted very well the experimental data then available on polypeptides (Moffitt and Yang, 1956). A transformation of this equation (section 2.2.9.), plotting

$$[\text{m}'] \cdot (\lambda^2 - \lambda_o^2) / \lambda_o^2 \quad \text{against} \quad \lambda_o^2 / (\lambda^2 - \lambda_o^2)$$

with $\lambda_o = 212\text{nm}$ ($\pm 2\text{nm}$) gives a straight line over the wavelength range 300 to 600nm. For poly- γ -benzyl-L-glutamate and poly-L-glutamic acid in several helix promoting solvents, b_o gave an average value of -630 whilst varied widely. It would appear then that $b_o \approx -630$ represents a reference value for a 100% right-handed helix and this value of b_o is currently accepted.

Moffitt's (1956) theoretical treatment neglected several important terms and his equation is now regarded as empirical. Visible ORD is influenced by the Cotton effects of the optically active absorption bands in the UV. Each dichroic band corresponding to a single Cotton effect is reduced to a one-term Drude equation in the region distant from the

absorption band. The helical polypeptide appears to have two Cotton effects one positive and one negative

SCHECHTER-BLOUT EQUATION: In order to account for the near UV and visible ORD, Schechter and Blout (1964), and Schechter, Carver and Blout (1964), proposed a four term Drude equation which is essentially a summation of the dispersions of the α -helix and random coil. The equation can be approximated by a two term Drude equation

$$[\mathbf{m}']_{\lambda} = A_{(\alpha,\rho)193} \frac{\lambda_{193}^2}{\lambda^2 - \lambda_{193}^2} + A_{(\alpha,\rho)225} \frac{\lambda_{225}^2}{\lambda^2 - \lambda_{225}^2}$$

where the subscript α refers to those terms associated with the Cotton effects of the α -helical portion of the molecule and the subscript ρ refers to terms associated with the random coil conformation. The analysis (section 2.2.9) leads to the evaluation of the coefficients $A_{(\alpha,\rho)193}$ and $A_{(\alpha,\rho)225}$ from the straight line resulting from

$$[\mathbf{m}']_{\lambda} \left(\frac{\lambda^2 - \lambda_{193}^2}{\lambda_{193}^2} \right) \quad \text{plotted against} \quad \left(\frac{\lambda^2}{\lambda^2 - \lambda_{225}^2} \right) .$$

3.8.2.3.

ESTIMATION OF HELIX CONTENT

Several ORD methods for estimating helical contents of proteins have been proposed. The b_0 method based on the Moffitt equation is by far the most widely used. The latest additions to the methods of calculating helical content are based on the Schechter-Blout equation (1964) and on the value of the trough at 233nm.

MOFFITT-YANG EQUATION - Most proteins, native and denatured, obey the simple Drude equation. The reason for recasting the ORD data into the Moffitt equation lies in the hypothesis that the polypeptide chain of the protein molecule has only partial helical content and the rest of the molecule is in a less ordered conformation which can be considered as being randomly coiled although the conformation may be held as rigidly as the helical part.

Definition of the reference scales for helical and coiled conformations for the b_o parameter of the Moffitt equation was originally fixed at $b_o = -630$ for the 100% helical conformation and $b_o = 0$ for the 100% randomly coiled conformation. These reference values were based on the studies with synthetic polypeptides. Thus on the basis of a linear relationship between the b_o value and the helical content,

$$\text{fraction of helix} = \frac{-b_o}{-630} .$$

The formula presumes to show the presence of a right-handed helical conformation. b_o values apparently do not distinguish between the α -helix and other helical conformations. The determination of the helix content of proteins from ORD studies has been confirmed by X-ray diffraction data, e.g. both suggest that tropomyosin has a high and ribonuclease a low helical content (Yang, 1967). Recent X-ray studies of lysozyme indicate the presence of about 35% helix which is in agreement with the ORD values. The helical segments in lysozyme are distorted towards the 3_{10} helix and it would appear that ORD is not sensitive enough to discriminate between the 3_{10} and α -helix. The b_o

method apparently cannot detect the presence of the β structure known to be present in lysozyme. The linear model relating helix content of the protein to the value of b_0 is a crude one as Moffitt's theoretical treatment did not take intrinsic residue rotations into consideration, but he predicted that the a_0 term was dependent on composition, solvent and temperature thus accounting for all the background rotations other than the helical backbone.

SCHECHTER-BLOUT EQUATION - On the basis of ORD data of poly-L-glutamic acid in water at pH 4 (helical) and pH 7 (coiled), Schechter and Blout (1964) have proposed that in their modified two term Drude equation, that

$$\% \text{ helix} = \frac{A_{193} + 750}{36.50}$$

$$\text{or} \quad \% \text{ helix} = \frac{-(A_{225} + 60)}{19.90}$$

for their two parameters A_{193} and A_{225} . Combining the two equations leads to the equation of a straight line

$$A_{225} = -0.55 A_{193} - 430 \quad .$$

Schechter and Blout (1964) have proposed that if the two values A_{193} and A_{225} for a protein molecule fall on the straight line only helical and randomly coiled conformations are present. If the values of the % helix content differ markedly, and A_{193} and A_{225} for the particular protein do not lie on the above straight line, structures other than helices and random coils are probably present in the molecule. For

lysozyme helical contents of 37% and 31% were calculated from A_{193} and A_{225} respectively compared with 32% from b_o . A_{193} and A_{225} are not independent of solvent effects but a variation in one of the parameters is compensated by an opposite variation in the other. The two equations which determine the helical content from A_{193} and A_{225} can be combined to give

$$\% \text{ helix} = \frac{A_{193} - A_{225} + 650}{55.8}$$

which is a good approximation of the helical content independent of solvent effects.

It would appear that any two term Drude equation only provides as much information as the Moffitt equation and appears to be in no way superior to it.

233nm TROUGH - The conformation dependent Cotton effects have provided a new method for characterising secondary structure of the protein molecule. The depth of the 233nm trough can be related linearly to the helical content of the protein, but there is an uncertainty in this quantitative aspect due to a lack of agreement in the reference values of $[m']_{\text{helix}}$ at the two wavelengths and similarly with $[m']_{\text{coil}}$. It has been assumed tentatively that

$$[m']_{233} (\text{helix}) \approx -11,900$$

$$\text{and } [m']_{233} (\text{coil}) \approx -2,000 \quad .$$

The helical content can therefore be calculated

$$\% \text{ helix} = \frac{-\left[\alpha\right]_{233} + 2000}{150}$$

(Blout et al., 1962).

3.8.3. INSTRUMENTATION

The Bendix/Bellingham and Stanley Polarmatic 62 recording spectropolarimeter (Gillham and King, 1961) was the instrument used for all the ORD studies. This spectropolarimeter employs the Faraday (or magneto-optic) effect, in which the direction of polarisation of light in a transparent medium is rotated by means of a magnetic field applied parallel to the beam. This effect is used to both modulate the beam rotationally at 380Hz, and to compensate for the rotation caused by the sample, thus allowing the photomultiplier tube to be subjected to a constant beam intensity. In the Bendix/Bellingham and Stanley design, the functions of monochromator and polariser are combined by using two crystalline quartz prisms both to disperse the radiation and polarise it. The two prisms (the polariser and analyser) are fixed in position. The only moving parts are two rotating mirrors which select the wavelengths of the light beam.

This instrument is estimated to read to ± 0.2 millidegrees (m°) in the wavelength range 185 to 600nm. However, it has been found that, because of the relatively weak light source (250 watt lamp as opposed to the 450 watt lamps in the other recording spectropolarimeters) the high noise level below 220nm makes precise ORD measurements of both proteins and nucleic acids in the far ultraviolet, impossible, due to their

high absorbance at low wavelengths. Adler and Fasman (1968) have reported that at 200nm the base line noise is $3m^{\circ}$ but a nucleic acid sample with an absorbance of 0.4 shows a noise level of over $10m^{\circ}$. Nucleic acid samples of this concentration have rotations of less than $40m^{\circ}$ near this wavelength. At high absorbance and lower wavelengths rotational artifacts may appear.

For all ORD measurements the temperature of the cell compartment was maintained at a constant temperature ($\pm 1^{\circ}$) by means of a Haake recirculating water bath.

The Bendix/Bellingham and Stanley polaromatic 62 was fitted with an Advance Electronics HR-96 X-Y recorder.

3.8.4.

INSTRUMENT CALIBRATION

The Bendix/Bellingham and Stanley Polarmatic 62 is fitted with a mercury lamp. The wavelength scale (calibrated in both wave numbers $(\nu) \times 10^{-3}$ and wavelength in nm) was checked using the line spectrum of mercury. No adjustment was ever necessary.

The rotational calibration of the instrument was checked frequently usually before any series of ORD measurements, with either Mann Ultra Pure or BDH Analar Grade sucrose in a filtered distilled water solution. A 0.6378% (w/v) solution of sucrose gives a rotation of 0.05° at 546nm (for full details see the instrument instruction manual), the ORD of the 0.6378% (w/v) sucrose or any other sucrose solution of known concentration is determined over the wavelength range 230 to 590nm. The ORD curve is analysed by the Drude equation (Drude, 1906).

$$[\alpha]_{\lambda} = \frac{K}{\lambda^2 - \lambda_c^2} .$$

A plot of $[\alpha]_{\lambda} \cdot \lambda^2$ versus $[\alpha]_{\lambda}$ yields a straight line (figure 3.29).

K is the intercept and λ_c^2 is the slope. The two constants $K = 21.75$ and $\lambda_c = 0.002269$ which were usually obtained from the sucrose solutions are in agreement with the published values (table 2.3)

STRAY LIGHT TEST - This was performed as described by Yang and Samejima (1963). To test for stray light inside the instrument of the rotatory dispersion of a sucrose solution was measured with and without a 0.004% (w/v) K_2CrO_4 solution in 0.05M-NaOH in series with sucrose in one of the compartments of a tandem cell. This potassium chromate solution has an absorption maximum at 373nm of 1.00 and a minimum of 0.07 at 313nm, in a 1cm path length cell. The ORD of sucrose was unaffected by the presence of this absorbing material thus ruling out the possibility of significant stray light inside the instrument. Stray light if present in large amounts would become most pronounced in the presence of an absorbing material due to the reduction of the light intensity at the chosen wavelength. This would be most serious with high concentrations of the absorbing material (Yang and Samejima, 1963).

SCATTERING TEST - E. coli RNA polymerase is a large protein molecule and can cause significant scattering of incident light. To test for the effect of scattering on rotation, the rotatory dispersion of a sucrose solution was measured with and without a 0.2% (w/v) Ludox ($M_w = 2.55 \times 10^6$) solution in 0.05M-NaCl in series with the sucrose. Over the wavelength

range 238 to 555nm the scattering solution had no effect on the ORD of the sucrose.

3.8.5.

DETERMINATION OF SELLMEIER COEFFICIENTS

The optical rotatory dispersion is dependent on the refractive index of the medium. Therefore to compare observed rotations in a variety of solvents, the rotations are reduced to values they would have in a vacuum by means of the Lorentz correction factor, $3/(n^2 + 2)$, where n is the refractive index of the solvent. To measure the reduced mean residue rotation of a protein $[R']$ at wavelength λ , the refractive index correction must be known (see section 2.2.). (Ideally, one would like to use a microscopic refractive index applicable for the immediate environment the optically active chromophore, however, this cannot be easily estimated and the macroscopic Lorentz factor is therefore employed for comparative purposes). The dispersion of the refractive index of the solvent i.e. its variation with the wavelength of light should be taken into account for the most accurate measurements. When these are not available, from tables such as the International Critical Tables, they can be approximated by the use of the Sellmeier equation,

$$n^2 = 1 + \frac{a \lambda^2}{\lambda^2 - \lambda_v^2}$$

where n is the refractive index, λ is the wavelength and λ_v^2 and \underline{a} are coefficients to be determined. This equation can be solved for \underline{a} and λ_v^2 by measurements of the refractive index of the solution at two wavelengths.

SELLMEIER COEFFICIENTS

TABLE 3.14.

Solution	n_1	λ_1 (nm)	n_2	λ_2 (nm)	a	λ_v^2
TMA	1.3340	589	1.3700	268	0.75771	9734
H ₂ O	1.3345	546	1.3403	436	0.75501	9881
A+0.01M-KCl	1.3353	546	1.3411	436	0.75744	9742
A+0.10M-KCl	1.3354	546	1.3412	436	0.75739	9859
A+0.2M-KCl	1.3356	546	1.3414	436	0.75792	9853
A+0.3M-KCl	1.3364	546	1.3425	436	0.75877	10317
A+0.5M-KCl	1.3386	546	1.3445	436	0.76545	9939
0.01M-tris HCl 0.50M-KCl	1.3386	546	1.3445	436	0.76545	9939
1% SDS	1.3392	546	1.3452	436	0.76661	10085
8M-urea	1.4022	546	1.4433	289	0.9272	12023
6M-GuHCl	-	-	-	-	0.9934	15067

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The Sellmeier coefficients for the solutions used in the ORD study of RNA polymerase were determined by any one of three different methods. Firstly from values of the refractive index of the solution at two different wavelengths or values of the Sellmeier coefficients obtained from the literature (see Table 3.14). Secondly by the method of Jovin, England and Bertsch (1969). The refractive index for TMA buffer at 20°C was measured relative to air with a Bellingham and Stanley Abbé refractometer in white light and computed as a ratio to the value of water measured under the same conditions. Refractive indices at two wavelengths were obtained by multiplying this ratio by the corresponding values for water at the two wavelengths. Thirdly, for all other solutions, the refractive indices at 436 and 546nm were determined at 20°C on a Brice-Phoenix differential refractometer.

A Digital PDP 8L computer was used to solve the simultaneous equations to determine the Sellmeier coefficients calculated from the refractive indices at two wavelengths (see appendix).

3.8.6.

ORD OF E. COLI MRE 600 RNA POLYMERASE

3.3.6.1.

INTRODUCTION

Nicholson (1971) has shown that the ORD of E. coli B core enzyme display low rotation values throughout the wavelength range 222 to 589nm. A single Cotton effect trough is present at 232nm ($[\text{m}']_{232} = -3.30^\circ$) and no aromatic bands are discernable. Analysis of the curve in the range 3 to 560nm by the procedure of Moffitt and Yang (1956) gives a straight line plot with a b_0 of -81. The value for the $[\text{m}']_{233}$ and the b_0 of -81

correspond to 12.8% and 13% helix respectively. Novak and Doty (1969) obtain a trough value of -4.20° at 232nm for E. coli core polymerase which they say corresponds to a helix content of 32%.

In this present study the optical rotatory dispersion of RNA polymerase in both:

- (i) low ionic strength buffers (prepared by both the Zillig (1966) and Burgess (1969a) procedures (sections 2.2.3 and 2.2.2)),
- (ii) high ionic strength buffer (prepared by the Burgess (1969a) procedure (section 2.2.3)),

was determined.

3.8.6.2.

ORD OF RNA POLYMERASE

Fraction 6 RNA polymerase prepared by the Zillig (1966) procedure was dialysed overnight (12h) at 4°C against several changes of TMA buffer, pH 7.5, to remove both the sucrose and glycerol of the storage solution which would interfere with the rotatory dispersion measurements. The protein concentration was determined spectrophotometrically (section 3.6.2). The ORD was measured at 20°C over the wavelength range 295 to 560nm and the resulting dispersion curve was analysed by both the Moffitt-Yang (1956) and Schechter-Blout procedures (figures 3.32 and 3.33), these gave straight line plots for the native enzyme. The results are shown in table 3.15. The % helix content was determined from the $[\text{m}']_{232}$ as well as the Moffitt-Yang and Schechter-Blout coefficients (table 3.16).

Fraction 6 RNA polymerase holoenzyme (subunit structure characteris

by SDS-polyacrylamide gel electrophoresis) prepared by the method of Burgess (1969a) and stored in buffer S (section 2.2.3), was equilibrated with a solution containing 0.01M-tris HCl, pH 7.9, and 0.5M-KCl by passage through a column of Sephadex G-25 previously equilibrated with the above buffer solution and used immediately. The protein concentration was determined from the ultraviolet spectrum as described previously (section 3.6.2). The ORD was measured over the wavelength range 220 to 560nm (figure 3.32). There was a trough at 232nm ($[\text{m}']_{232} = -4.30^\circ$). The ORD over the wavelength range 294 to 560nm was analysed by both the Moffitt-Yang and Schechter-Blout analyses (section 2.2.9) and straight line plots were obtained (figure 3.33 and 3.34). The Moffitt-Yang and Schechter-Blout coefficients are shown in table 3.15 and the derived helical contents are shown in table 3.16.

The optical rotatory dispersion studies show that RNA polymerase holoenzyme in buffers of low ionic strength (TMA + 0.022 and 0.11M- NH_4Cl) has a helix content of 31% to 32% measured from the b_0 by the method of Urnes and Doty (1961). The helix content is 28 to 30% measured from the A_{225} and 37 to 38% measured from the A_{193} coefficient. From b_0 (Blout) the helix is 41 to 42% and from $[\text{m}']_{232}$ it is 19%. The mean helix calculated from both A_{193} and A_{225} is 37 to 38%.

Similar results were obtained with RNA polymerase in 0.01M-tris HCl pH 7.9 and 0.5M-KCl. The enzyme has a helical content of 22% calculated from $[\text{m}']_{232}$, 33 to 34% calculated from b_0 (Doty) and A_{193} , 39% and 41% from b_0 (Blout) and A_{225} , and 38% as the mean of A_{193} and A_{225} . This is very similar to that of RNA polymerase in TMA buffer purified by the

ORD ANALYSES OF NATIVE RNA POLYMERASE

TABLE 3.15.

	μ	$[\eta]_{232}^{20}$	Moffitt-Yang		Schechter-Blout	
			a_o	b_o	A_{193}	A_{225}
TMA buffer, pH 7.5 *	0.04	-	-84 -68	-205 -197	798 776	-661 -631
TMA buffer + 0.11M-NH ₄ Cl	0.15	-4,300°	-63	-194	767	-620
0.01M-trisHCl, pH 7.9 0.5M-KCl.	0.51	-4,300°	-169	-213	762	-714

* two different preparations of RNA polymerase

TABLE 3.16

HELIX CONTENT OF RNA POLYMERASE

Solution	% Helix					
	$[\eta]$ 232	b_o (Doty)	B_o (Blout)	A_{193}	A_{225}	H_{mean}
TMA, pH 7.5	19	31	37	42	29	37
		32	38	42	30	38
TMA + 0.11M-NH ₄ Cl	-	31	37	41	28	37
0.01M-tris HCl, pH 7.9 0.50M-Kcl.	20	34	39	41	33	38

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Zillig (1966) procedure.

All the ORD curves were consistent with a right-handed helical structure. Although all of these RNA polymerase preparations gave similar results there was very poor agreement among the helix content determined by the different procedures. These gave RNA polymerase holoenzyme a helix content anywhere between 19 to 42%.

3.8.7.

EFFECT OF IONIC STRENGTH ON THE CONFORMATION OF RNA POLYMERASE

3.8.7.1.

Richardson (1966a), using sedimentation velocity and sedimentation equilibrium studies, showed that at low ionic strength *E. coli* RNA polymerase has a sedimentation coefficient of 21S to 24S and a molecular weight of about 880,000. At high ionic strength, the enzyme dissociates into two 13S monomers (molecular weight = 440,000 daltons). This dissociation process is reversible. Stevens et al. (1966) have studied the sedimentation properties of RNA polymerase and their results are in agreement with those of Richardson (1966a). In addition to the enzyme sedimenting at 22S in low ionic strength buffer, they find that the enzyme sediments at intermediate S values at intermediate ionic strengths. This suggests that the two forms of the RNA polymerase molecule are in rapid equilibrium (Schachman, 1959). Berg and Chamberlin (1970) have performed sedimentation studies with both RNA polymerase core enzyme and holoenzyme and their conclusions are very similar to those of Stevens et al. (1966).

Anderson and Abraham (1970) have reported that the ultraviolet spectrum of RNA polymerase is greatly influenced by the ionic strength of

the solution. As the enzyme is transferred from a solution of high ionic strength to one of lower ionic strength so the A_{280}/A_{260} decreases. This spectral change is most marked around the absorption minimum at 251nm. If this observed spectral change was caused solely by the exposure of certain hidden aromatic residues of the protein, the effect would have been greater around 280nm. However, Anderson and Abraham (1970) suggest that a conformational change of the RNA polymerase resulting in the exposure of certain aromatic amino acids and secondary changes in the polypeptide backbone such as a helix-coil transition) could lead to the spectral changes which they observe.

3.8.7.2.

EFFECT OF IONIC STRENGTH ON CONFORMATION

Preliminary ORD experiments (section 3.8.5.2) showed that the conformation (% helix) of RNA polymerase in low and high ionic strength buffer were similar although the helix content of the enzyme in the buffer of ionic strength = 0.51 was the marginally higher. To examine this observation more fully, several samples of the same preparation of RNA polymerase, prepared by the Burgess (1969a) procedure (section 2.2.3) were dialysed against several changes of buffer A containing varying concentrations of KCl, for 12h at 4°C. The concentration of protein in each of the samples was determined spectrophotometrically (after ultrafiltration) on a Cary 15 spectrophotometer (section 3.6.2), and by the Lowry (1951) procedure. The ORD of each of the RNA polymerase samples was measured in the Polarmatic 62 at 20°C. The Moffitt-Yang and Schechter-Blout coefficients of each of the protein solutions were

TABLE 3.17 EFFECT OF BUFFER IONIC STRENGTH ON THE ORD OF RNA POLYMERASE

Solution	μ	Moffitt-Yang		Schechter-Blout	
		a_o	b_o	A_{193}	A_{225}
A + 0.01M-KCl	0.02	-77	-191	742	-615
A + 0.10M-KCl	0.11	-179	-211	772	-215
A + 0.20M-KCl	0.21	-120	-206	820	-707
A + 0.30M-KCl	0.31	-104	-234	840	-720
A + 0.50M-KCl	0.51	-127	-193	719	-643

TABLE 3.18. HELIX CONTENT OF RNA POLYMERASE IN SOLUTIONS OF VARYING IONIC STRENGTH

Solution	μ	% HELIX				
		b_o (Doty)	b_o (Blout)	H_{193}	H_{225}	H_M
A + 0.01	0.02	30	36	41	28	36
A + 0.10	0.11	33	39	42	33	38
A + 0.20	0.21	33	38	43	33	39
A + 0.30	0.31	37	41	44	33	40
A + 0.50	0.51	31	37	40	29	36

calculated from the visible rotatory dispersion (section 2.2.9) over the wavelength range 294 to 580nm (figures 3.35 to 3.38). The Sellmeier coefficients for each of the solutions (table 3.14) were determined as described in section 3.8.4. The results are shown in Tables 3.17 and 3.18. These show that increasing the ionic strength of the buffer over the range were the 23S form of RNA polymerase dissociates to the 13S monomer leads to no gross conformational change of the enzyme as suggested by Anderson and Abraham (1970). Figure 3.9 shows a plot of b_o (a direct measure of helix content (Urnés and Doty, 1961)) against KCl concentration in buffer A. There is a very slight, but significant, increase in b_o with increasing ionic strength of the buffer (31.8% to 33.2% over the ionic strength range 0.01 to 0.51).

It would appear, therefore, that *E. coli* MRE 600 RNA polymerase undergoes no large conformational change (helix-coil transition) in moving from the monomer (13S) to dimer (23S) form of the enzyme.

When the Moffitt-Yang a_o coefficients are plotted against ionic strength (figure 3.40) there is a gradual decrease in the a_o value (i.e. it becomes increasingly more negative) as the ionic strength increases. The points are quite widely scattered unlike the b_o 's. The a_o term in the Moffitt-Yang equation is greatly influenced by the composition of the solvent (section 3.8.2.2).

3.8.8.

RNA POLYMERASE IN DENATURING SOLVENTS

In the investigation into the subunit structure of *E. coli* MRE 600 RNA polymerase by the technique of SDS-polyacrylamide gel electrophoresis (section 3.3.1) the protein subunits were completely dissociated and

denatured by known protein denaturing solutions - 1% (w/v) SDS in 0.01M-sodium phosphate, pH 7.1, 8M-urea and 6M-guanidine hydrochloride. Each solution contained 1% (v/v) 2-mercaptoethanol to reduce any disulphide bonds present in the protein species. The ORD of each of the samples of RNA polymerase in the denaturing solutions was performed after dialysis against the appropriate solvent for 48h and 20°C. The protein concentration was determined spectrophotometrically as described previously (section 3.6.2) assuming a specific absorbance of 0.65/mg/ml, the same as that of the native enzyme (this could lead to a possible source of error, but this should not be greater than 5%). The Sellmeier constants for the SDS solution were determined on the differential refractometer, for the 8M-urea solution from Adler and Fasman (1968) and for the 6M-guanidine hydrochloride solution from Tanford et al. (1967). The ORD of each of the solutions was measured over the wavelength range 294 to 560nm and Moffitt-Yang and Schechter-Blout analyses were performed on each of the solutions (figures 3.41 and 3.42 respectively). The Moffitt-Yang and Schechter-Blout coefficients are shown in table 3.19 and helix contents in table 3.20.

RNA polymerase in the 1% (w/v) SDS solution undergoes an increase in helix content when measured from the Moffitt-Yang b_0 by the method of Urnes and Doty (1961). Similar results have been obtained by Jirgensons (1966) for several other proteins in SDS solutions. The values for the helix content determined from the Schechter-Blout A coefficients are almost identical suggesting that only the helical and random coil conformations are present. It would appear then that SDS (anionic detergent) leads to a more compact conformation of the E. coli RNA

TABLE 3.19 ORD ANALYSES OF RNA POLYMERASE IN DENATURING SOLVENTS

Solution	Moffitt-Yang		Schechter-Blout	
	a_o	b_o	A_{193}	A_{225}
1% (w/v) SDS	-337	-234	766	-864
8M-urea	-742	-68	-157	-545
6M-GuHCl	-704	-14	-384	-347

All solutions contain 2-mercaptoethanol

TABLE 3.20 HELIX CONTENT OF RNA POLYMERASE IN VARIOUS DENATURING
SOLVENTS

Solution	% HELIX				
	b_o (Doty)	b_o (Blout)	H_{193}	H_{225}	H_{mean}
1% SDS	37	42	42	40	41
8M-urea	11	21	16	24	18
6M-GuHCl	2	14	10	14	11

All solutions contained 2-mercaptoethanol

polymerase enzyme or its subunits.

Both 8M-urea and 6M-guanidine hydrochloride, in the presence of reducing agents such as 2-mercaptoethanol, are known to denature protein molecules by unfolding them into the random coil conformation (Tanford, 1961). In 8M-urea RNA polymerase still has some helical conformation present (around 11%), this could be because of the ORD measurements being performed before the slow process of the protein molecule unfolding has been completed. From the results in table 3.20, it would appear that in 6M-guanidine hydrochloride all the RNA polymerase subunits exist as near random coils with little or no helix conformation present in the molecules. The ORD spectrum in the region 290 to 560nm falls within the range reported by Tanford et al. (1967) for a number of proteins in 6M-guanidine hydrochloride. Also the Moffitt-Yang parameters are in good agreement with those determined for various other proteins in the same solvent, the small value of b_0 and large negative a_0 being consistent with a random coil structure (Urnes and Doty, 1961). The results suggest that RNA polymerase retains no ordered structure in 6M-guanidine hydrochloride, 1% (v/v) 2 mercaptoethanol.

3.8.9.

CONFORMATION OF RNA POLYMERASE

From the data in tables 3.16 and 3.18 it can be seen that there is poor agreement among the various estimates of helical content of native RNA polymerase over the range of ionic strengths 0.01 to 0.51. Disagreement between helical contents calculated from the A coefficients of the Schechter-Blout equation implies the presence of structures other

than random coils and helices (Blout et al., 1967). Only proteins and polypeptides composed of helical and random coil conformations lie on the line described by the equation

$$A_{225} = -0.55 A_{193} - 430$$

(Schechter and Blout, 1964). Figure 3.43 shows the points for the A coefficients of native and denatured RNA polymerase and their relationship is the above equation.

It can be seen that the native RNA polymerase molecule, both in high and low ionic strength buffers, has other structural features, probably β -conformation and hydrophobic regions, as well as helical and random coil conformations.

RNA polymerase in 1% (w/v) SDS, 1% (v/v) 2 mercaptoethanol, 0.01M-sodium phosphate, pH 7.1, appears to consist of only helices and random coil structures as the A coefficients for the denatured enzyme place it on the above line (figure 3.43). RNA polymerase denatured by 8M-urea still shows the presence of structures other than helices and random coils (figure 3.43). In 6M-guanidine hydrochloride, 1% (v/v) 2-mercaptoethanol, the RNA polymerase subunits exist as almost complete random coils as the values of A_{193} and A_{225} of the denatured enzyme lie quite close to those of poly-L-glutamic acid in the random coil conformation (figure 3.43).

3.9.

INTERACTION OF RNA POLYMERASE WITH DNA

3.9.1.

INTRODUCTION

A necessary first step in the initiation of RNA synthesis is the interaction between RNA polymerase and DNA. It has been shown that RNA polymerase can bind to DNA even in the absence of the four ribonucleoside triphosphates (Jones and Berg, 1966; Pettijohn and Kamiya, 1967; Fox, Gumpert and Weiss, 1965, Richardson, 1966b; Sternberger and Stevens, 1966). Under certain conditions the association is sufficiently strong enough to allow separation of bound enzyme from free enzyme by lengthy procedures such as zone sedimentation or zone electrophoresis (Fox et al., 1965; Kodoya et al., 1964). With the use of these methods, as well as others, the following observations concerning the binding of RNA polymerase to DNA have been made.

The binding reaction is fast and a DNA-RNA polymerase complex is detectable only seconds after the addition of enzyme to the DNA solution (Richardson, 1969). The binding is reversible (Richardson, 1966b) but there may be specific sites to which the enzyme binds more irreversibly (Bremer, Konrad and Bruner, 1966; Stead and Jones, 1967). The association is sensitive to ionic strength and pH (Richardson, 1966b). Magnesium ions are not required for binding (Fox et al., 1965; Richardson, 1966b). The binding reaction is specific and the amount of enzyme that can bind to a particular DNA species is much less than expected, if the binding reaction were completely non-specific (Richardson, 1969).

A simple model for this binding reaction has been put forward by Chamberlin (1970). RNA polymerase

binds at random sites on the DNA which the enzyme encounters by diffusion. This binding is in most instances reversible and the number of potential binding sites on DNA is large, probably any nucleotide sequence has an appreciable affinity for the enzyme. This process leads ultimately to an encounter between the RNA polymerase and a specific site on the DNA. The number of such sites (promoters) on a specific DNA molecule is believed to be small. Binding at such a promoter site leads to a specific reaction with RNA polymerase in which the strands of the DNA are opened over a short region (Zillig et al., 1970; Hinkle and Chamberlin, 1970). Kosogonov et al. (1971) have provided some evidence for the partial disruption of the native DNA helix on binding RNA polymerase. The nature of the physical changes undergone by RNA polymerase on its interaction with DNA are not yet known but in carrying out the various steps in the synthesis of RNA, RNA polymerase must certainly undergo a series of changes in its conformation and polypeptide chain interactions. Observations that changes in the sensitivity of the enzyme to salt, proteases and inhibitors occur when the enzyme binds to DNA and when it is synthesising RNA have been interpreted as demonstrating conformational changes. Khesin et al. (1967) have shown that *E. coli* RNA polymerase becomes more resistant to the hydrolytic action of trypsin and to dissociation from the DNA in the presence of high salt, and that this resistance increases upon the addition of the four ribonucleoside triphosphates which allow RNA synthesis. Novak and Doty (1968) using micrococcal RNA polymerase have also shown that there is increased resistance of the enzyme to proteases in the RNA synthesis.

RNA-DNA-RNA polymerase complex.

Novak (1968) has also investigated the occurrence of conformational changes in the micrococcal RNA polymerase in isolated binary DNA-RNA polymerase and ternary DNA-RNA polymerase-RNA complexes by the physical techniques of circular dichroism, fluorescence quenching and tritium-hydrogen exchange. The results suggest that transcription but not binding requires conformational changes in the RNA polymerase enzyme.

More recently Novak and Doty (1970) have examined the conformation of *E. coli* RNA polymerase purified by the method of Burgess (1969a) (core enzyme) in binary and ternary complexes by the related techniques of circular dichroism and optical rotatory dispersion. They found that the binding of RNA polymerase to native calf thymus DNA did not result in the disruption but rather "the straining" of large helical regions in the polymerase. The ORD and CD curves for the DNA-RNA polymerase complexes at 25°C were the sum of the curves of the individual components. Thermal ORD studies showed that the helical regions in the DNA bound RNA polymerase were more susceptible to thermal disruption than they were in the free enzyme. Such a large decrease in thermal stability of the RNA polymerase helix regions was not found for the enzyme in a single stranded DNA complex or for the RNA polymerase in native DNA-RNA polymerase complexes.

3.9.2.

RESULTS

Experiments similar to those of Novak and Doty (1970) were devised in order to examine the interaction of *E. coli* MRE 600 RNA polymerase (holoenzyme) with DNA by the technique of optical rotatory dispersion.

All ORD measurements were performed on the Bendix/Bellingham and Stanley Polarmatic 62 spectropolarimeter fitted with a Haake recirculating water bath, accurate to $\pm 1^\circ$, to control the temperature of the cell compartment. All ORD measurements were carried out using either Bellingham and Stanley 1cm path length cells (ORD of calf thymus ^{DNA} and enzyme alone) or in quartz tandem cells ($2 \times 1\text{cm}$) for the mixing studies.

RNA polymerase holoenzyme was purified from E. coli MRE 600 by the method of Burgess (1969a) (section 2.2.3). All RNA polymerase concentrations were determined spectrophotometrically as described in section 3.6.2. The concentration of calf thymus DNA was determined from the ultraviolet spectrum at 260nm ($E_{\text{cm}}^{1\%} = 200$, Samejima and Yang, 1965).

Measurements of the specific rotation of calf thymus DNA in a buffer containing 0.1M-tris HCl, pH 7.9, and 0.1M-KCl showed that the ORD at both 20°C and 45°C was identical, and similar, to that published (Samejima and Yang, 1965) (figure 3.44). The UV ORD of E. coli RNA polymerase in the same buffer was similar to that previously determined (section 3.8.6.2) and was also found to be the same at both 20°C and 45°C (figure 3.45). Therefore raising the temperature from 20°C to 45°C has no apparent effect on the conformation of either RNA polymerase or calf thymus DNA.

Using a tandem cell in the thermostatted cell compartment of the Polarmatic 62, the composite spectrum of RNA polymerase in series with calf thymus DNA, both in 0.1M-tris HCl, 0.1M-KCl, at 20°C was obtained. A 1:1.5 weight ratio of calf thymus DNA to RNA polymerase was used (Richardson, 1969). This DNA:RNA polymerase ratio is known to lead to the complete binding of the enzyme to DNA (Jones and Berg, 1966). The

ORD was measured over the wavelength range 220 to 320nm and is shown in figure 3.46. The absorbance did not exceed 1.5 at all wavelengths measured. The curve obtained is the expected composite one with a DNA peak at 290nm, and DNA trough at 257nm and the protein trough at 232nm.

Without removing the tandem cell from cell holder in the cell compartment both the DNA and RNA polymerase solution were withdrawn, mixed, and allowed to stand for 10min at 20°C. The mixed solution was then returned to both compartments of the tandem cell. The ORD over the wavelength range 220 to 320nm was again measured. As can be seen in figure 3.46, the height of the DNA peak at 290nm had not altered, but the value of the trough at 232nm had become less negative indicating that the RNA polymerase had undergone a conformational change, corresponding to a decrease in helix content. The helix content of E. coli RNA polymerase as measured from the value of the trough at 232nm (Blout et al., 1962) is 21% ($[\text{m}']_{232} = -4.5^\circ$). The decrease in the value of the trough ($\Delta[\text{m}']_{232} = -0.88^\circ$) is a decrease of 3% in the helix content of the enzyme molecule.

The tandem cell containing the mixture of E. coli RNA polymerase and calf thymus DNA was then heated to 45°C. The ORD over the wavelength range 220nm to 320nm was again measured. A further decrease in the depth of the 232nm trough had occurred, the $[\text{m}']_{232}$ had changed from -4.5° (composite ORD) to -3.4° . This is a decrease in helix content of around 9% for the enzyme, i.e. the helix content of the enzyme was now 12%. On cooling from 45°C to 20°C there was no reversal to the original ORD curve of the DNA-RNA polymerase complex at 20°C, in fact a further

slight decrease in the value of the trough at 232nm had occurred $\left[[\text{m}']_{232} = -3.0^\circ \right]$. The helix content of the RNA polymerase was then only 8% as measured by the $[\text{m}']_{232}$.

These results show that

- (i) there is no detectable conformational change in the structure of DNA in the presence of bound RNA polymerase,
- (ii) RNA polymerase undergoes a change in conformation on binding to DNA
- (iii) the conformation of RNA polymerase is less stable to heating when bound to DNA,
- (iv) thermal denaturation of RNA polymerase occurs on heating from 20°C to 45°C ,
- (v) this thermal denaturation of RNA polymerase cannot be reversed by cooling.

To investigate further the interaction of RNA polymerase with DNA, experiments should be performed using both core enzyme and holoenzyme and a native bacteriophage DNA to provide a more significant insight into the interaction of RNA polymerase with DNA.

FIG. 3.1

FIGURE 3.1. RELEASE OF RNA POLYMERASE FROM E.COLI

The 200ml stainless steel vessel of the Zillig cell mill was used. The buffer : bacteria : beads ratio was as described in section 2.2.2. Homogenisation was performed at 4°C. Aliquots (0.5ml) were removed at 1min intervals and diluted with 1.5ml of homogenisation buffer before centrifugation at 2,000g for 10min at 4°C to remove the glass beads. The supernate was then assayed for RNA polymerase activity by the Zillig assay procedure (section 2.2.2.).

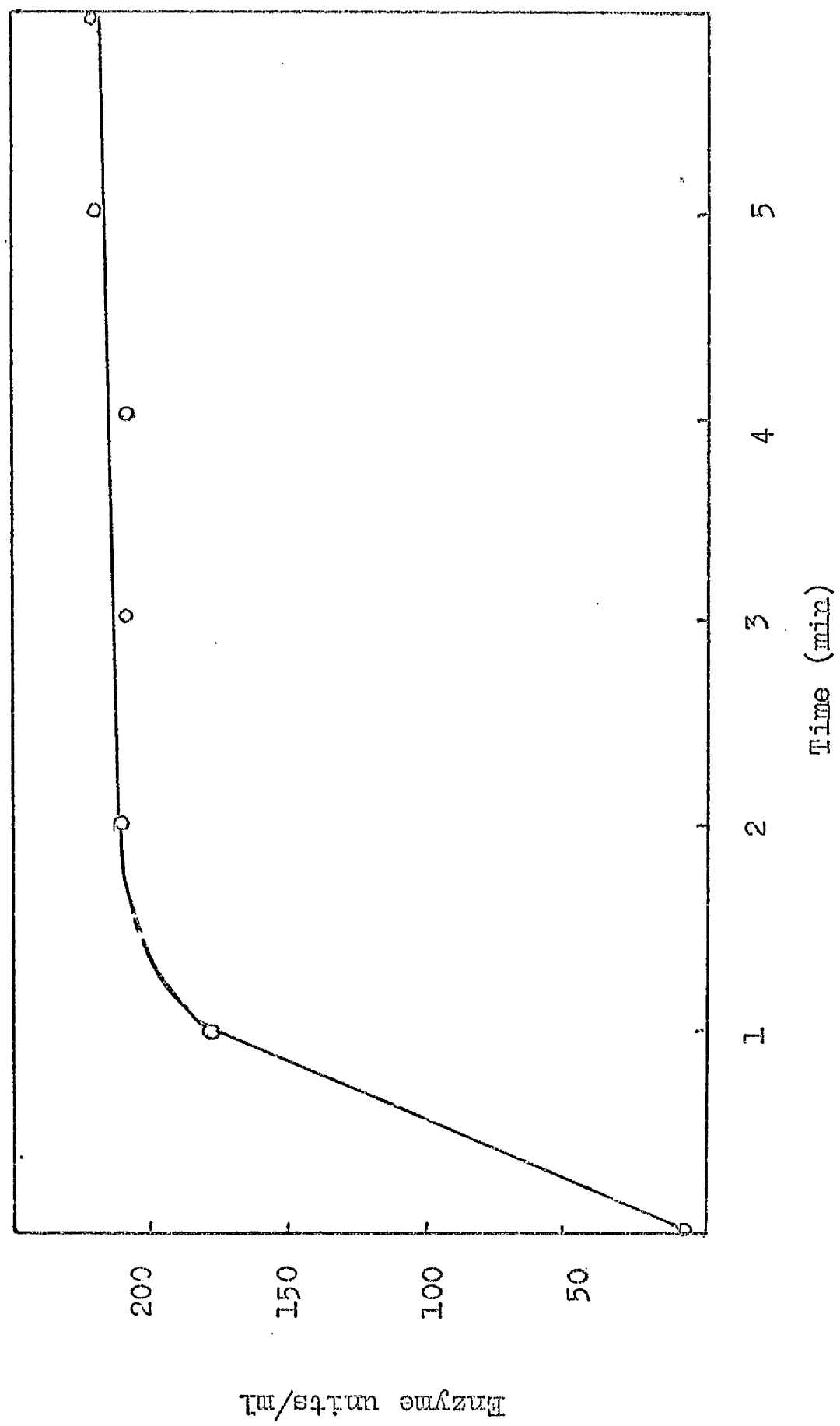


FIGURE 3.2. VISCOSITY OF THE CRUDE EXTRACT TREATED
WITH DEOXYRIBONUCLEASE

E. coli MRE 600 were homogenised as described in section 2.2.3 in buffer G. The glass beads were allowed to settle before DNase (electrophoretically pure) was added to the bacterial extract. The viscosity of the aliquots of the extract to which the DNase had been added was measured at various time intervals at 4°C using a large-bore Ostwald viscometer.

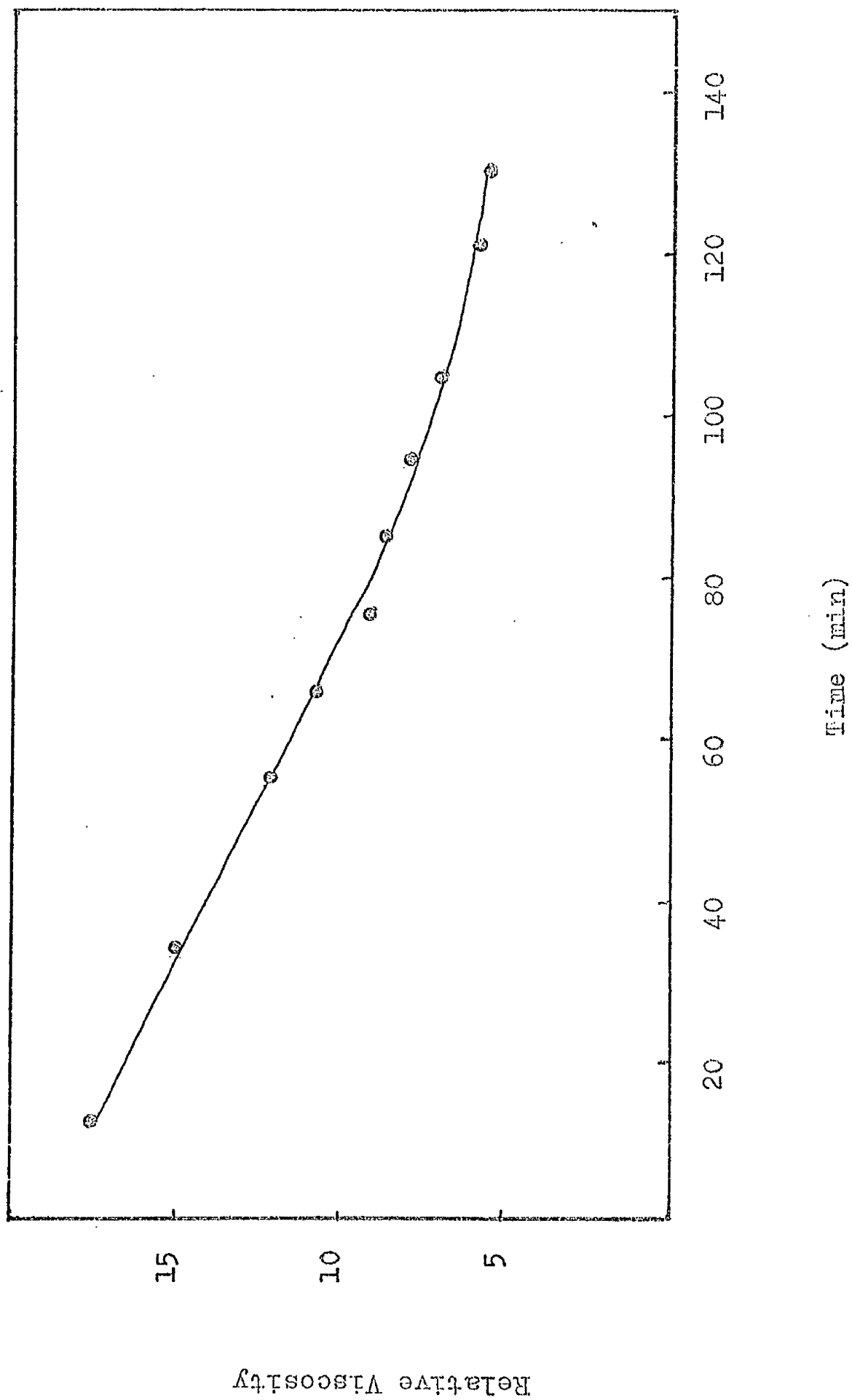


FIG. 3.3

FIGURE 3.3. RIBONUCLEASE ASSAY

This was performed as described by Zillig, Fuchs and Millette (1966).

A 2ml-volume of the standard RNA polymerase assay was prepared (section 2.2.3) and the enzyme used to start the reaction. 0.1ml aliquots of the reaction mixture were removed at 2min time intervals and precipitated by addition to 0.1ml of 10% (w/v) TCA. After a 10min synthesis period, the reaction was stopped by the addition of 0.1µmole/ml of actinomycin D and the fate of the synthesised radioactive RNA was followed by the removal of further 0.1ml aliquots and precipitation with TCA at various times of further incubation. The amount of RNA synthesised was measured as the amount of ^{14}C -ATP incorporated into the acid-insoluble product (RNA). There was no detectable RNase activity.

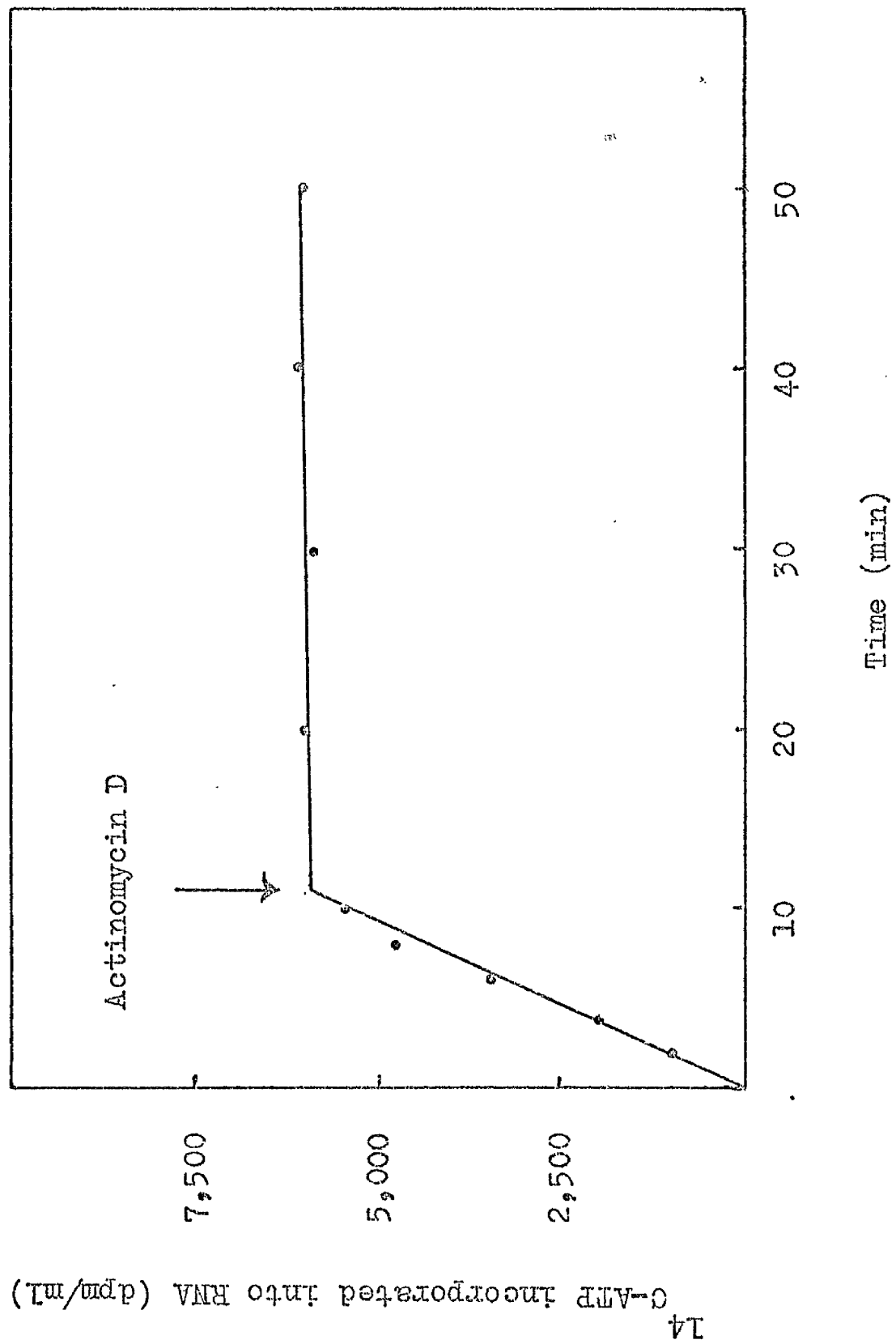


FIG. 3.4

FIGURE 3.4. DEOXYRIBONUCLEASE ASSAY

The assay, performed at 37°C, in a Zimm viscometer with a Haake recirculating water bath, was based on that of Lehman, Roussos and Pratt (1962) and contained in 3ml, 0.06M-trisHCl, pH 7.5, 0.006M-MgCl₂ and 150µg of λ DNA. The viscosity of the solutions was determined at various time intervals after the addition of 19µg of fraction 6 RNA polymerase. A control assay was performed with 1µg of pancreatic DNase. The results show that RNA polymerase has no detectable DNase activity.

o——o 19µg of fraction 6 RNA polymerase.

o——o 1µg of pancreatic DNase.

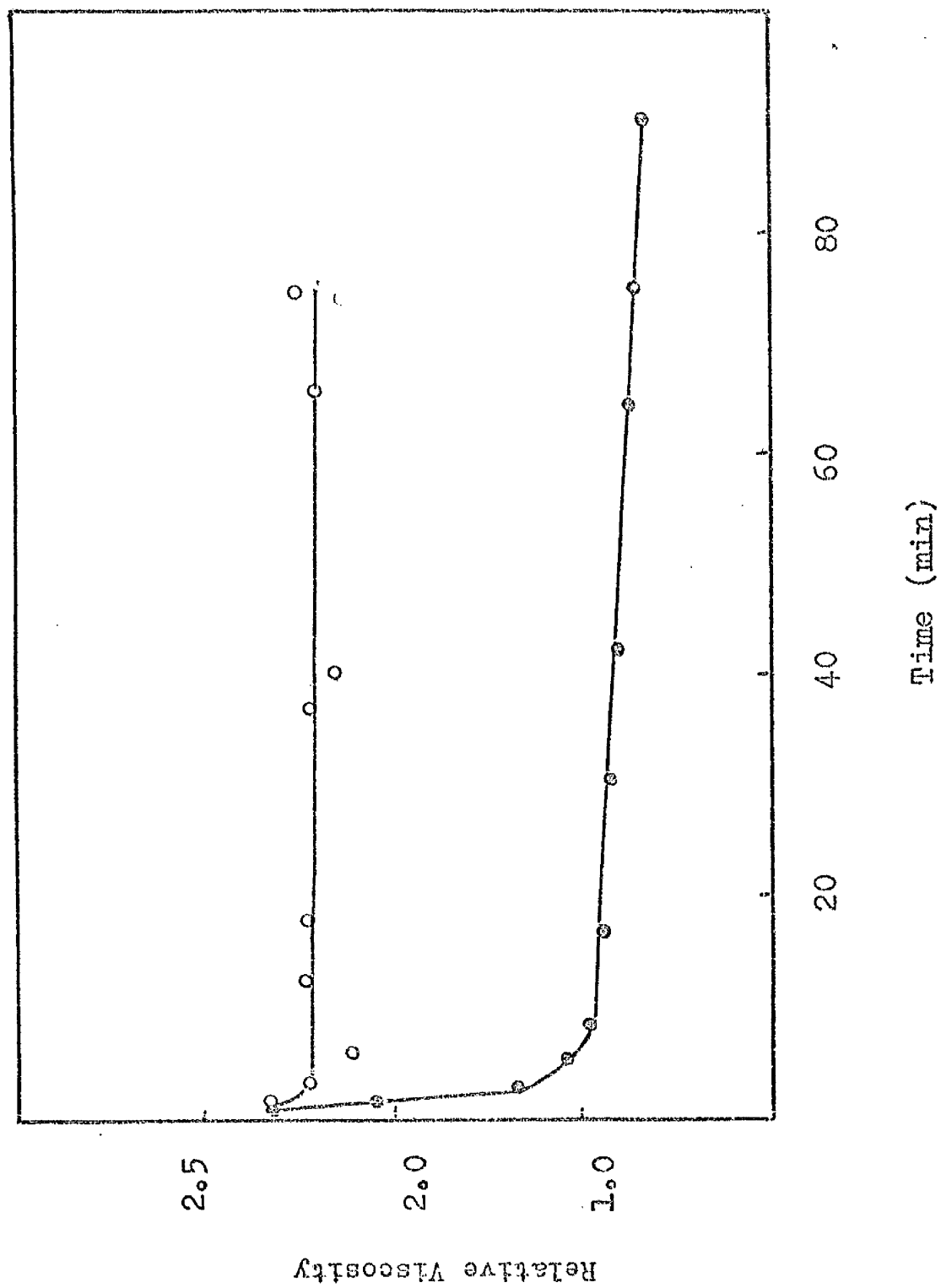
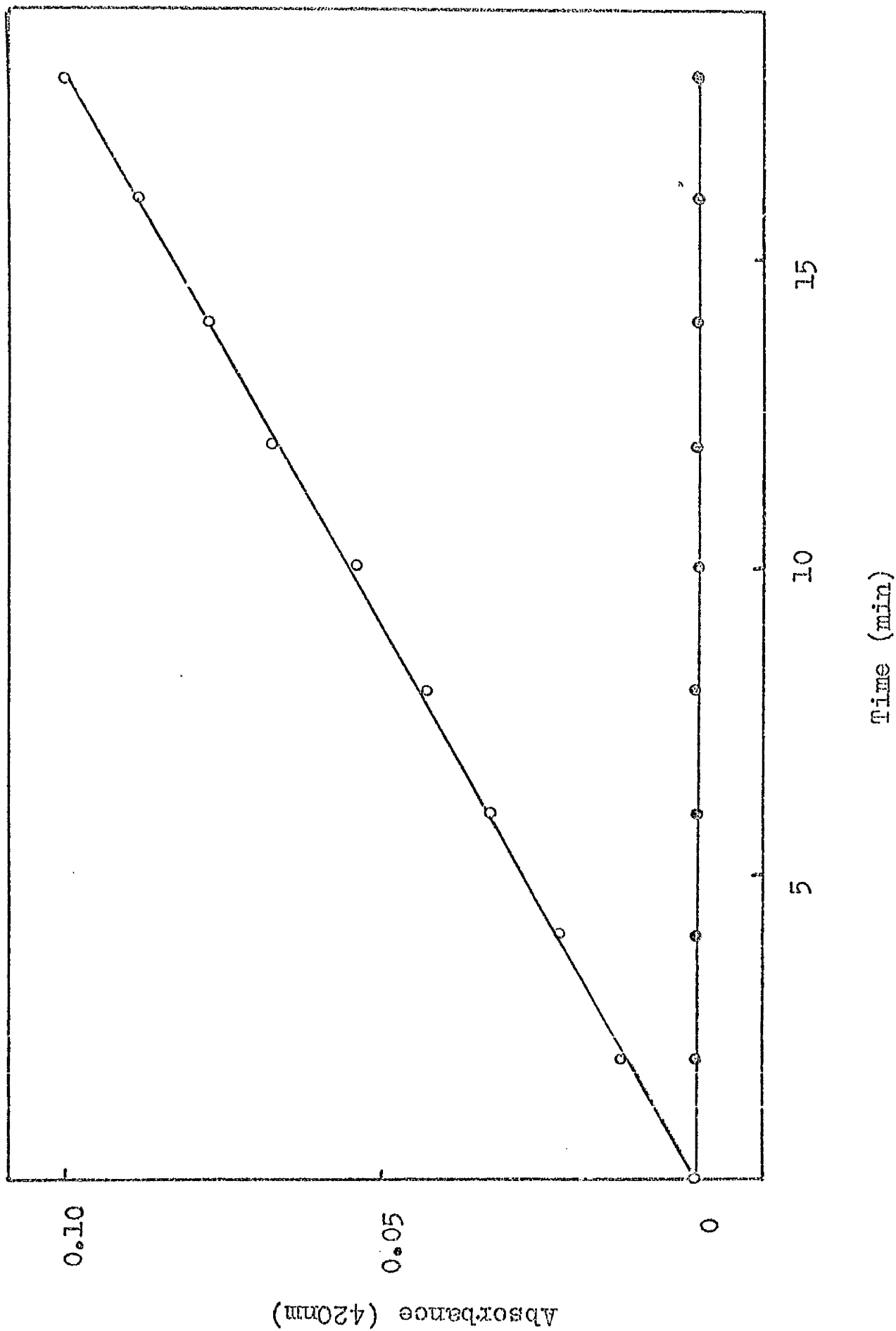


FIG. 3.5

FIGURE 3.5. ALKALINE PHOSPHATASE ASSAY

Alkaline phosphatase activity was measured by the increase in absorbance at 410nm after incubating 200 μ g of fraction 6 RNA polymerase (o—o) in a 3ml solution containing 0.2mg/ml para-nitrophenyl phosphate, 0.1M-trisHCl, pH 7.9, at 23⁰C. A sample containing 5 μ g of E.coli alkaline phosphatase (o—o) as a control was also measured. The absorbance was measured on a Cary 15 recording spectrophotometer. The results show that there is no detectable alkaline phosphatase activity present in fraction 6 RNA polymerase.



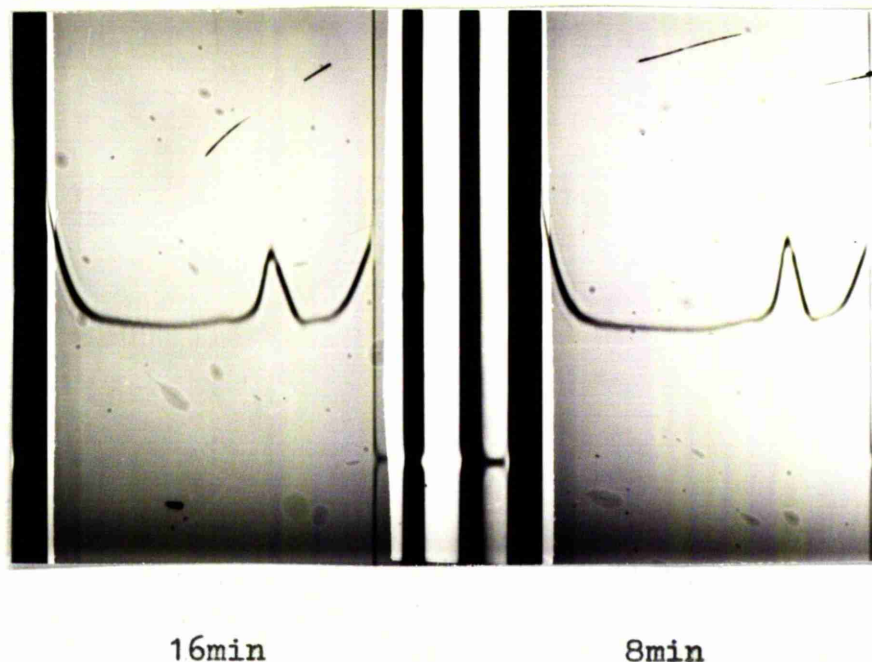


FIGURE 3.6 SCHLIEREN PHOTOGRAPH OF RNA POLYMERASE

Sedimentation pattern of RNA polymerase in TMA buffer, pH 7.5, (section 2.2.2) at 20°C. The schlieren photographs using a single sector cell were taken approximately 8min and 16min after reaching a speed of 56,100rev/min. The protein concentration was 3.2mg/ml and the phase plate angle 80°. The RNA polymerase had an $S_{20,w}$ of 23.3.

FIG. 3.7

FIGURE 3.7. UV SEDIMENTATION VELOCITY PROFILE OF
RNA POLYMERASE

This figure shows tracings of sedimentation velocity patterns of pure RNA polymerase holoenzyme (core enzyme patterns are similar). Samples of fraction 6 enzyme were dialysed overnight (16h) against buffer A + 0.5M-KCl (2.2.3). Absorption optics were used to scan at 280nm. Sedimentation is from left to right. The top reference edge, meniscus and base are marked by R, M and B respectively. The sample was centrifuged at 44,000rev/min (the times given are those after coming to speed). The temperature was 19.7°C. The initial protein concentration was 0.32mg/ml. The $S_{20, \text{buffer}}$ was 12.7. No other sedimenting species is detectable.

Time (mins)

Absorbance at 280nm

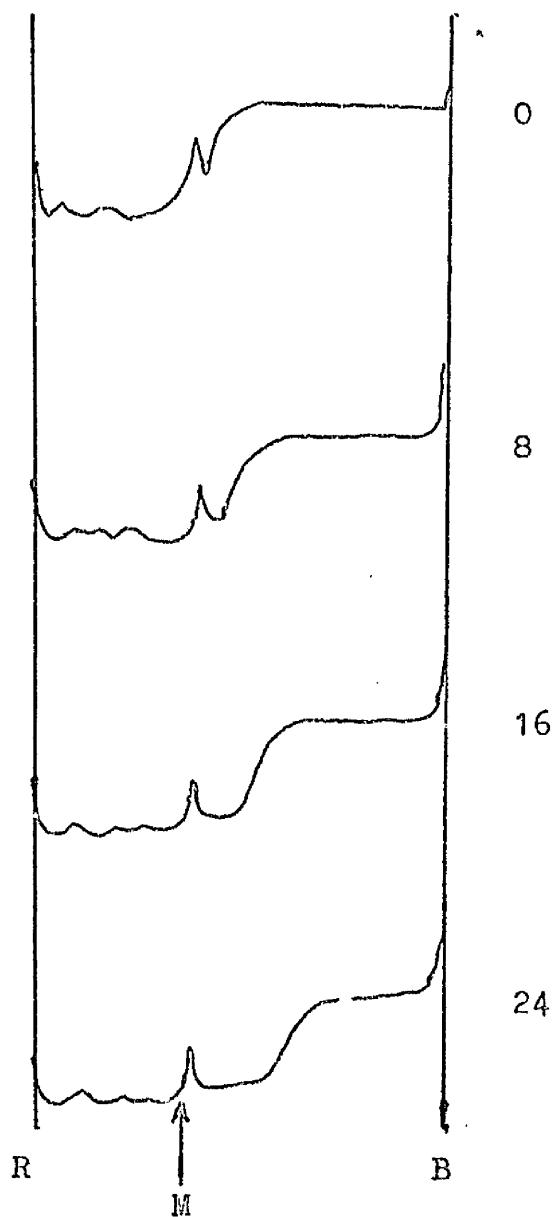


FIG. 3.8

FIGURE 3.8 POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA POLYMERASE
BY THE ORNSTEIN AND DAVIS PROCEDURE.

A 50 μ g sample of fraction 6 RNA polymerase was dialysed against 0.0025M-tris-glycine buffer, pH 8.3 for 16h at 4 $^{\circ}$ C before application in 10% (w/v) sucrose to the upper gel (see section 2.2.6.1). Electrophoresis was performed at 1.5mA for 3h. The gel was stained with 1% (w/v) amido black in 7% (v/v) acetic acid for 6h before destaining in 7% (v/v) acetic acid.

CATHODE

upper gel: 2.5% (w/v)

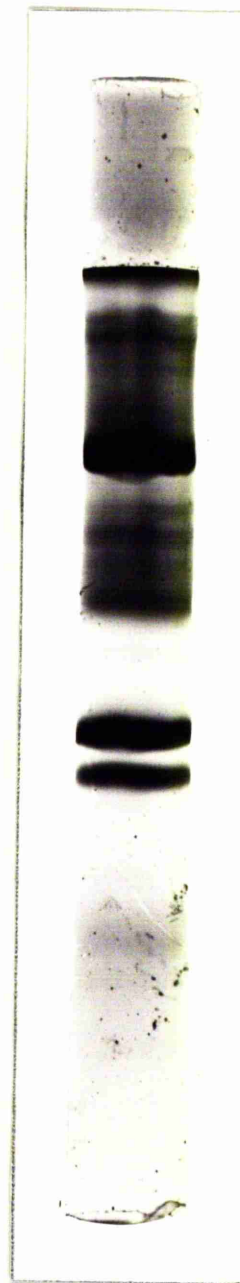
polyacrylamide

major protein species

two minor protein species

lower gel: 7.5% (w/v)

polyacrylamide



ANODE

FIG. 3.9

FIGURE 3.9 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
RNA POLYMERASE

SDS-polyacrylamide gel electrophoresis of RNA polymerase holoenzyme and core enzyme by the method of Shapiro et al. (1967) as described in section 2.2.6.3. 25 μ g of protein was applied to each tube.

- A RNA polymerase holoenzyme stained with amido black.
- B RNA polymerase core enzyme prepared by phosphocellulose chromatography of holoenzyme, stained with amido black.
- C RNA polymerase holoenzyme stained with coomassie blue.

Only trace protein impurities can be detected.

β, β'

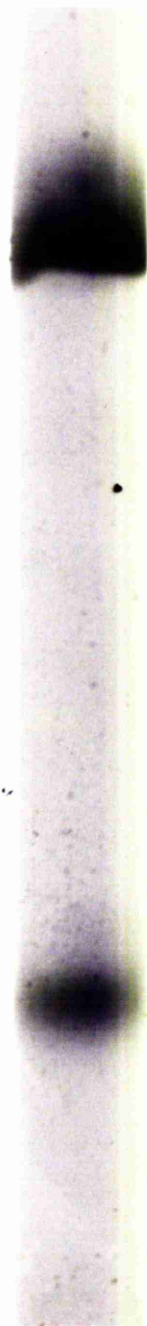
σ

θ

α



A



B



C

β, β'

σ

θ

α

FIG. 3.10

FIGURE 3.10 8M-UREA POLYACRYLAMIDE GEL ELECTROPHORESIS
OF RNA POLYMERASE

The polyacrylamide gel electrophoresis was performed by the method of Jovin et al. (1964) as described in section 2.2.6.2. RNA polymerase holoenzyme and core enzyme were dialysed 16h at 20°C against 8M-urea. 50µg of holoenzyme and 40µg of core enzyme were applied to the 7.5% (w/v) polyacrylamide gels which were run for 3.5h at 1.5mA per tube. Staining was for 2h with amido black.

A RNA polymerase holoenzyme

B RNA polymerase core enzyme

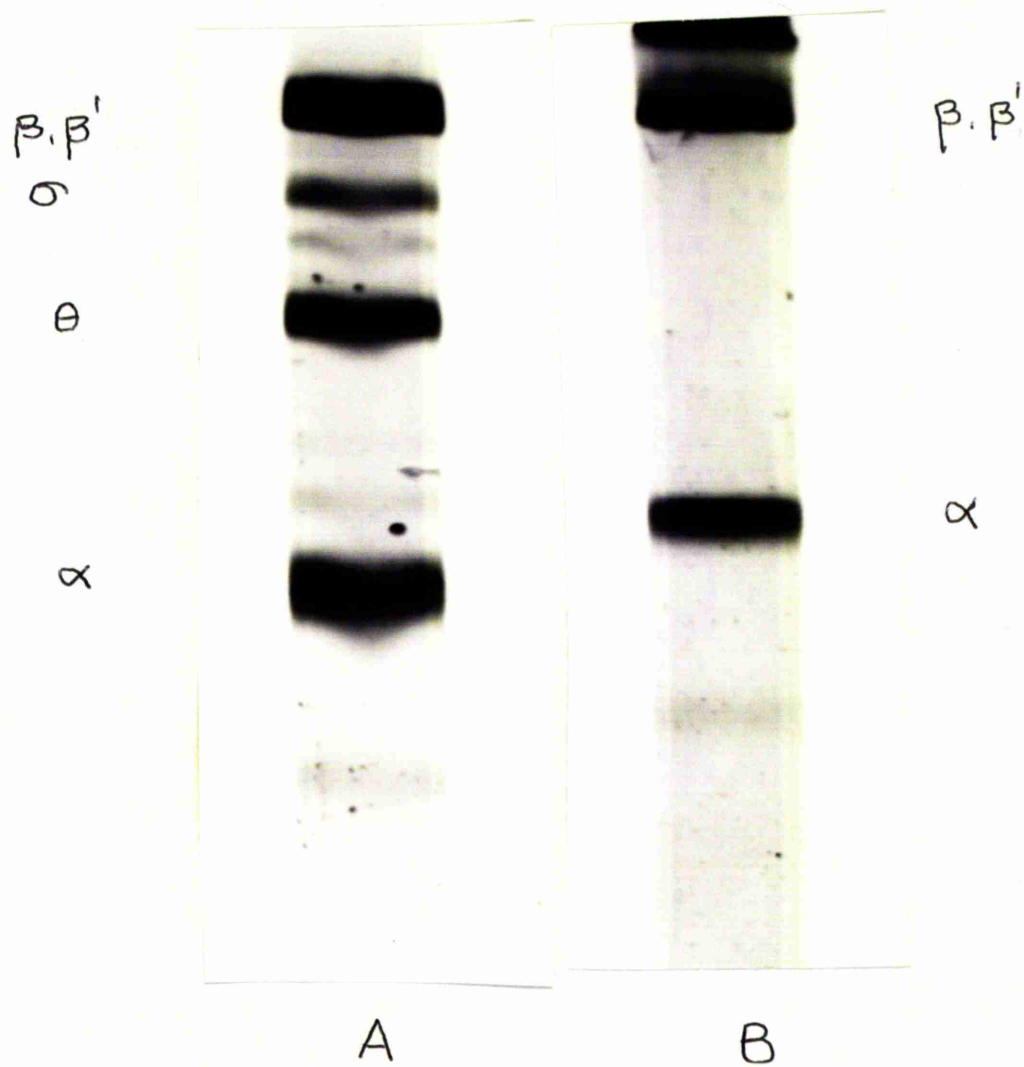


FIG. 3.11

FIGURE 3.11 STANDARD CURVE FOR THE ESTIMATION OF THE
MOLECULAR WEIGHT OF RNA POLYMERASE SUBUNITS
BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins of known molecular weight were electrophoresed on 5% (w/v) polyacrylamide (A) and 10% (w/v) polyacrylamide gels (B) and their mobilities relative to the tracking dye determined. Plotting the logarithm of the molecular weight against mobility yields straight lines for both gels. The marker proteins were sheep γ -globulin, bovine serum albumin, sheep γ -globulin H-chain, ovalbumin, pepsin, sheep γ -globulin L-chain, lysozyme and ribonuclease.

Molecular Weight

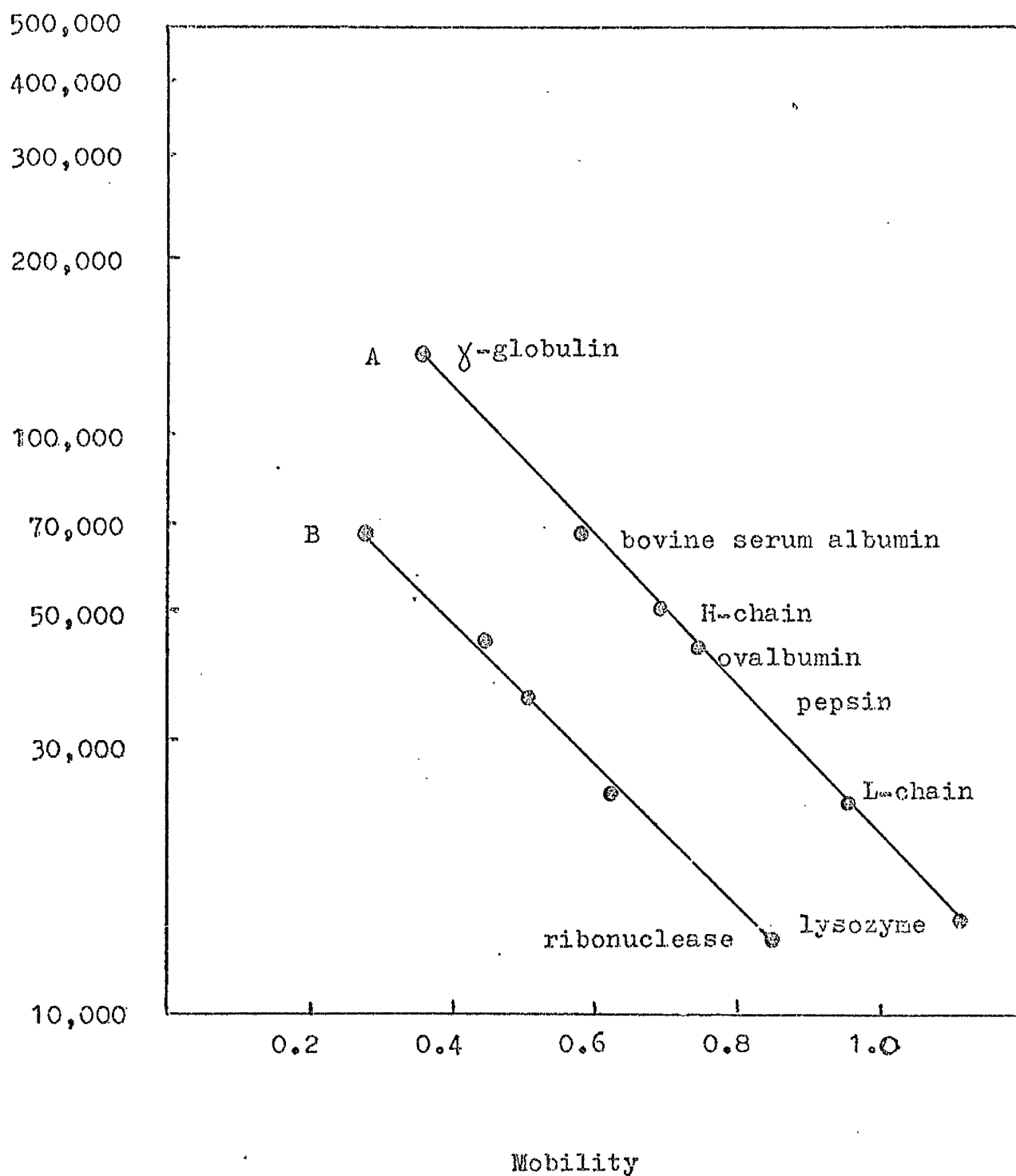


FIG. 3.12

FIGURE 3.12 RELATIONSHIP OF THE DEGREE OF AMIDO BLACK
STAINING TO THE AMOUNT OF PROTEIN APPLIED
TO SDS-POLYACRYLAMIDE GELS

Various volumes of a 400 μ g/ml bovine serum albumin solution in 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.1M-sodium phosphate, pH 7.1, were applied to a 5% (w/v) polyacrylamide gel and electrophoresed for 3.5h by the standard procedure (section 2.2.6.3). The protein was fixed, stained for 2h with amido black and destained in 7% (v/v) acetic acid. The gels were then scanned in a Vitatron recording densitometer. The areas under each protein peak were obtained by two different methods --

- (1) by determining the area under each peak by assuming them to be triangular in shape, (o—o),
- (2) by cutting out a profile of each of the peaks traced onto paper of uniform thickness and weighing each on a Stanton balance (o—o).

Both methods gave almost identical results.

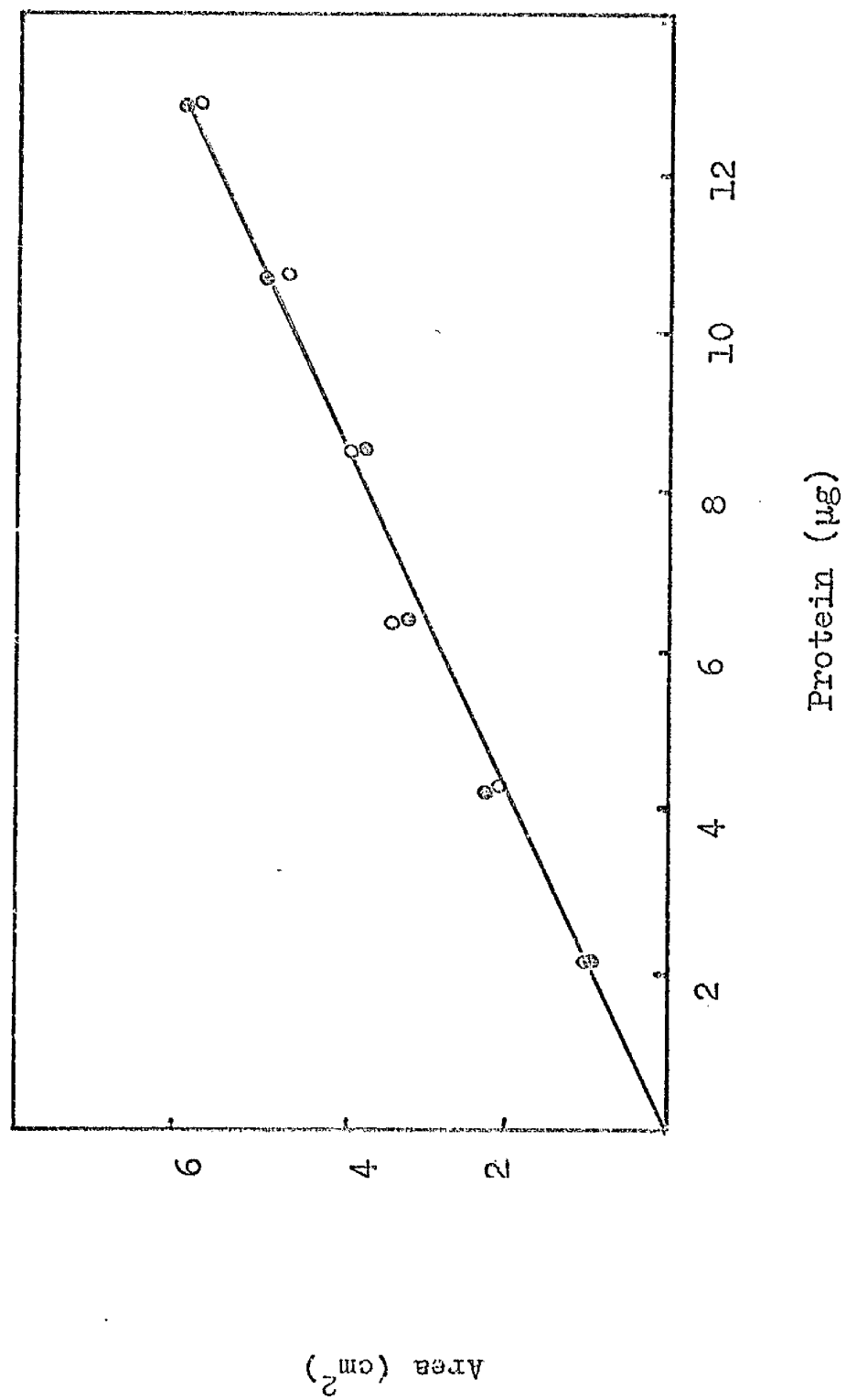


FIG. 3.13

FIGURE 3.13 DENATURATION STUDIES ON RNA POLYMERASE

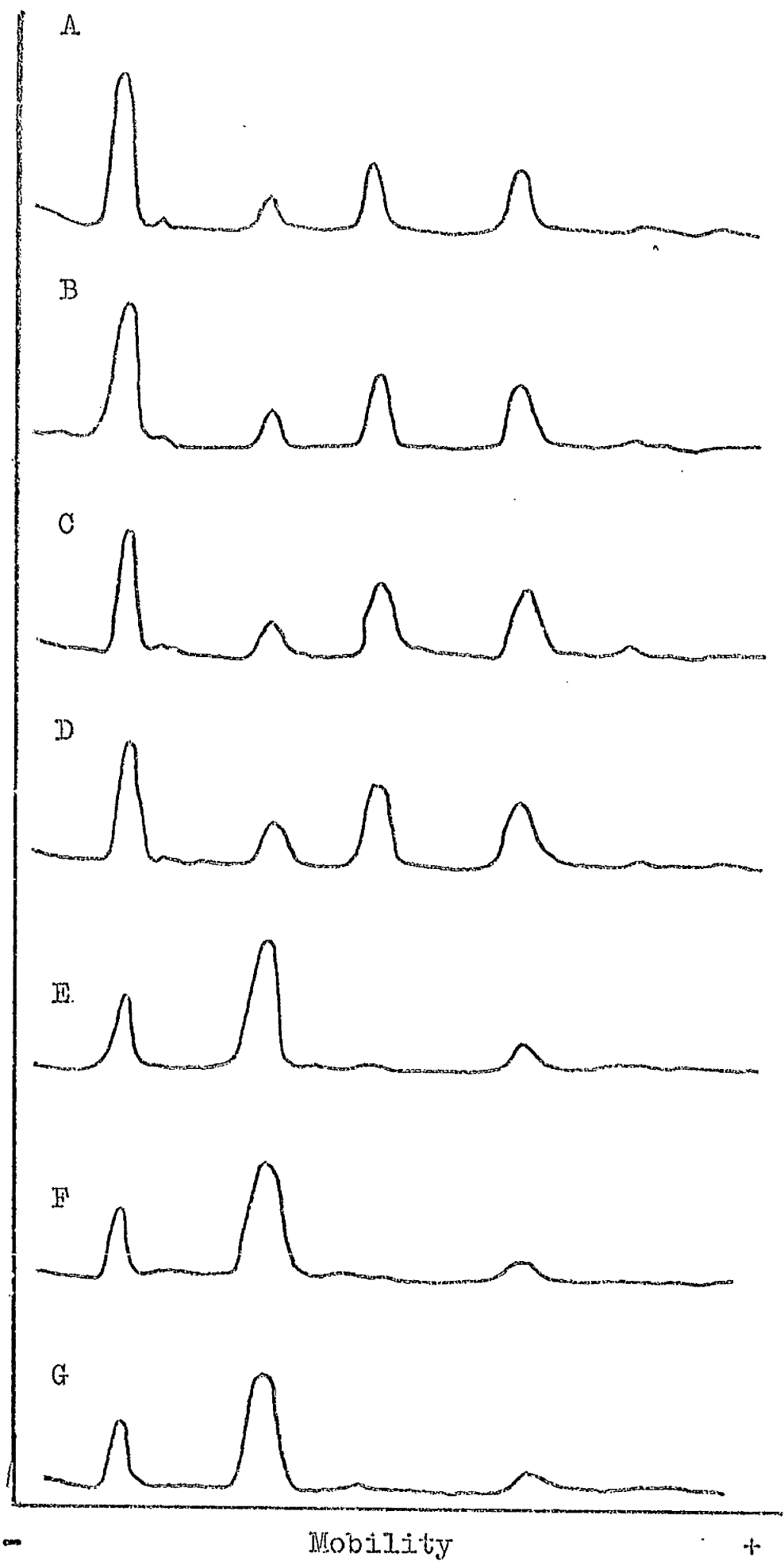
These profiles are taken from Vitatron densitometer traces. RNA polymerase holoenzyme samples were treated for 24h at 20°C with 6M-guanidine hydrochloride (A), 8M-urea (B), and 0.01M-NaOH (C), dialysed against 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 0.1M-sodium phosphate, pH 7.1, for 16h, then 20µg samples of each, including a control (D), which had received no denaturation pretreatment were electrophoresed and analysed by the procedure described in section 2.2.6.3. Denaturation led to no breakdown or dissociation of the σ subunit nor any other RNA polymerase subunit.

E and F show a similar experiment with 6M-guanidine hydrochloride and 8M-urea respectively on peak B protein from phosphocellulose chromatography of holoenzyme (see section 3.3.3) by the method of Bautz et al. (1970). G was a control and received no pretreatment. No dissociation of σ occurred.

The RNA polymerase holoenzyme sample used in the above experiments (A to D) had a lower σ content than that usually prepared.

Electrophoresis is from left to right.

Relative Absorbance



Relative Absorbance

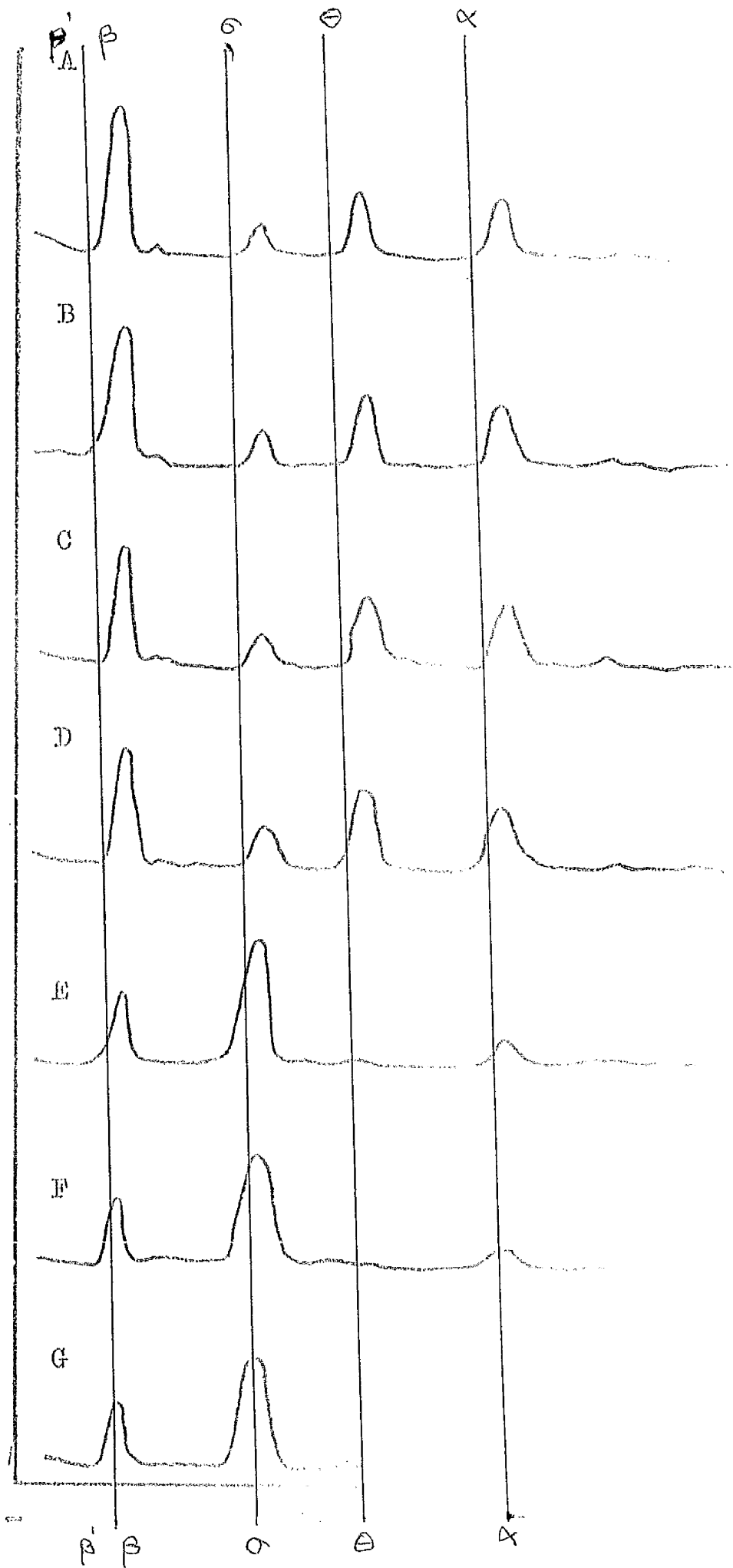


FIG. 3.14

FIGURE 3.14 GRADIENT ELUTION OF RNA POLYMERASE FROM
A COLUMN OF PHOSPHOCELLULOSE

10mg of RNA polymerase holoenzyme was dialysed 16h at 4°C against buffer C + 0.05M-KCl and applied to a 1.5X8.0cm column of Whatman PC 11 phosphocellulose prepared as described in section 2.2.4.1 and equilibrated with buffer C + 0.05M-KCl. The RNA polymerase was pumped onto the column at a rate of 6ml/h. When all the washthrough material (peak A) had come through, the column was then eluted at 10ml/h with a 0.05 to 0.40M-KCl gradient in buffer C. 2.2ml fractions were collected.

●—● Absorbance at 280nm on an LKB Uvicord II.

----- [KCl] in buffer C.

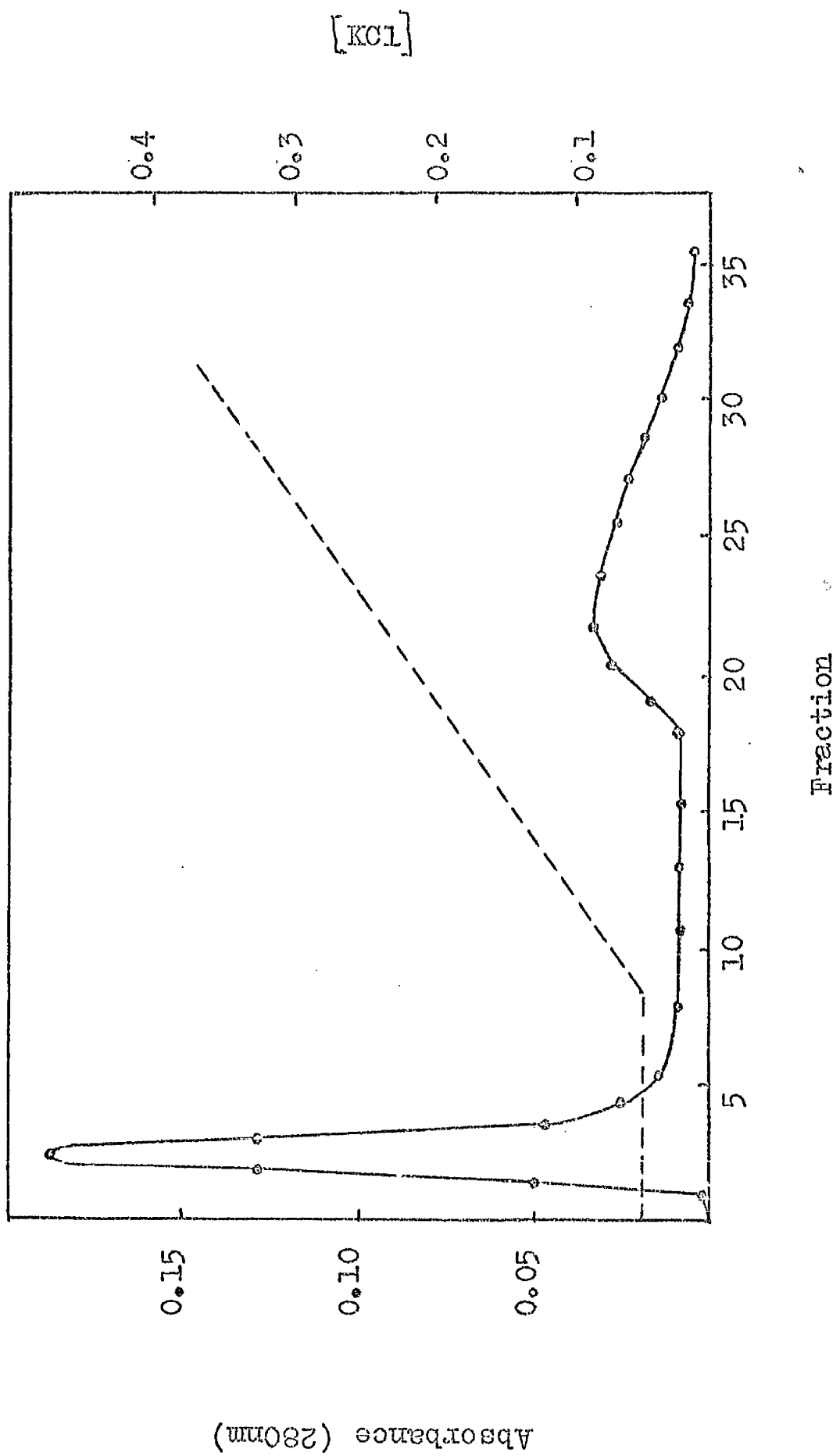


FIG. 3.15

FIGURE 3.15 STEPSWISE ELUTION OF RNA POLYMERASE FROM
A COLUMN OF PHOSPHOCELLULOSE

10mg of RNA polymerase was dialysed against buffer C + 0.05M-KCl for 16h at 4°C before application to a 2.5X4.0cm column of Whatman PC 11 phosphocellulose prepared as described in section 2.2.4.1. The enzyme was pumped onto the column at 6ml/h and when all of the flowthrough material (peak A) had been eluted the column was washed with buffer C + 0.25M-KCl until all of peak B was eluted, then finally peak C was eluted with buffer C + 0.40M-KCl, both at a flow rate of 24ml/h. 2.2ml fractions were collected.

○—○ Absorbance at 280nm in an LKB Uvicord II.

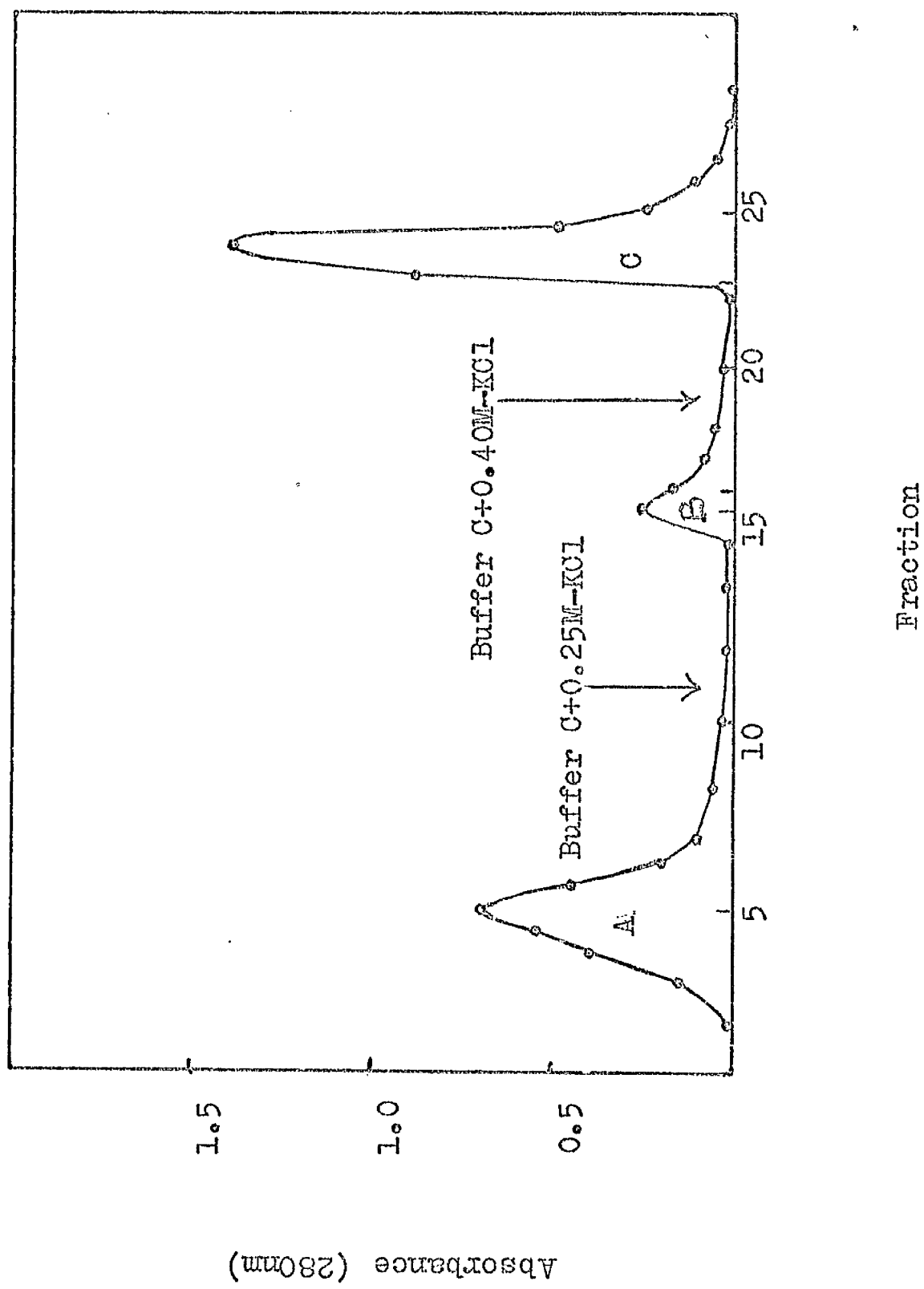
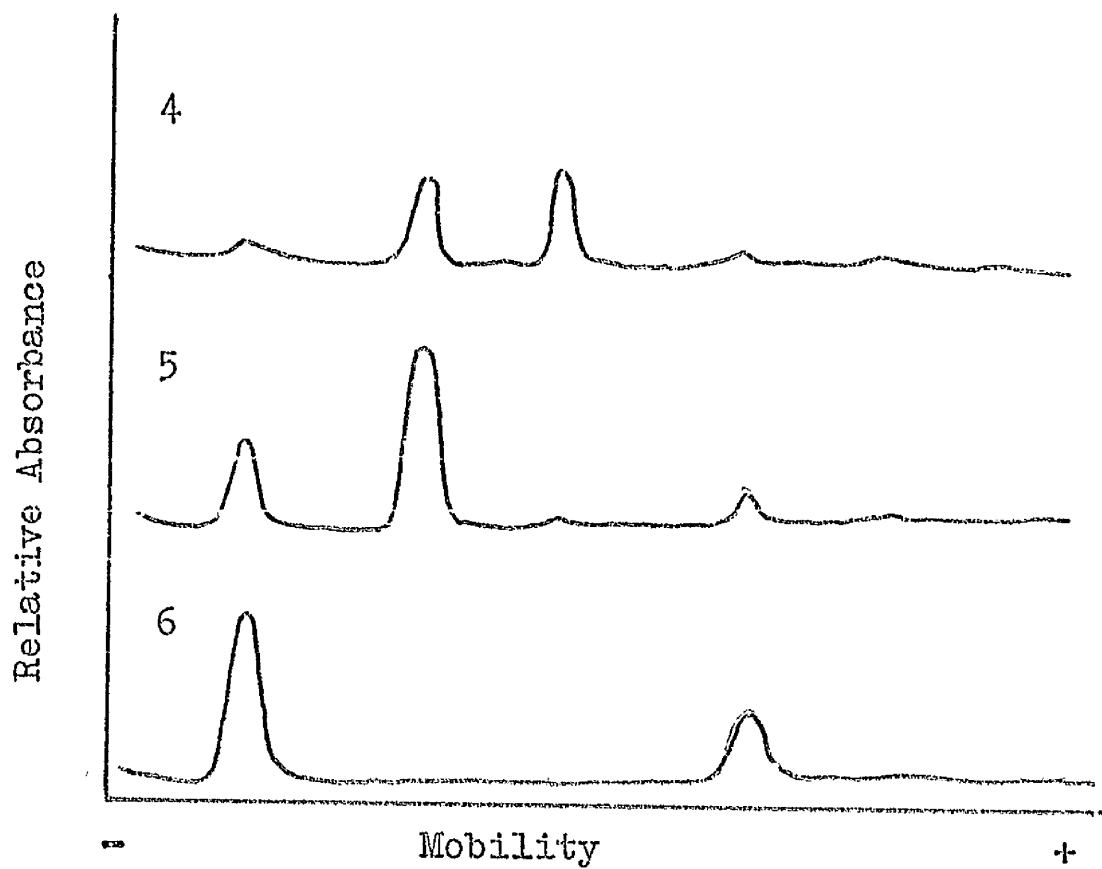
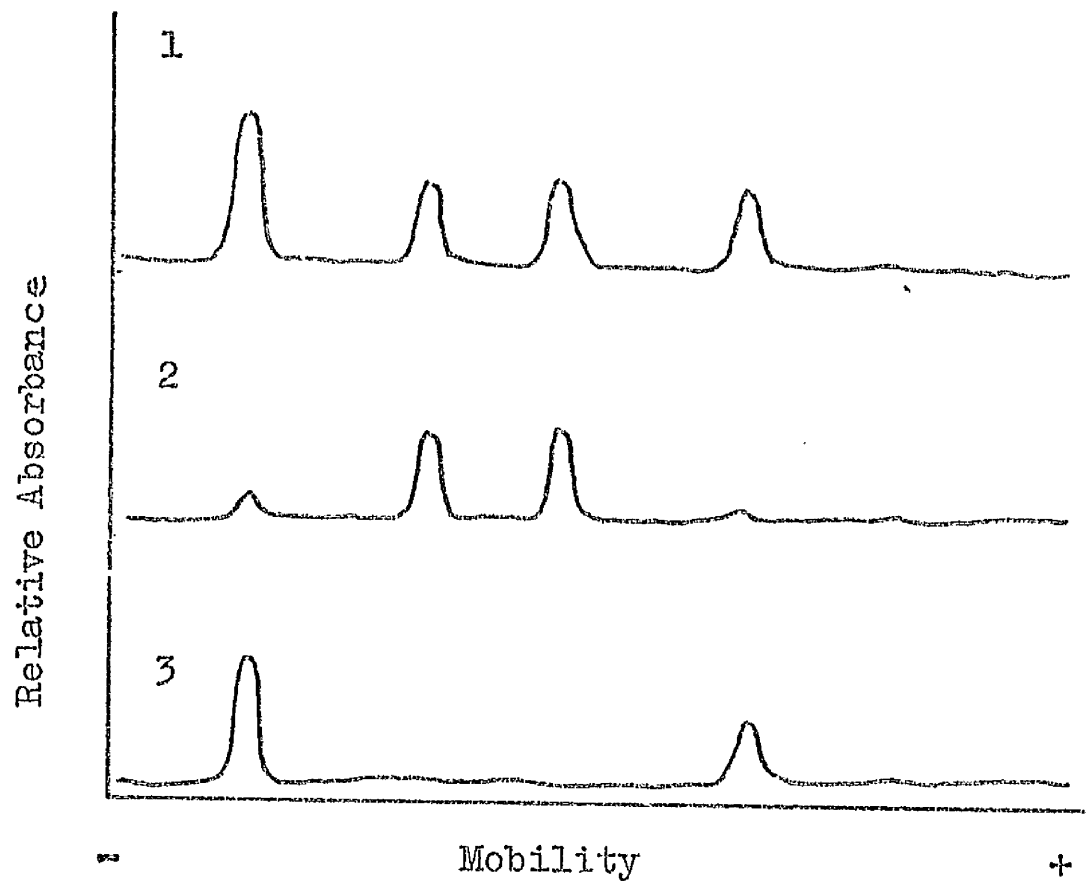


FIG. 3.16

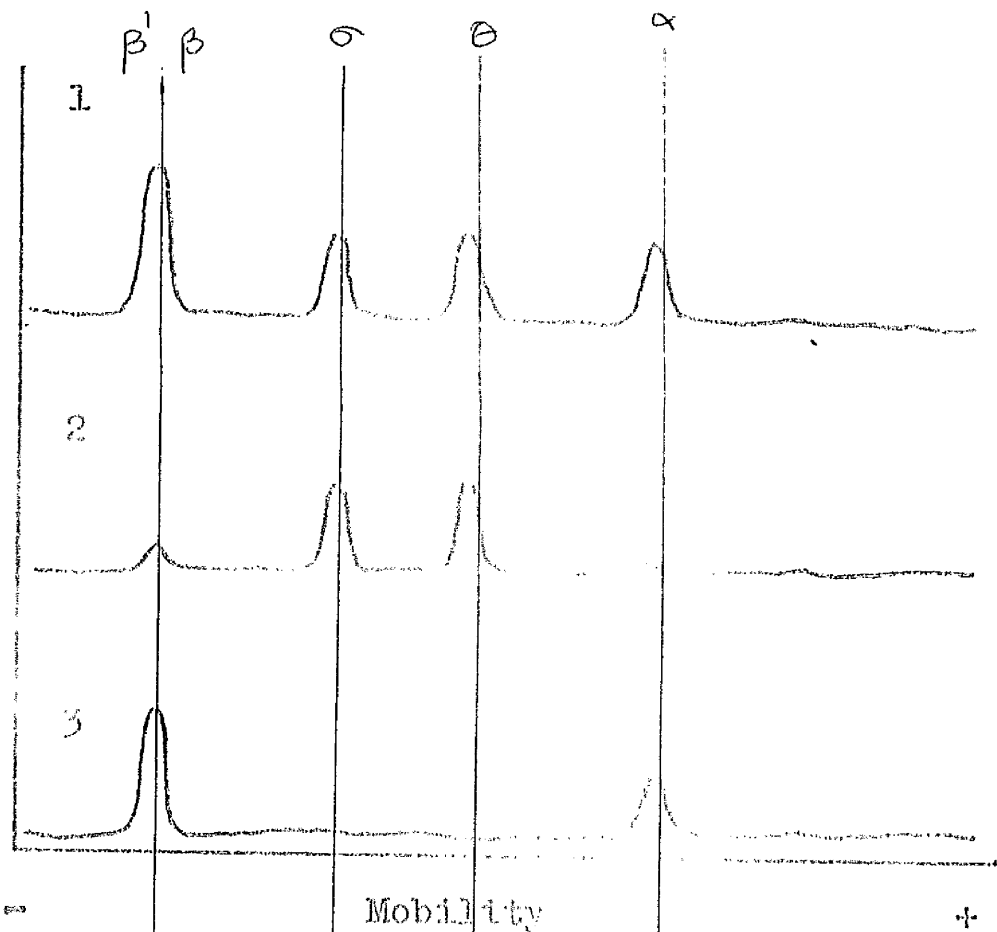
FIGURE 3.16 SDS-POLYACRYLAMIDE GELS OF THE ELUATE FROM
PHOSPHOCELLULOSE CHROMATOGRAPHY OF RNA POLMERASE

All SDS-polyacrylamide gel profiles are taken from Vitatron recording densitometer traces.

- (1) RNA polymerase holoenzyme,
- (2) Peak A from the gradient elution of the phosphocellulose column showing that mainly θ and σ are present with only a trace amount of the other subunits.
- (3) Peak B from the gradient elution from the phosphocellulose column, only the core enzyme subunits are present- β' , β and α .
- (4) Peak A from the stepwise elution of the phosphocellulose column. This peak contains mainly θ and σ with trace core enzyme subunits.
- (5) Peak B from the stepwise elution of the phosphocellulose σ is the predominant subunit, β and α are also present.
- (6) Peak C from the stepwise elution of the phosphocellulose column, only the core enzyme subunits β' , β and α are present.



Relative Absorbance



Relative Absorbance

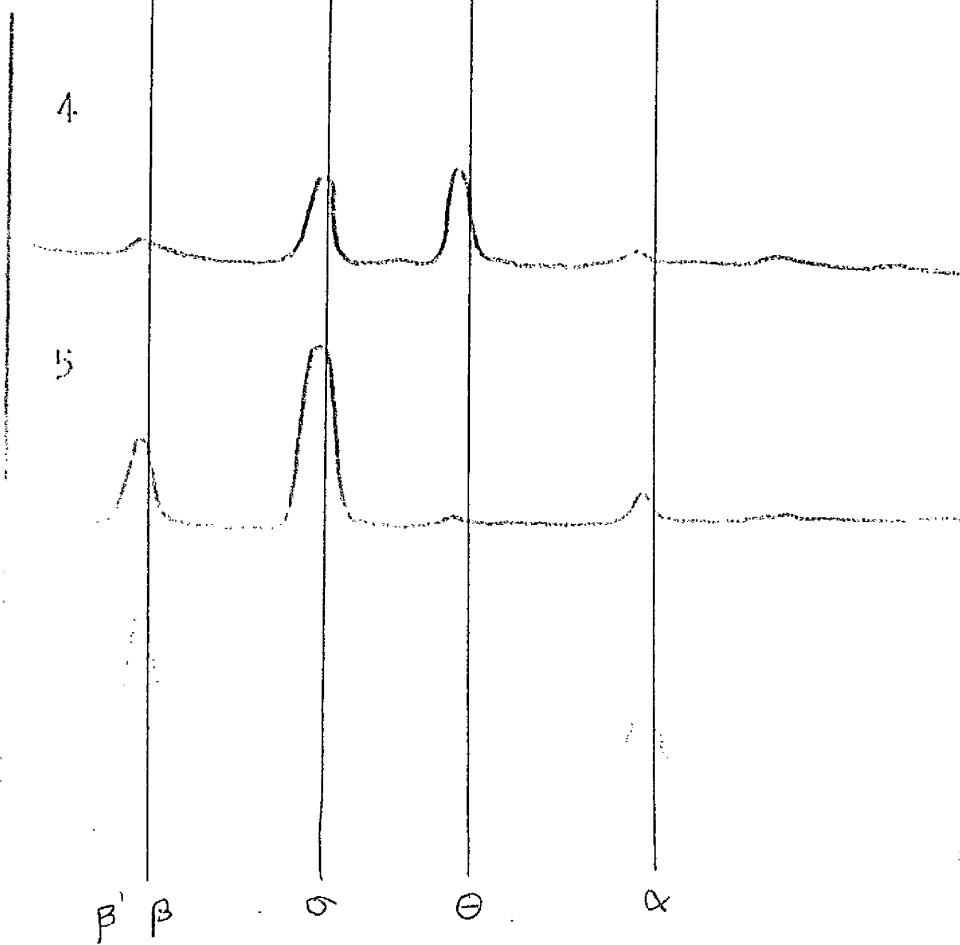


FIG. 3.17

FIGURE 3.17 HYDROXYAPATITE CHROMATOGRAPHY OF RNA POLYMERASE

10mg of RNA polymerase holoenzyme was dialysed 16h against 0.04M-sodium phosphate buffer, pH 7.0, containing 5% (v/v) glycerol and 1mM-dithiothreitol before application to a 1.5X8.0cm column of hydroxyapatite (Biogel HTP) equilibrated with the same buffer (section 2.2.4.3). The RNA polymerase was eluted at 24ml/h with a gradient of 0.04 to 0.16M-sodium phosphate, pH 7.0, in 5% (v/v) glycerol, 1mM-dithiothreitol. 2.2ml fractions were collected.

o-----o Absorbance at 280nm in a 1cm path length cell in a Cary 15 recording spectrophotometer.

----- M-sodium phosphate, pH 7.0.

[sodium phosphate]

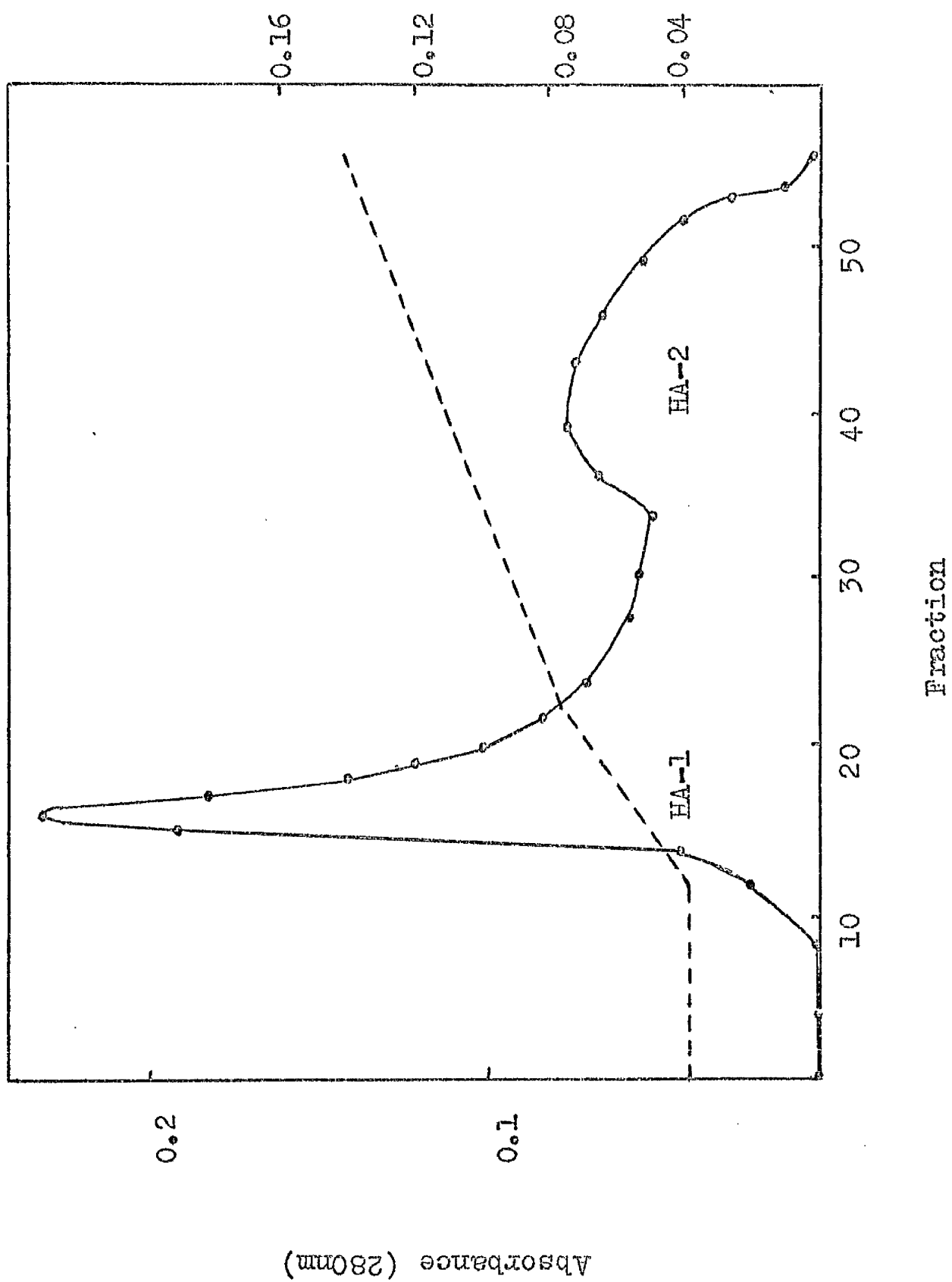


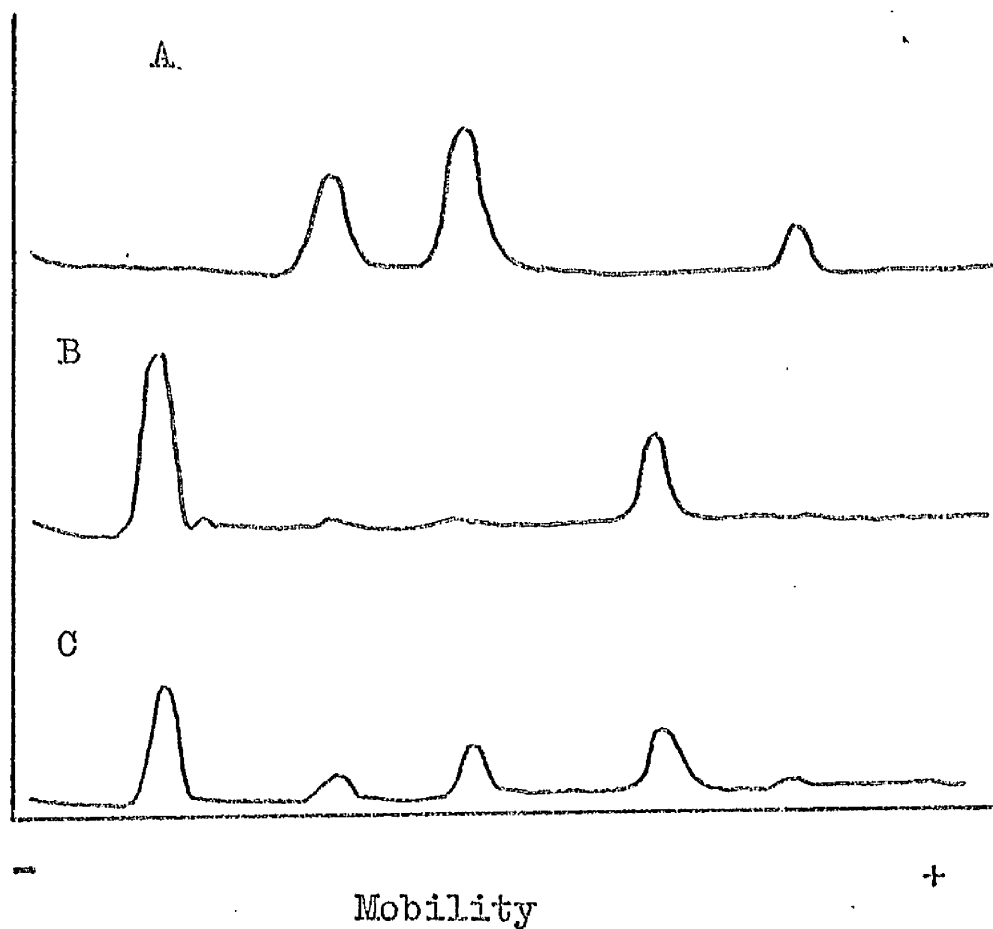
FIG. 3.18

FIGURE 3.18 SDS-POLYACRYLAMIDE GELS OF THE ELUATE FROM
HYDROXYAPATITE CHROMATOGRAPHY OF RNA POLYMERASE

These gel profiles are taken from the Vitatron recording densitometer traces of the SDS-polyacrylamide gels.

- (A) Peak HA-1 from the hydroxyapatite chromatography of RNA polymerase. This peak contains mainly θ and σ subunits of the enzyme, some γ is also present.
- (b) Peak HA-2 from the hydroxyapatite chromatography of RNA polymerase, mainly core enzyme subunits are present- β' , β and α but there is also trace θ and σ .
- (C) Native holoenzyme before hydroxyapatite chromatography.

Relative Absorbance



Relative Absorbance

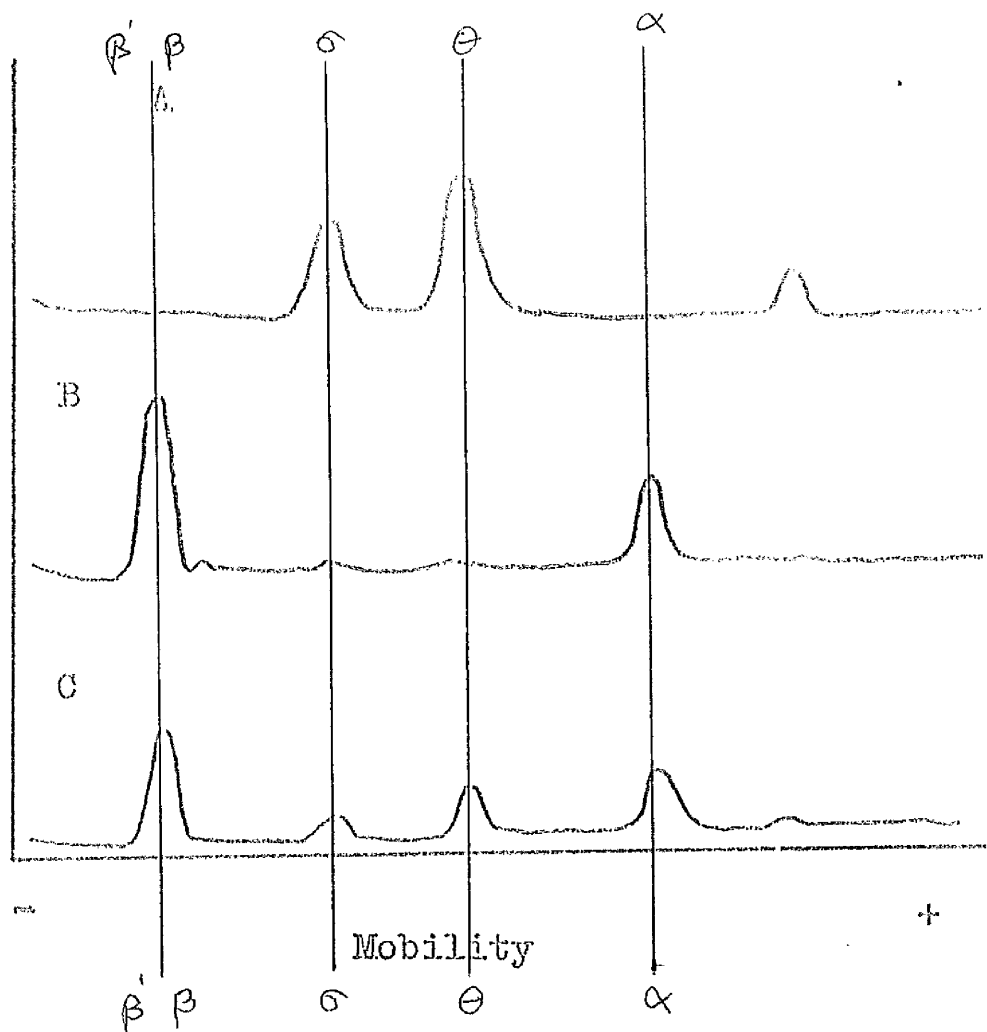


FIG. 3.19

FIGURE 3.19 ANALYTICAL DEAE-CELLULOSE CHROMATOGRAPHY OF
RNA POLYMERASE

10mg of RNA polymerase holoenzyme was dialysed 16h at 4°C against buffer A before application to a column of DEAE-cellulose equilibrated with buffer A prepared as described in section 2.2.4.2. The RNA polymerase was applied at 24ml/h and washed in with 20ml of buffer A before elution with buffer A + 0.16M-KCl and buffer A + 0.23M-KCl at the same rate. 2.2ml fractions were collected.

○——○ Absorbance at 280nm in a 1cm path length cell in
Cary 15 recording spectrophotometer.

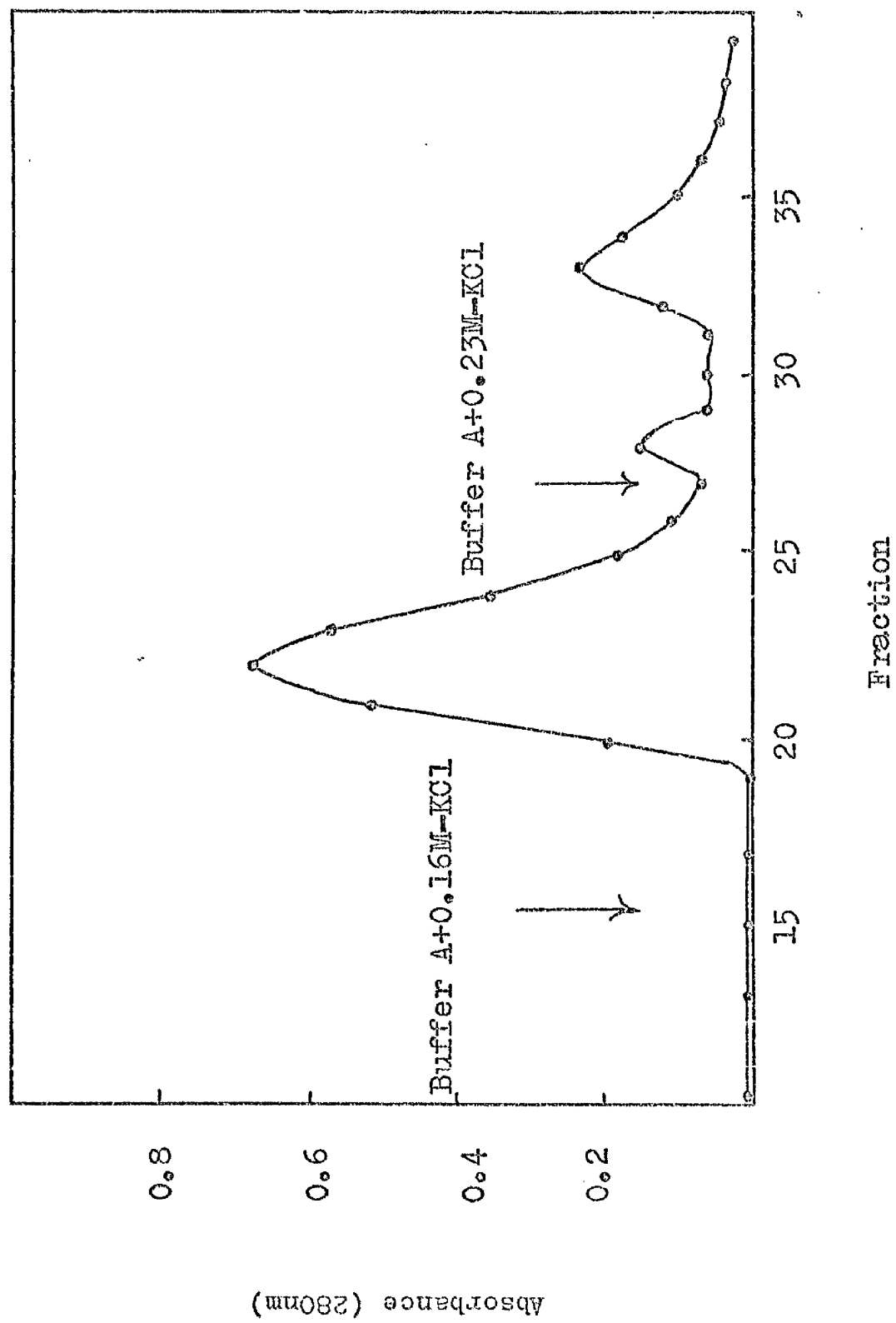


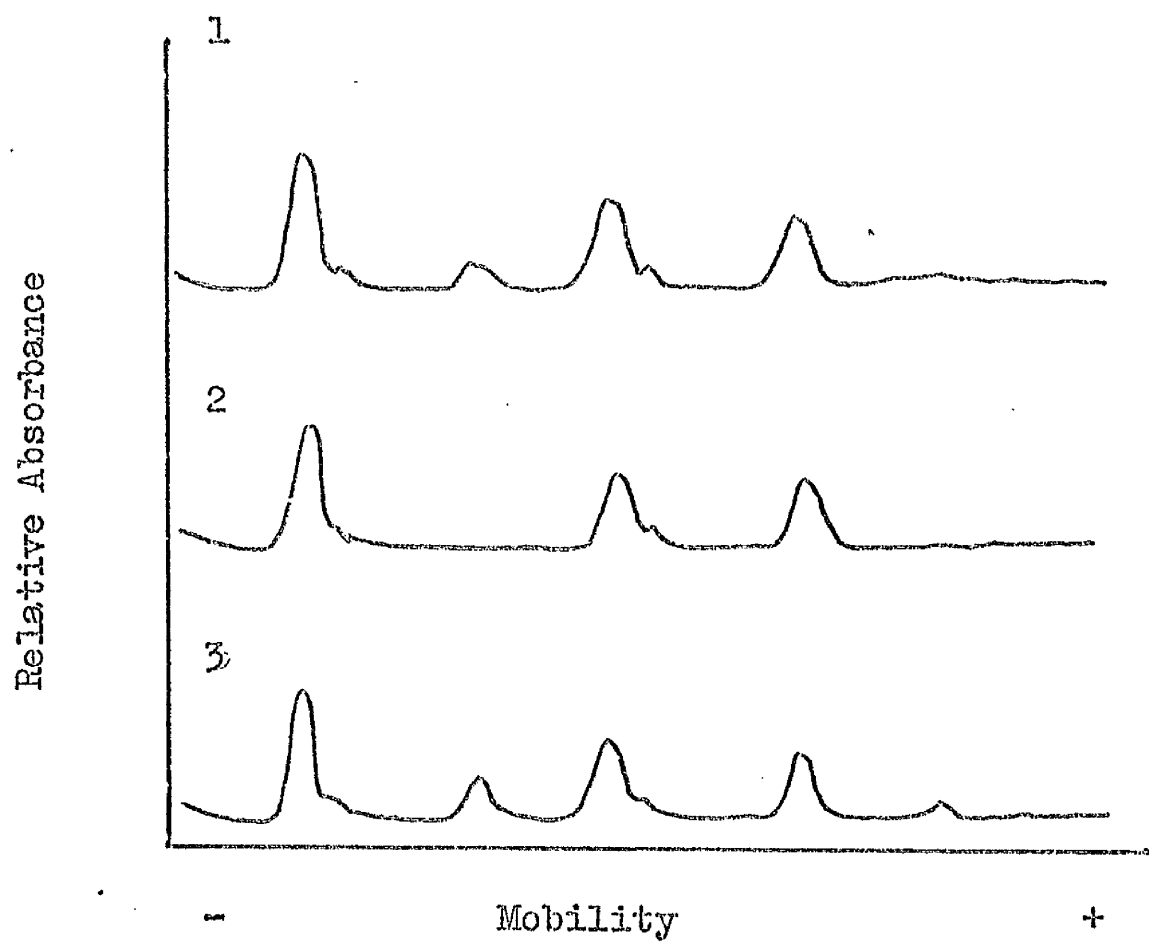
FIG. 3.20

FIGURE 3.20 SDS-POLYACRYLAMIDE GELS OF THE ELUATE FROM
THE ANALYTICAL DEAE-CELLULOSE CHROMATOGRAPHY
OF RNA POLYMERASE

- (1) SDS-polyacrylamide gel profile of the RNA polymerase holoenzyme applied to the DEAE-cellulose column.
- (2) Gel profile of peak B material. The subunits β' , β , α and θ are present, σ is absent.
- (3) Gel profile of peak A material. All the subunits β' , β , α , σ and θ are present. The ratio of σ to θ has increased.

These SDS-polyacrylamide gel profiles are taken from Vitatron recording densitometer traces.

Electrophoresis is from left to right.



Relative abundance

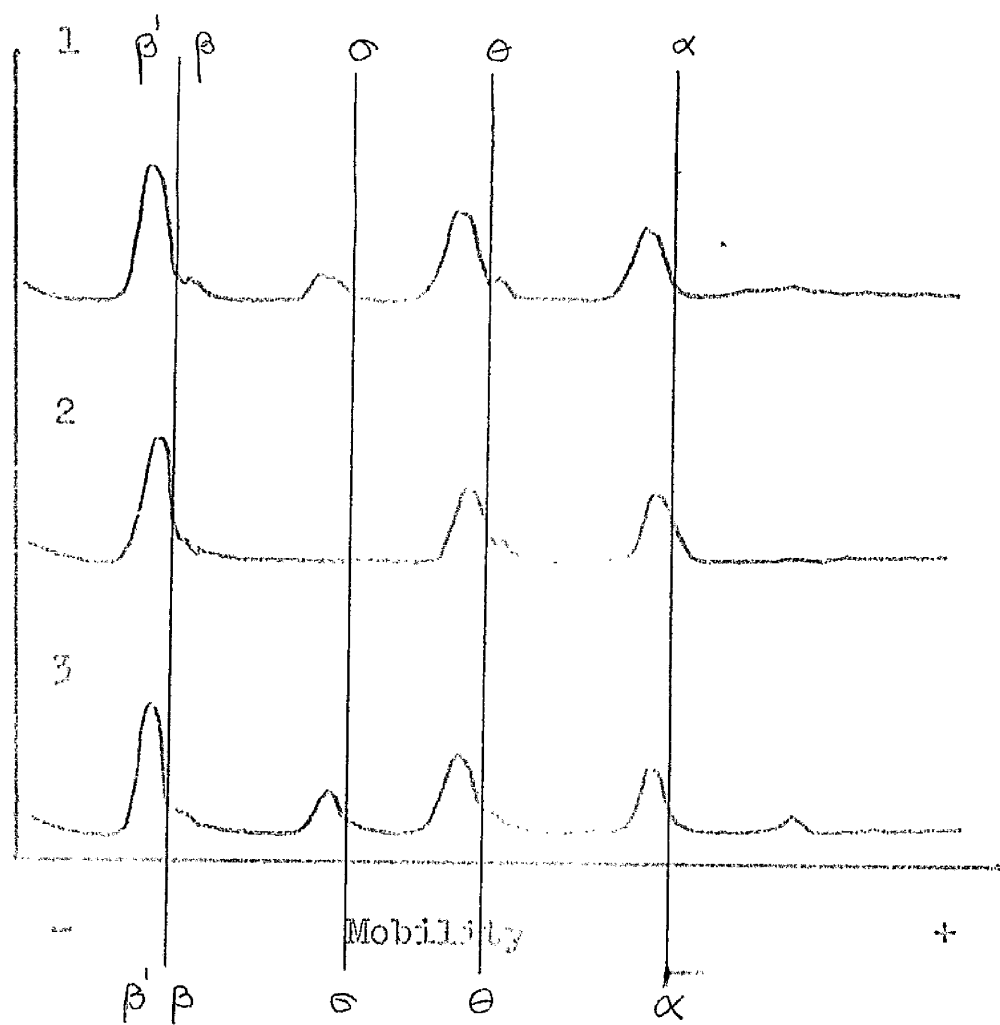
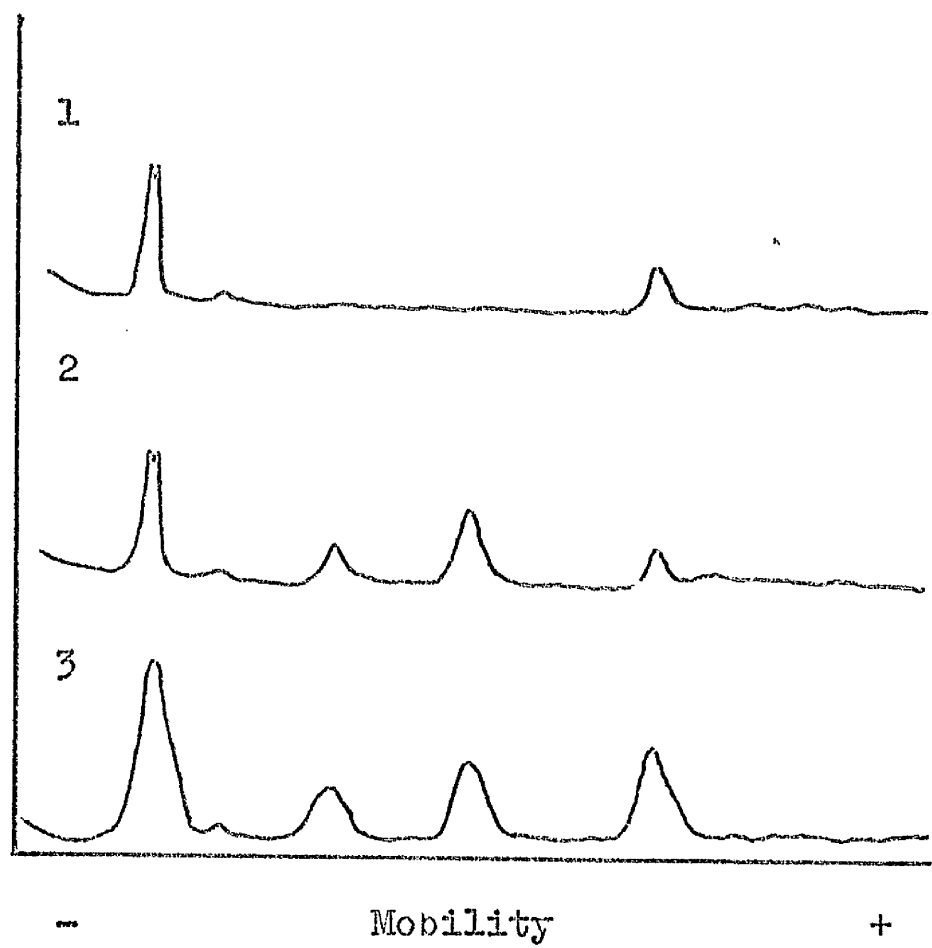


FIG. 3.21

FIGURE 3.21 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS :
SIGMA-RELEASE EXPERIMENT

- (1) SDS-polyacrylamide gel electrophoresis of the supernate in the control reaction tube which contained no ribonucleoside triphosphates and in which no RNA synthesis occurred. The subunits present are β' , β and α . As the amount of DNA added was sufficient to bind all of the RNA polymerase molecules these subunits must either be free core enzyme which has lost the ability to bind to DNA or they are free RNA polymerase subunits.
- (2) Supernate of the reaction mixture actively synthesising RNA. Here too we have β' , β and α in the same concentration as the control tube (1), but we also have σ and θ present which have been released from the holoenzyme by active RNA synthesis. These two subunits σ and θ are present in the same ratio as they are in the RNA polymerase holoenzyme used for the experiment. This indicates that both σ and θ are released to the same extent by RNA synthesis.
- (3) RNA polymerase holoenzyme.

Relative Absorbance



Relative Absorbance

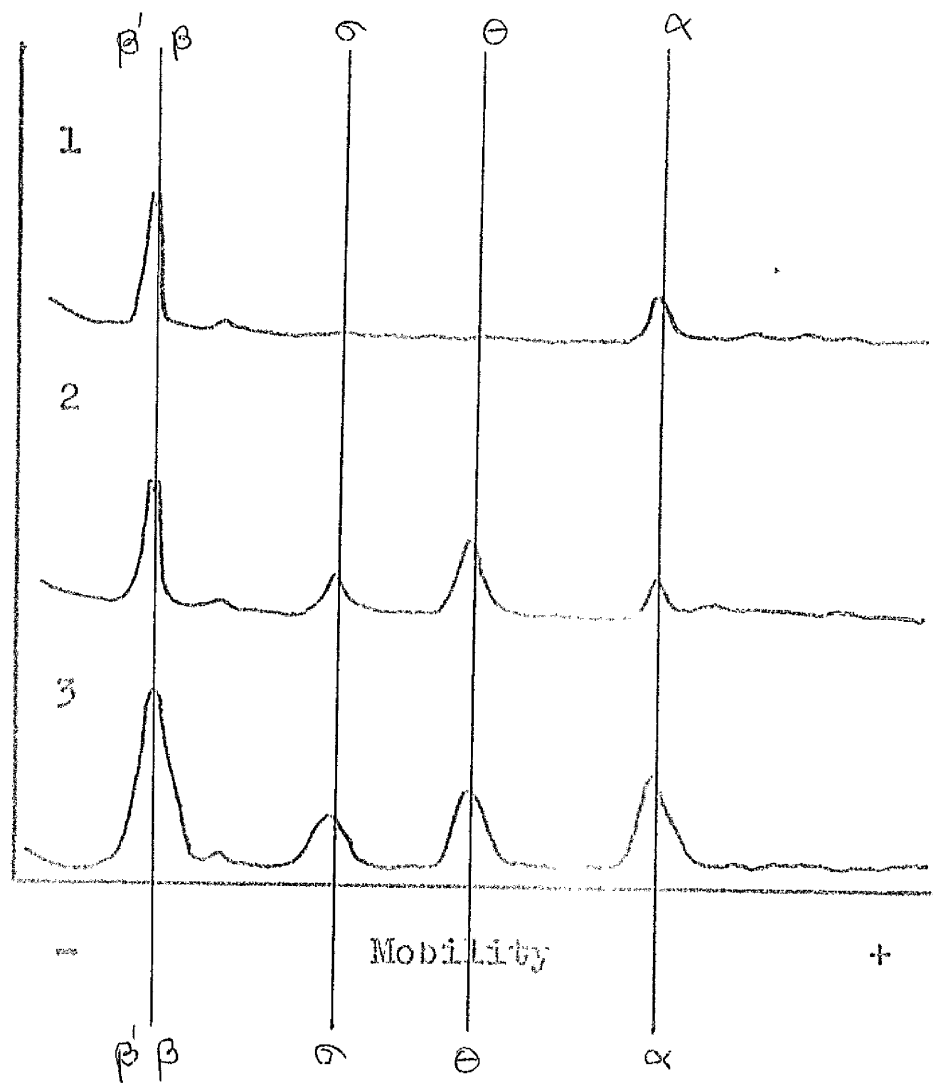


FIG. 3.22

FIGURE 3.22 ANALYSIS OF THE BIOGEL A5M AGAROSE ELUATE

400mg of fraction 4 RNA polymerase (see section 2.2.3) in 10ml of buffer C were applied to a 5X95cm column of Biogel A5M equilibrated with buffer C and eluted at 60ml/h with the same buffer. 6ml fractions were collected.

0.2ml aliquots of the large non-RNA polymerase protein peak were added to a standard RNA polymerase holoenzyme ^3H -GTP incorporation assay (section 2.2.3) using E.coli DNA as template. Complete inhibition of RNA synthesis was achieved by fractions coinciding with the protein peak.

o——o Absorbance at 280nm in the 3mm path length cell of an LKB Uvicord II.

o---o ^3H -GTP (dpm) incorporated into RNA.

³H-GTP incorporated into RNA (dpm)

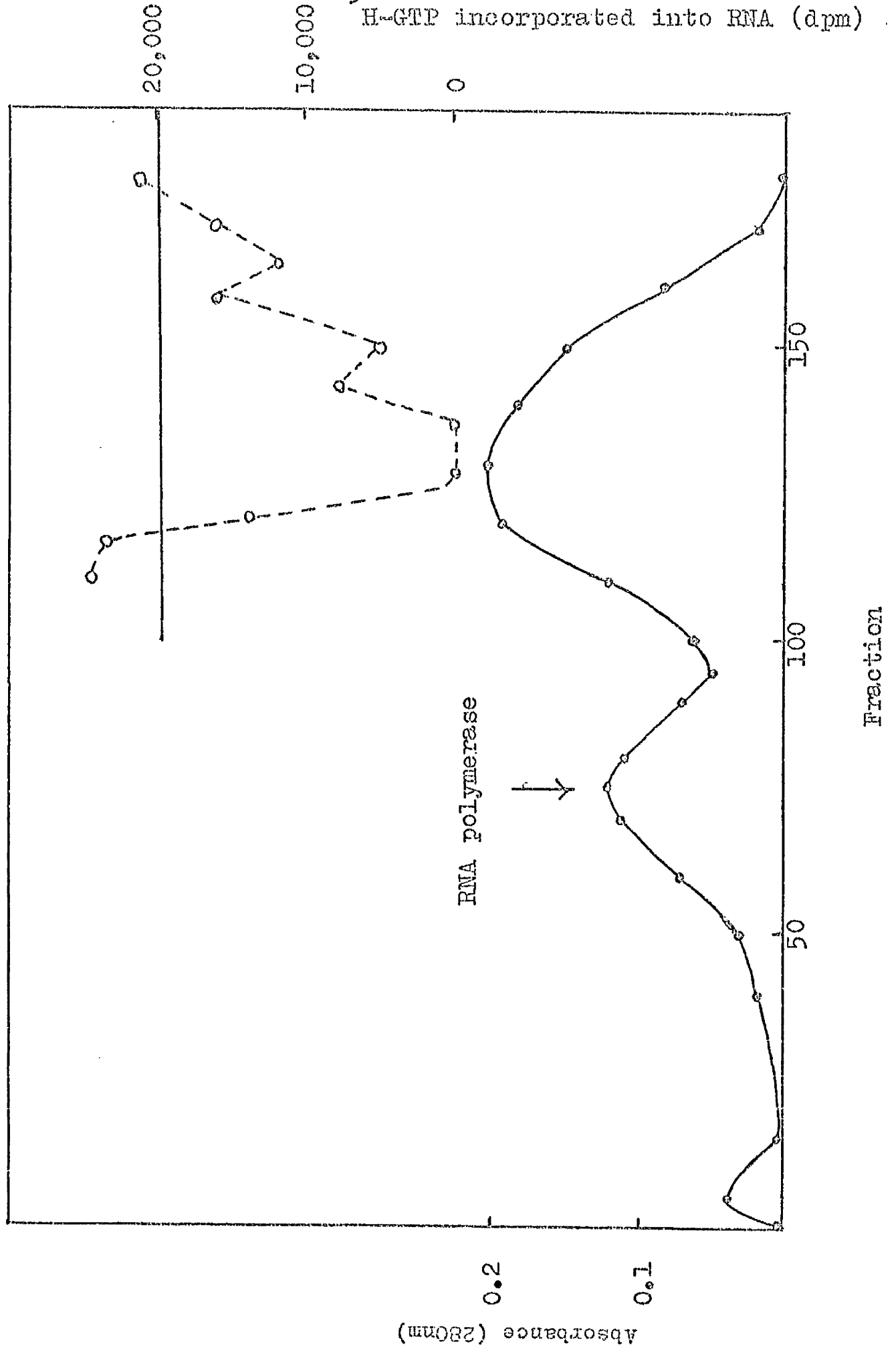
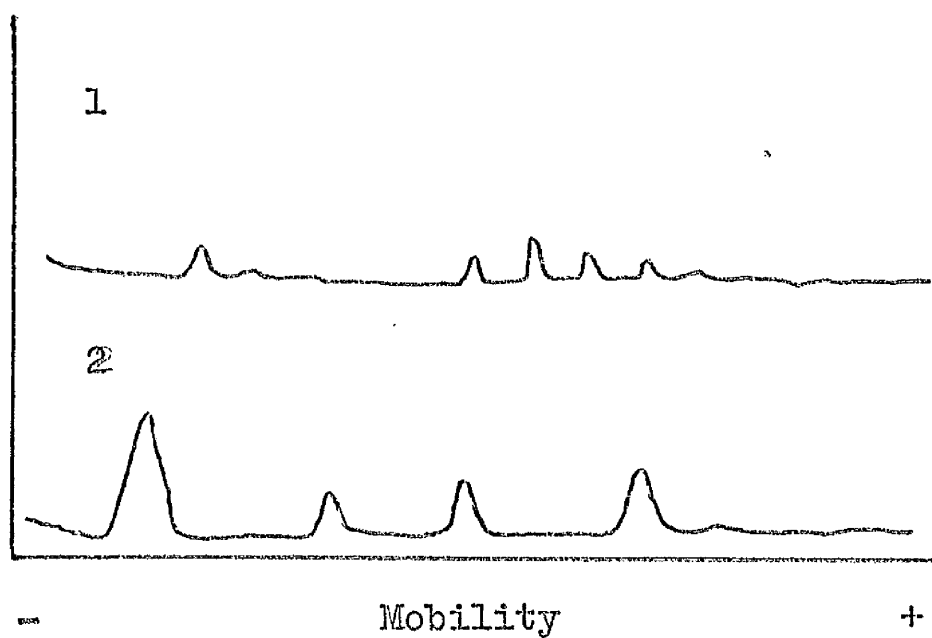


FIG. 3.23

FIGURE 3.23 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
THE RNA POLYMERASE INHIBITOR PROTEIN

- (1) A 20 μ g sample of the inhibitor protein peak prepared and electrophoresed as described in section 2.2.6.3. There are many polypeptide species present.
- (2) 20 μ g of RNA polymerase holoenzyme electrophoresed in a parallel gel . . There are apparently no common polypeptide species.

Relative Absorbance



Relative Absorbance

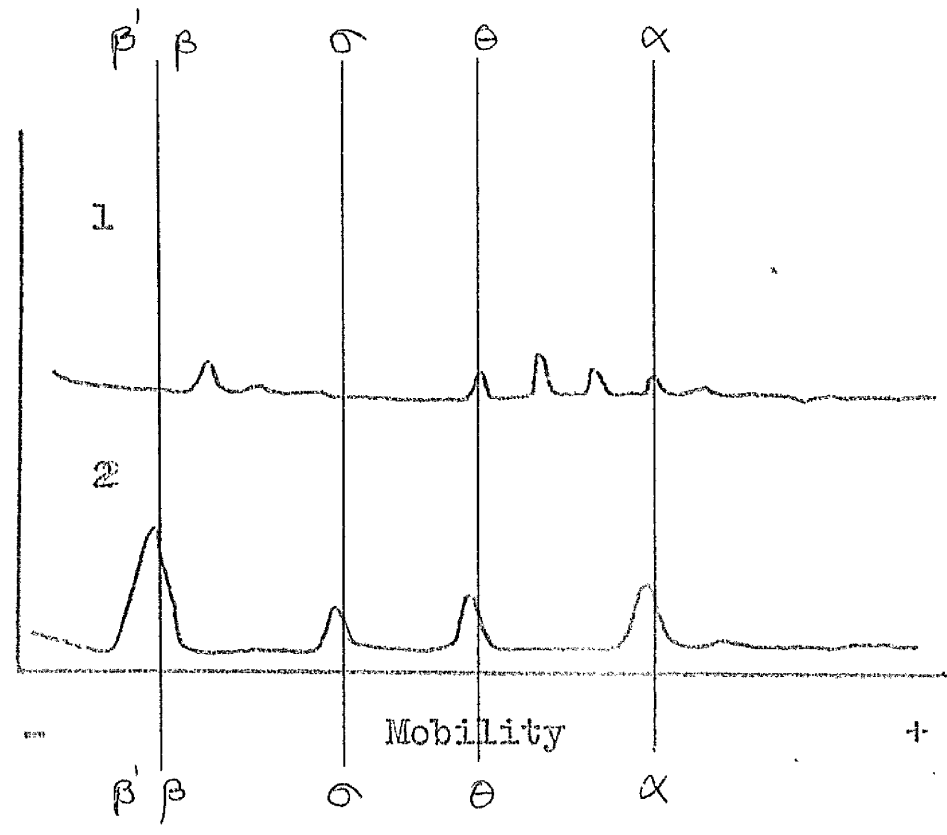


FIG. 3.24

FIGURE 3.24 DEAE-CELLULOSE CHROMATOGRAPHY OF RNA POLYMERASE
INHIBITOR PROTEIN

Protein (200mg) from an ammonium sulphate precipitation of the RNA polymerase inhibitor peak from the Biogel A5M column was dialysed 16h at 4°C against buffer A before application to a 2.5X8.0cm column of DEAE-cellulose equilibrated with buffer A. The protein was washed into the DEAE-cellulose with 20ml of buffer A before elution with a 0.0M-KCl to 0.4M-KCl gradient in buffer A at 24ml/h. 6ml fractions were collected.

o——o Absorbance at 280nm in a 1cm path length cell in
a Cary 15 recording spectrophotometer.

o---o ³H-GTP (measured as dpm) incorporated into RNA
by the standard RNA polymerase assay procedure
with T7 DNA as template (section 2.2.3).

----- Molar KCl concentration in buffer A.

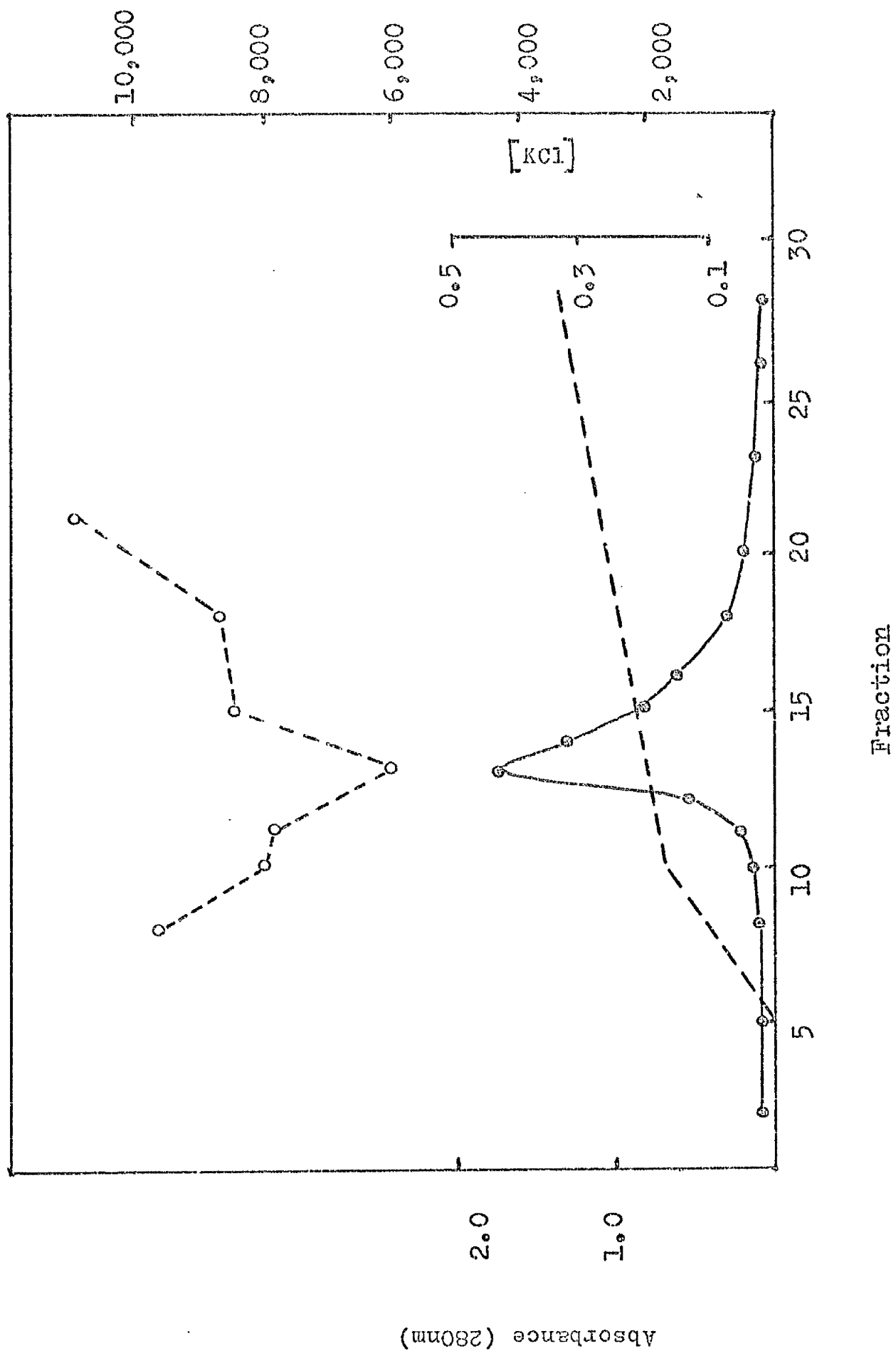


FIG. 3.25

FIGURE 3.25 ULTRA-VIOLET SPECTRUM OF RNA POLYMERASE
CORE ENZYME

The ultra-violet spectrum of RNA polymerase core enzyme in buffer A + 0.50M-KCl obtained by phosphocellulose chromatography (section 3.3.3) of RNA polymerase holoenzyme prepared by the method of Burgess (1969a), is characterised by a maximum at 275.5nm and a minimum at 251nm. The A_{280}/A_{260} is 1.87 and the protein concentration, determined spectrophotometrically by the method described in section 3.6.2, is 0.84mg/ml.

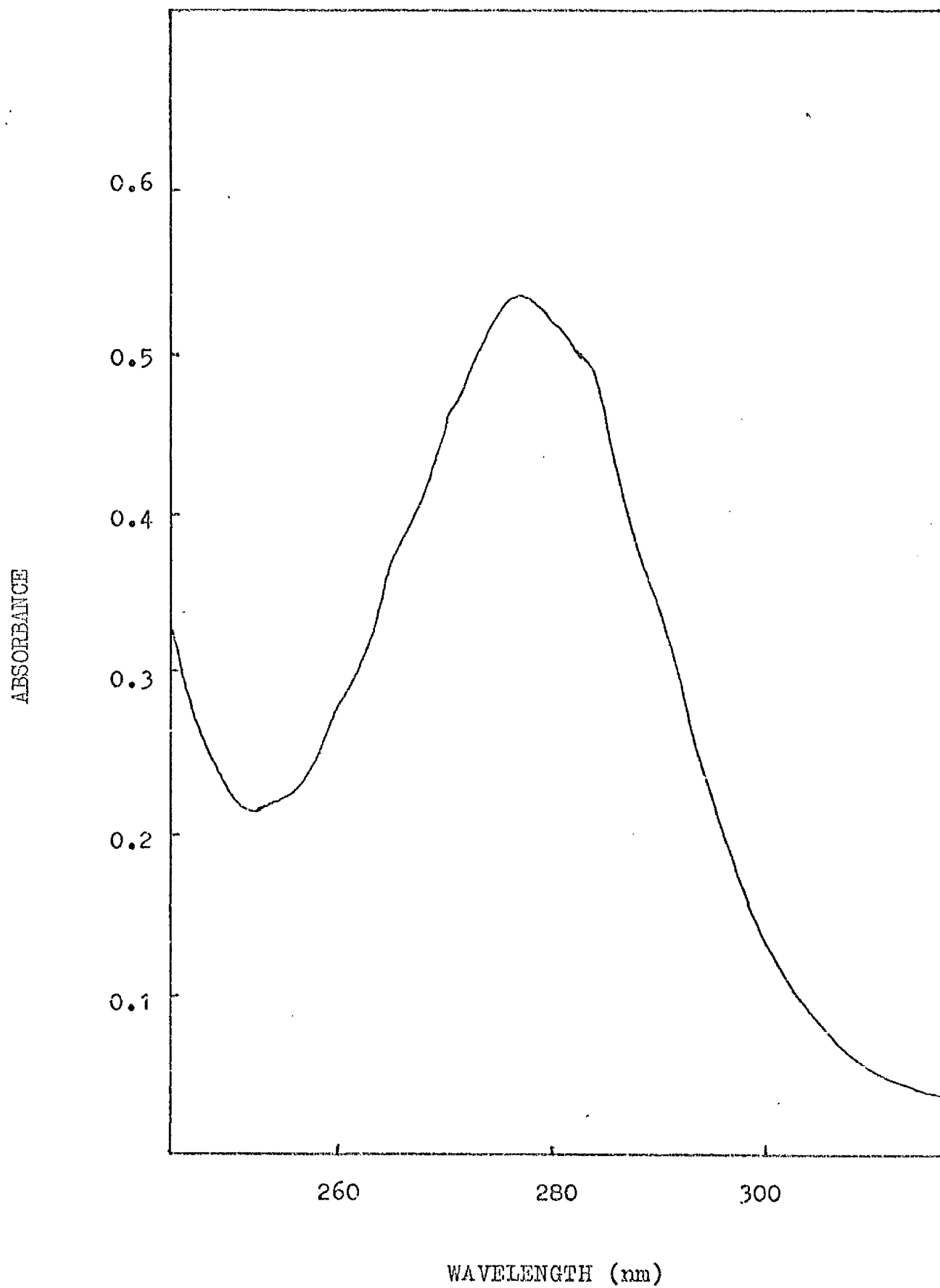


FIG. 3.26

FIGURE 3.26 ULTRA-VIOLET SPECTRUM OF RNA POLYMERASE
HOLOENZYME

The ultra-violet spectrum of RNA polymerase holoenzyme in buffer A + 0.50M-KCl prepared by the method of Burgess (1969a), is characterised by a maximum at 275.5nm and a minimum at 251nm. The A_{280}/A_{260} is 1.60 and the protein concentration, determined from the ultra-violet spectrum by the method described in section 3.6.2, is 0.74mg/ml.

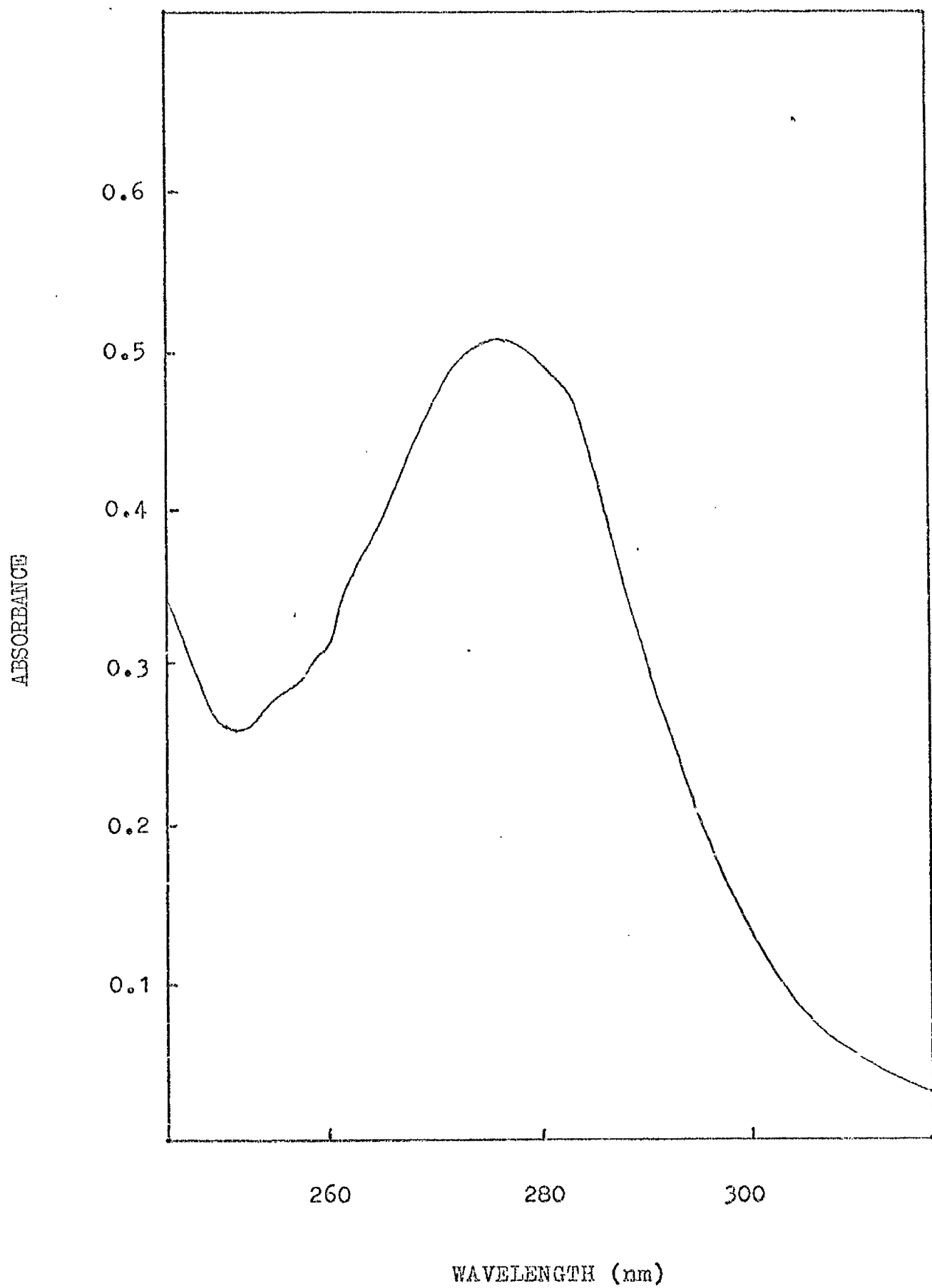


FIG. 3.27

FIGURE 3.27 RELATIONSHIP OF A_{280}/A_{260} OF RNA POLYMERASE
TO PER CENT NUCLEIC ACID CONTAMINATION

The relationship of the A_{280}/A_{260} of E.coli MRE 600 RNA polymerase to the per cent (w/w) deoxyribonucleic contamination. This curve is based on a specific absorbance of 0.65/mg/ml for the enzyme (section 3.6.2). and an A_{280}/A_{260} of 1.85, the highest value obtained for the core enzyme (section 3.6.2). This gives a better measure of the nucleic acid contamination than the Warburg and Christian (1941) formula.

0—0 Relationship of the A_{280}/A_{260} of E.coli
MRE 600 RNA polymerase to per cent DNA
contamination.

⊙—⊙ Warburg and Christian (1941) data.

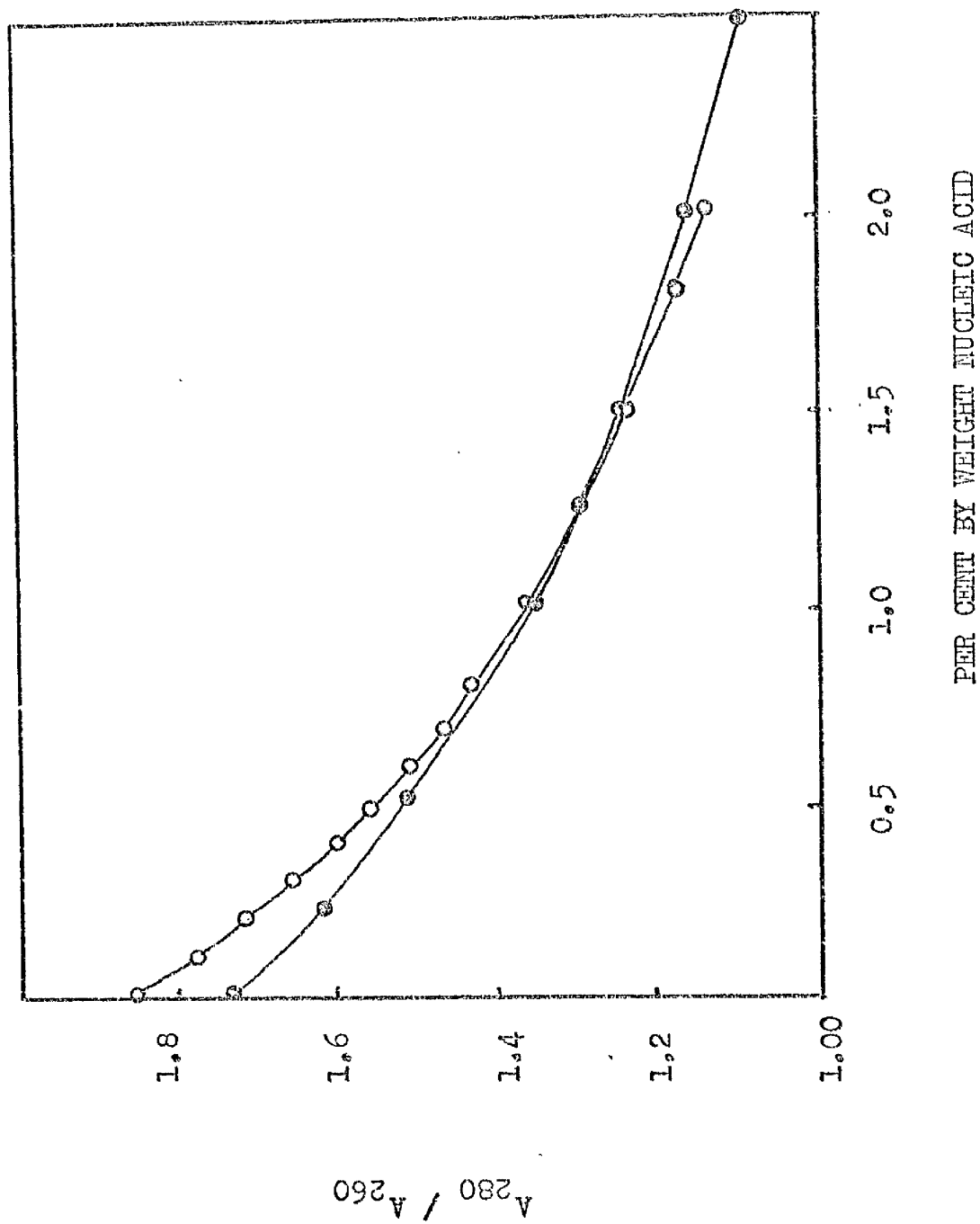


FIG. 3.28

FIGURE 3.28 SPECTROPOLARIMETER CALIBRATION : THE ORD
OF SUCROSE

The Bendix/Bellingham and Stanley Polaromatic 62 spectropolarimeter was calibrated by determining the optical rotatory dispersion of a 0.6378% (w/v) sucrose solution at 20°C in a 1cm path length cell over the wavelength range 240nm to 580nm. This sucrose solution gives a rotation of 50 millidegrees at 546nm in a 1cm path length cell (Harris et al., 1932) (see section 3.8.4).

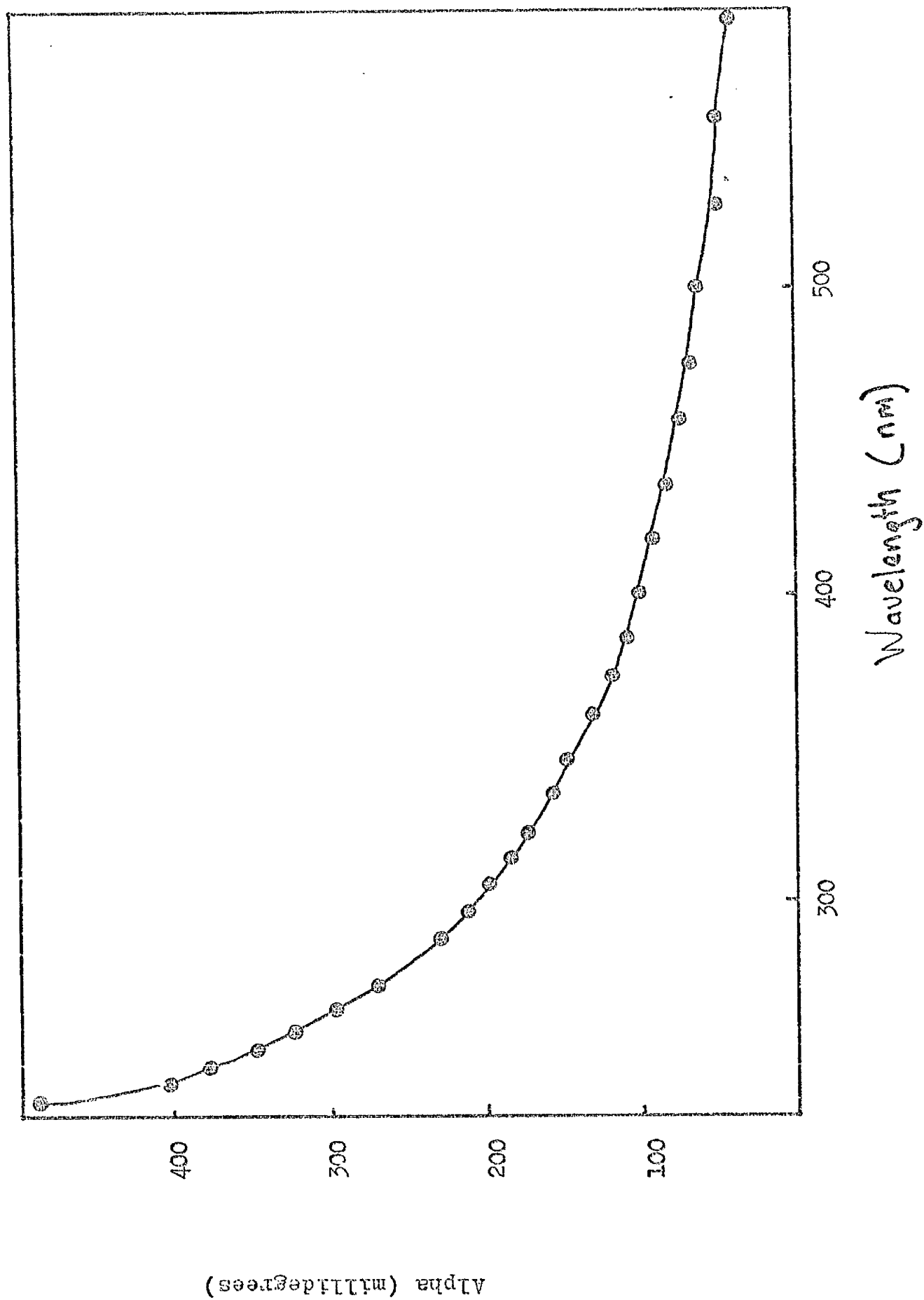


FIG. 3.29

FIGURE 3.29 SPECTROPOLARIMETER CALIBRATION : DRUDE PLOT
OF THE ORD OF SUCROSE

Drude plot of $[\alpha]_{\lambda} \lambda^2$ versus $[\alpha]_{\lambda}$ for a 0.6378% (w/v) sucrose solution over the wavelength range 240nm to 580nm. The value obtained for the intercept, $k = 21.75$ and the square root of the slope $\lambda_c = 0.002269$ are similar to values in the literature (see table 2.3). The wavelength is in microns.

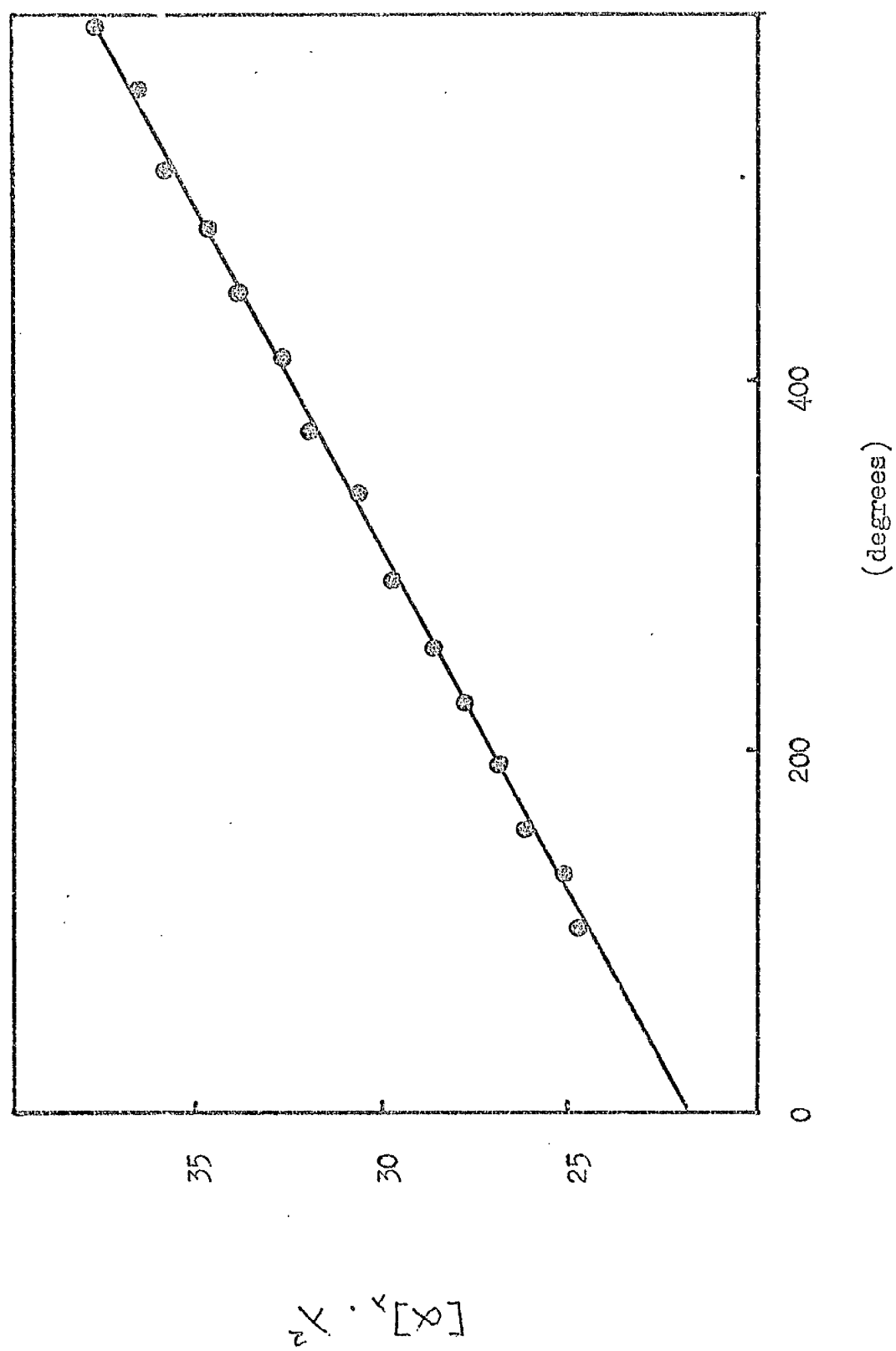


FIG. 3.30

FIGURE 3.30 STRAY LIGHT TEST

Optical rotatory dispersion of a 0.6378% (w/v) sucrose solution in a 1cm path length cell over the wavelength range 230nm to 590nm with and without the presence of a 0.004% (w/v) K_2CrO_4 solution in 0.05M-NaOH in series in a 1cm path length cell. The ORD of the sucrose solution was unaffected by the absorbing K_2CrO_4 solution.

⊙——⊙ ORD of the 0.6378% (w/v) sucrose solution

○——○ Absorbance of the K_2CrO_4 solution.

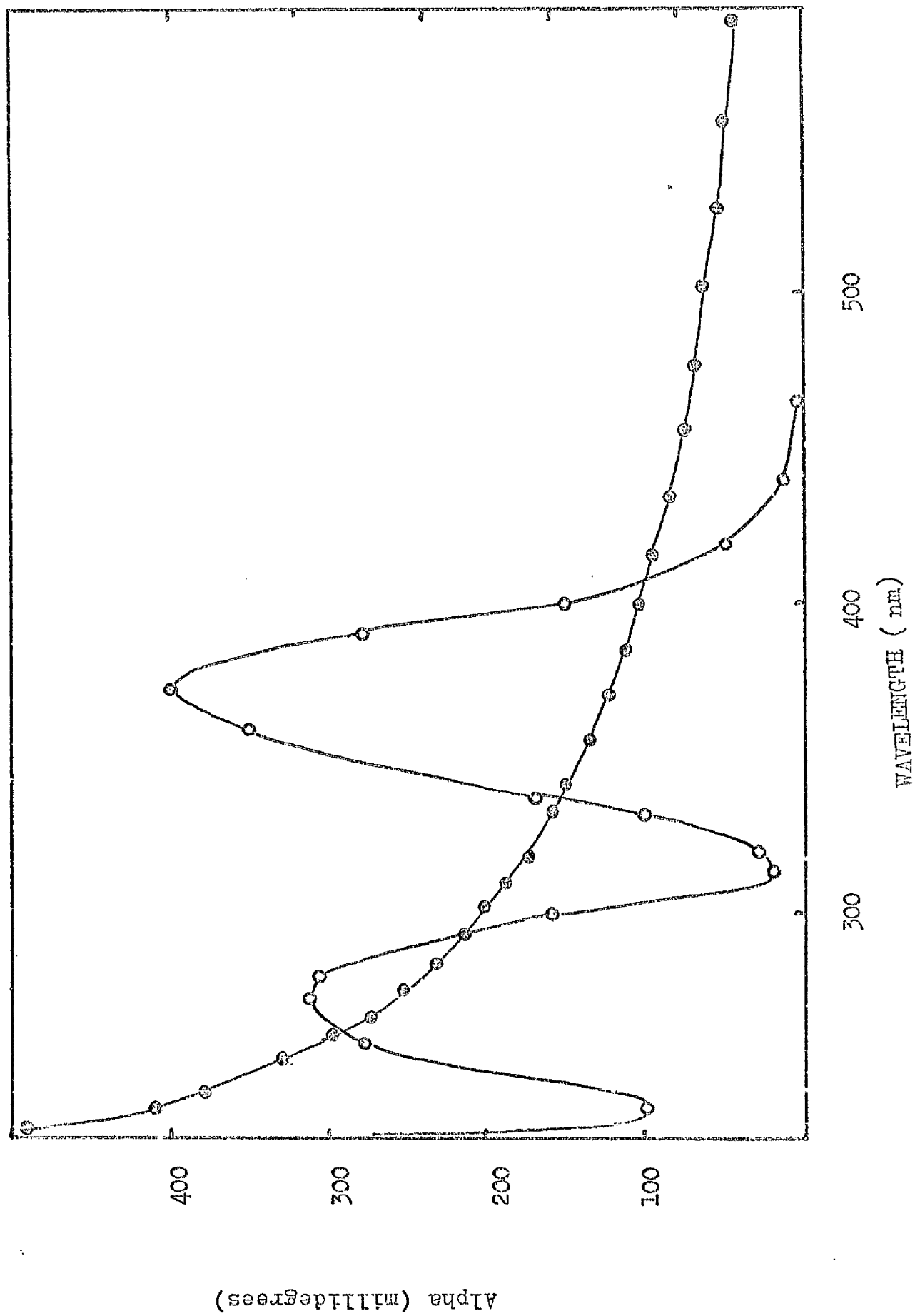


FIG. 3.31

FIGURE 3.31 SCATTERING TEST

Optical rotatory dispersion of a 0.6378% (w/v) sucrose solution in a 1cm path length cell with and without a 0.2% (w/v) Ludox solution in 0.05M-NaCl in series in a 1cm path length cell. The ORD of the sucrose was unaffected by the presence of the scattering material (Ludox).

⊙——⊙ ORD of the 0.6378% (w/v) sucrose solution.

○——○ Turbidity of the Ludox solution.

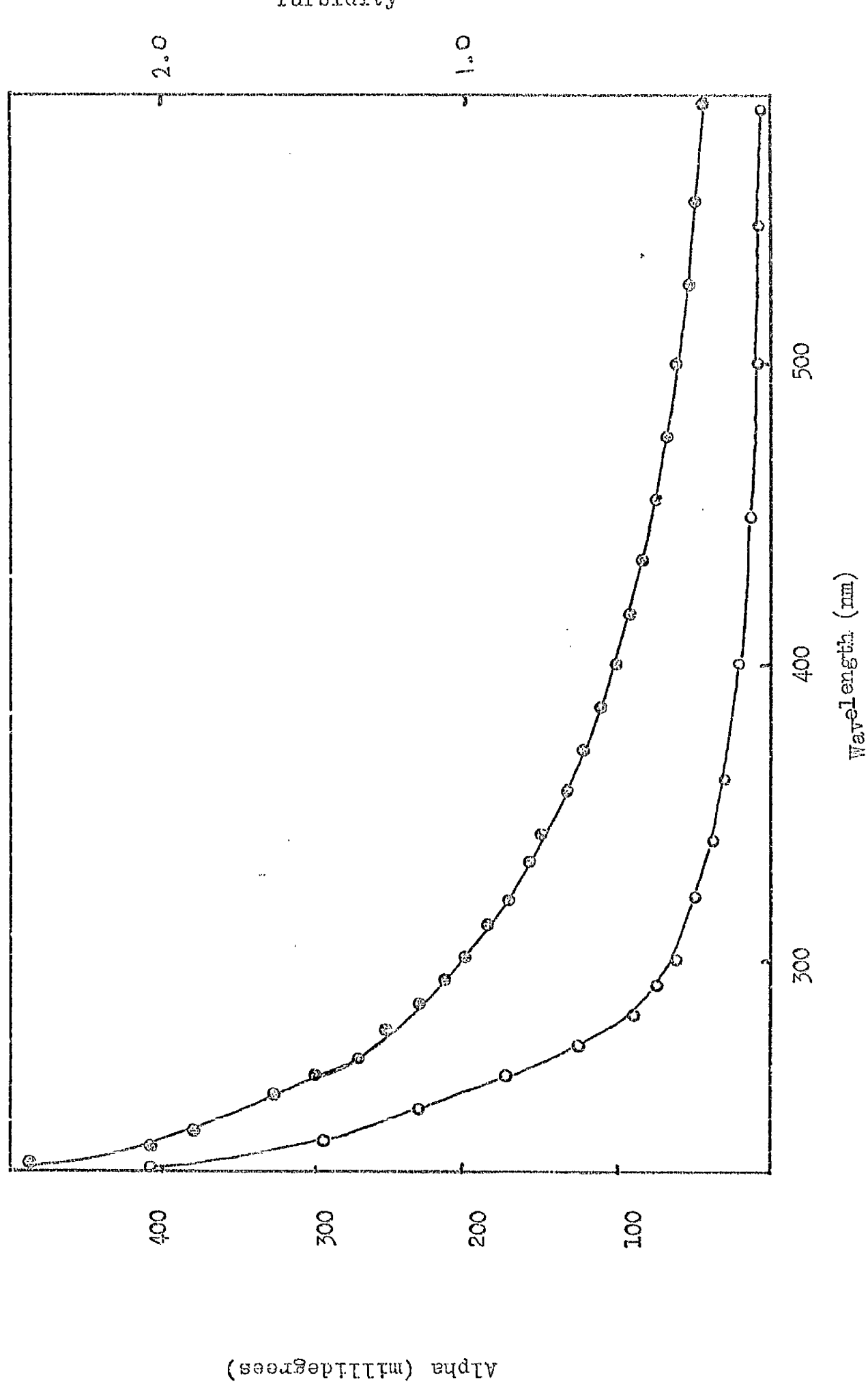


FIG. 3.32

FIGURE 3.32 OPTICAL ROTATORY DISPERSION OF RNA POLYMERASE

Optical rotatory dispersion of RNA polymerase holoenzyme prepared by the Burgess (1969a) procedure as described in section 2.2.3. The enzyme was equilibrated with a buffer containing 0.01M-tris HCl, pH 7.9, 0.50M-KCl by Sephadex G-25 chromatography in the same buffer as described in section 3.8.6.2. The ORD was determined over the wavelength range 220nm to 530nm. The protein concentration was 0.51mg/ml.

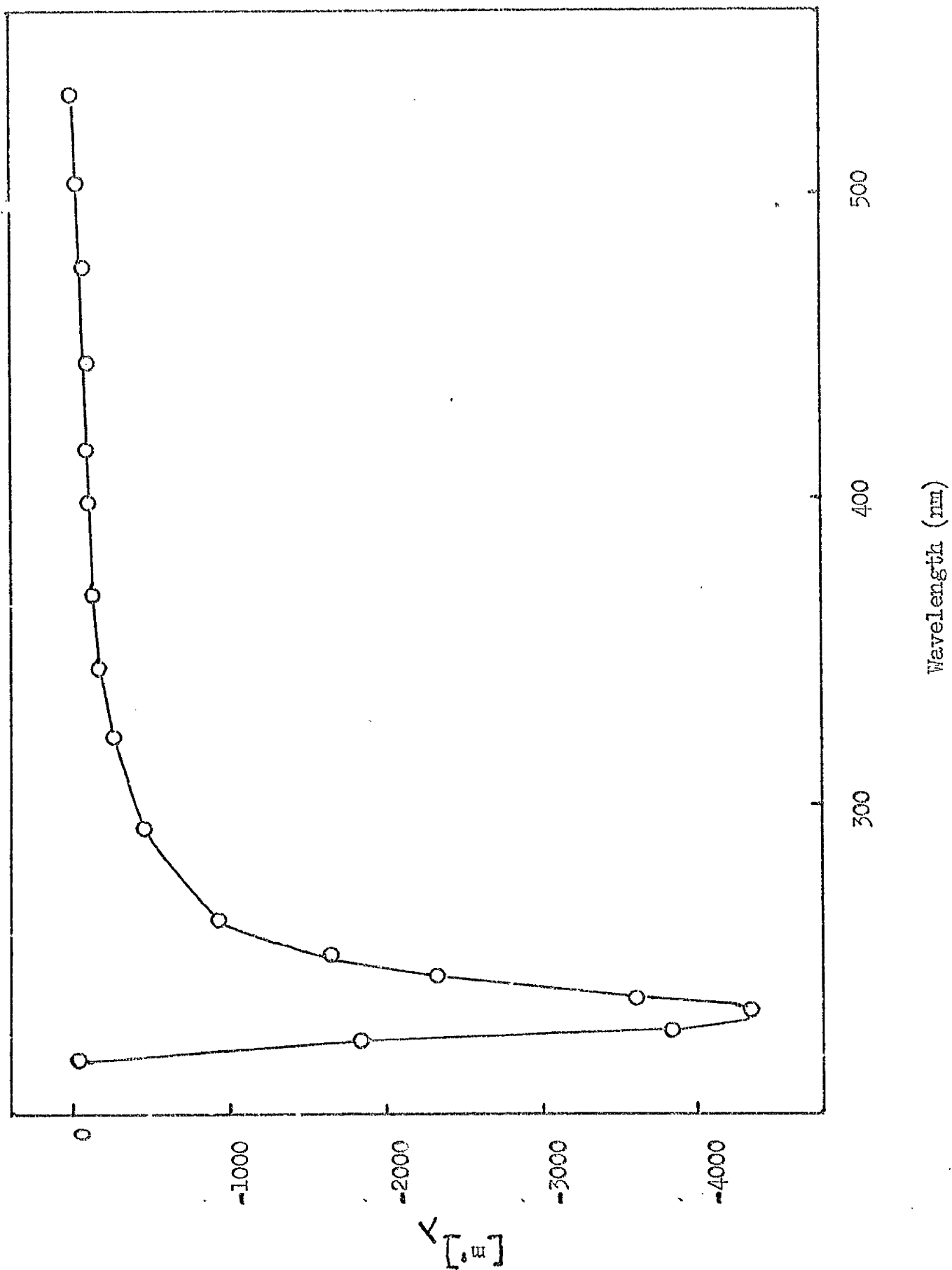


FIG. 3.33

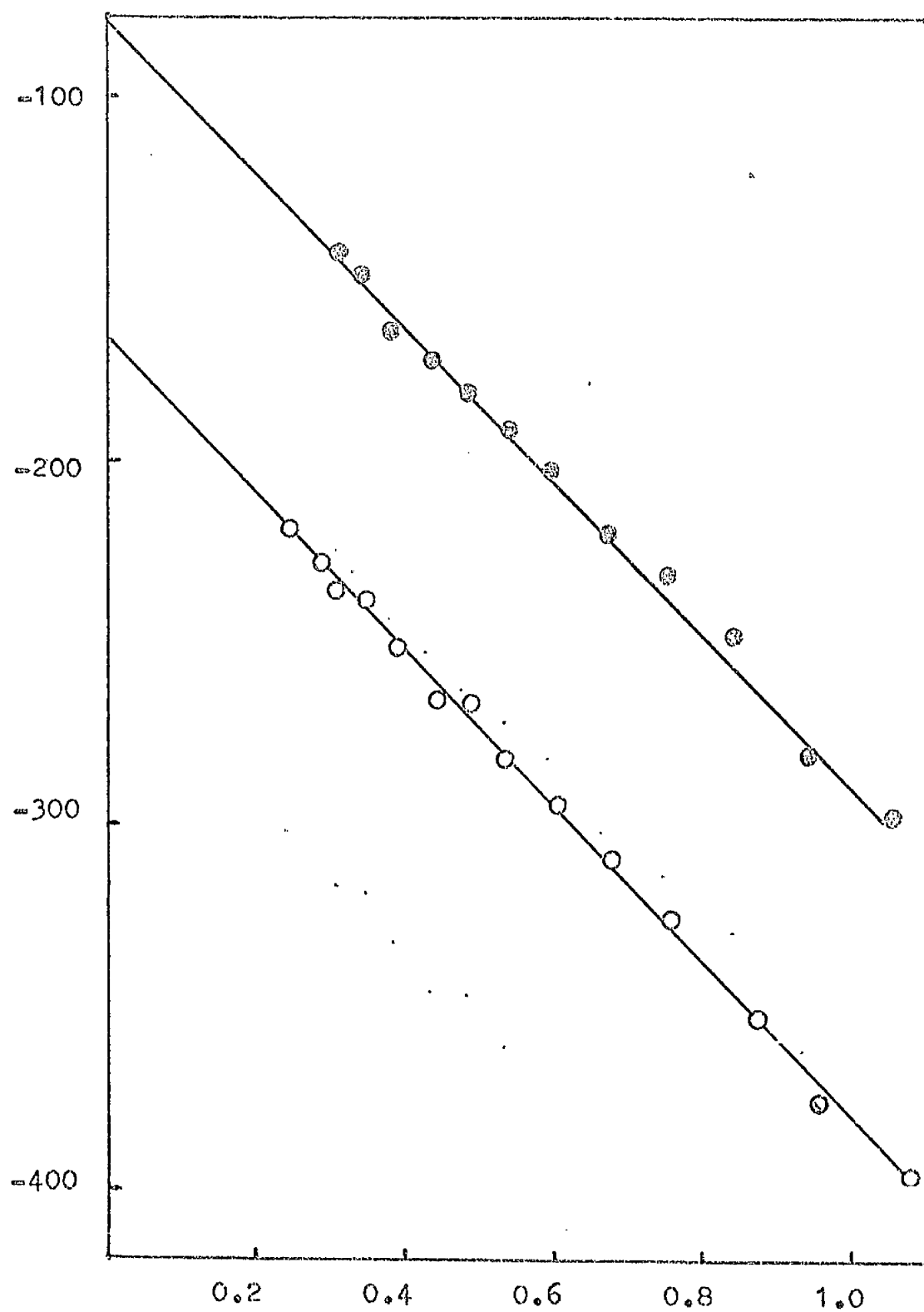
FIGURE 3.33 MOFFITT-YANG PLOT OF THE ORD OF RNA POLYMERASE

Moffitt-Yang plot of the optical rotatory dispersion of native RNA polymerase holoenzyme prepared by both the Zillig (1966) and Burgess (1969a) procedures as described in sections 2.2.2 and 2.2.3 respectively. The ORD was determined over the wavelength range 294nm to 560nm.

○—○ RNA polymerase in TMA buffer, pH 7.5.

○—○ RNA polymerase in 0.01M-trisHCl, pH 7.9,
0.50M-KCl.

$$[m']_{\lambda} \cdot (\lambda^2 - \lambda_0^2) / \lambda_0^2$$



$$\lambda_0^2 / (\lambda^2 - \lambda_0^2)$$

FIG. 3.34

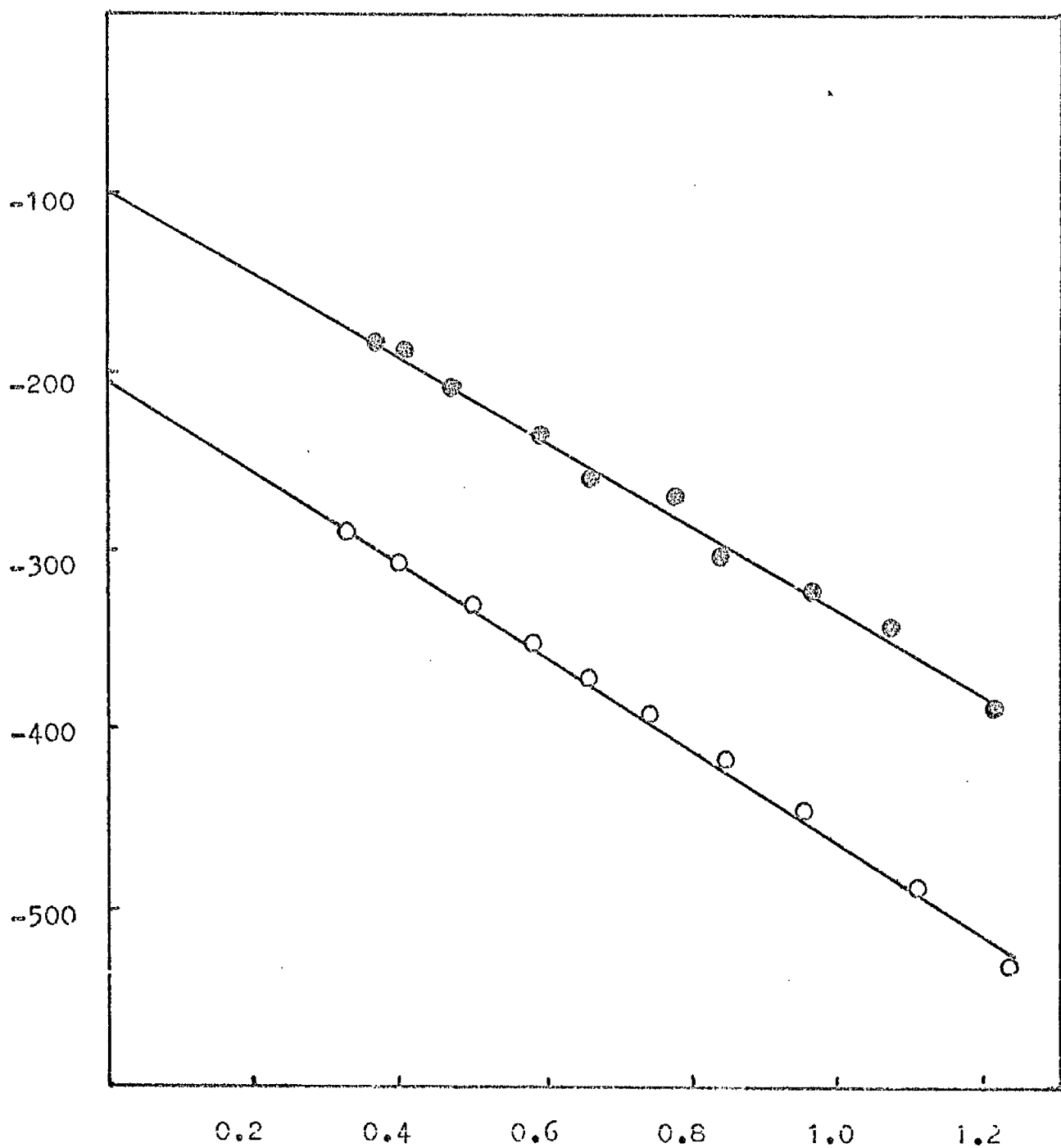
FIGURE 3.34 SCHECHTER-BLOUT PLOT OF THE ORD OF NATIVE
RNA POLYMERASE

Schechter-Blout plot of the optical rotatory dispersion of native RNA polymerase holoenzyme prepared by both the Zillig (1966) and Burgess (1969a) procedures as described in sections 2.2.2 and 2.2.3 respectively. The analyses were based on data over the wavelength range 294nm to 560nm.

e——o RNA polymerase in TMA buffer, pH 7.5.

o——o RNA polymerase in 0.01M-trisHCl, pH 7.9,
0.50M-KCl.

$$[n']_{\lambda} \cdot (\lambda^2 - \lambda_{193}^2) / \lambda_{193}^2$$



$$\lambda_{225}^2 / (\lambda^2 - \lambda_{225}^2)$$

FIG. 3.35

FIGURE 3.35 MOFFITT-YANG PLOT OF THE ORD OF RNA POLYMERASE
IN BUFFER A + 0.01M, 0.10M AND 0.20M-KCl

Moffitt-Yang plot of the optical rotatory dispersion of
RNA polymerase holoenzyme in buffers of varying ionic
strength over the wavelength range 294nm to 530nm.

- A RNA polymerase in buffer A + 0.01M-KCl.
- B RNA polymerase in buffer A + 0.20M-KCl.
- C RNA polymerase in buffer A + 0.10M-KCl.

$$[m']_{\lambda} (\lambda^2 - \lambda_0^2) / \lambda_0^2$$

-100

-200

-300

0.2

0.4

0.6

0.8

1.0

$$\lambda_0^2 / (\lambda^2 - \lambda_0^2)$$

A

B

C

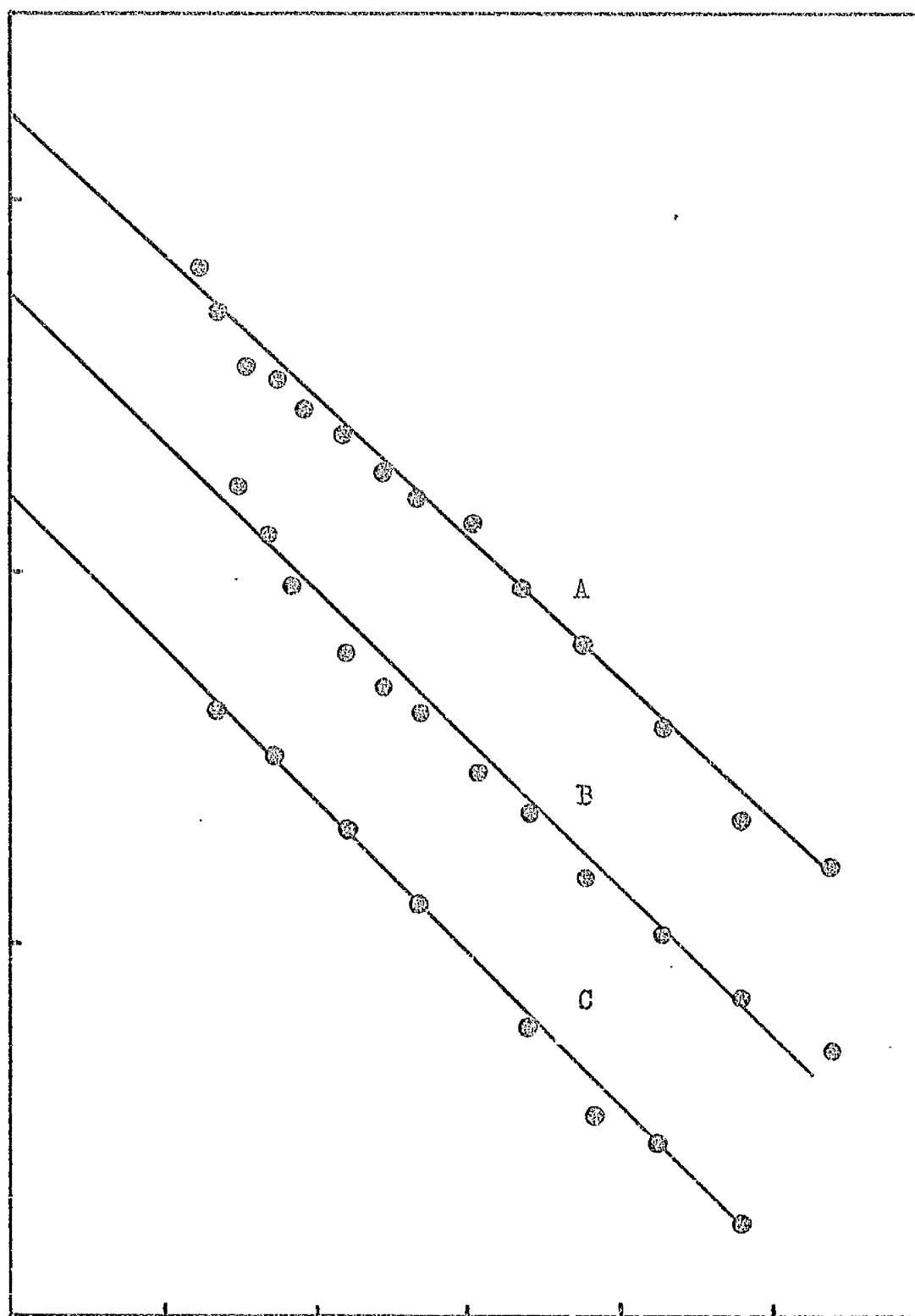


FIG. 3.36

FIGURE 3.36 MOFFITT-YANG PLOT OF RNA POLYMERASE IN
BUFFER A + 0.3M AND 0.5M-KCl

Moffitt-Yang analyses of RNA polymerase holoenzyme in
buffer A + 0.3M-KCl and buffer A + 0.5M-KCl based on
their optical rotatory dispersion over the wavelength
range 294nm to 530nm.

○—○ buffer A + 0.3M-KCl

○—○ buffer A + 0.5M-KCl

[m]. $(\lambda^2 - \lambda_0^2) / \lambda_0^2$

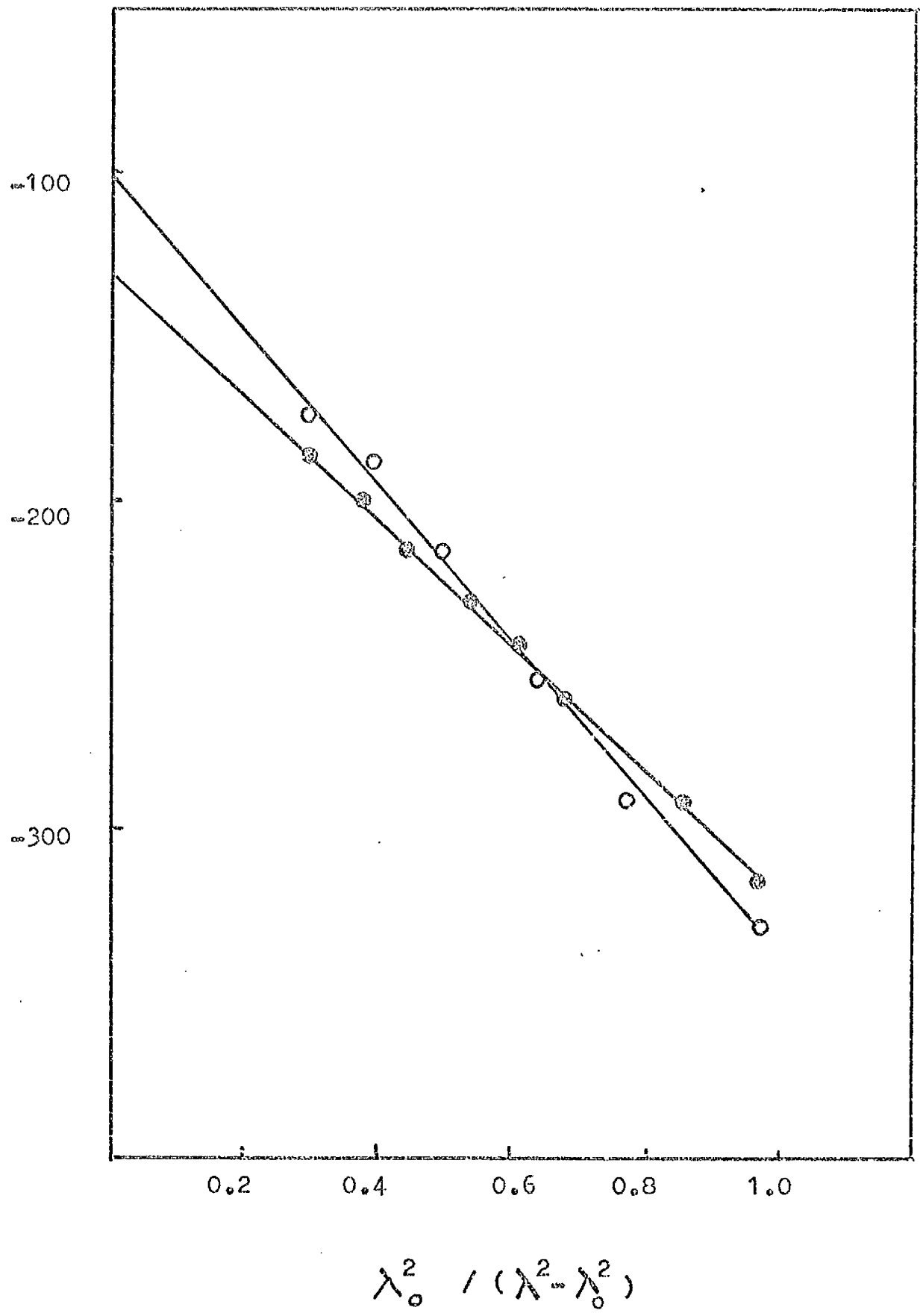


FIG. 3.37

FIGURE 3.37 SCHECHTER-BLOUT PLOT OF RNA POLYMERASE IN
BUFFER A + 0.01M, 0.10M AND 0.20M-KCl

Schechter-Blout analyses of RNA polymerase holoenzyme in buffer A + 0.01M-KCl, buffer A + 0.10M-KCl and buffer A + 0.20M-KCl based on their optical rotatory dispersion over the wavelength range 294nm to 530nm.

A buffer A + 0.01M-KCl

B buffer A + 0.10M-KCl

C buffer A + 0.20M-KCl

$$[m]. (\lambda^2 - \lambda_{193}^2) / \lambda_{193}^2$$

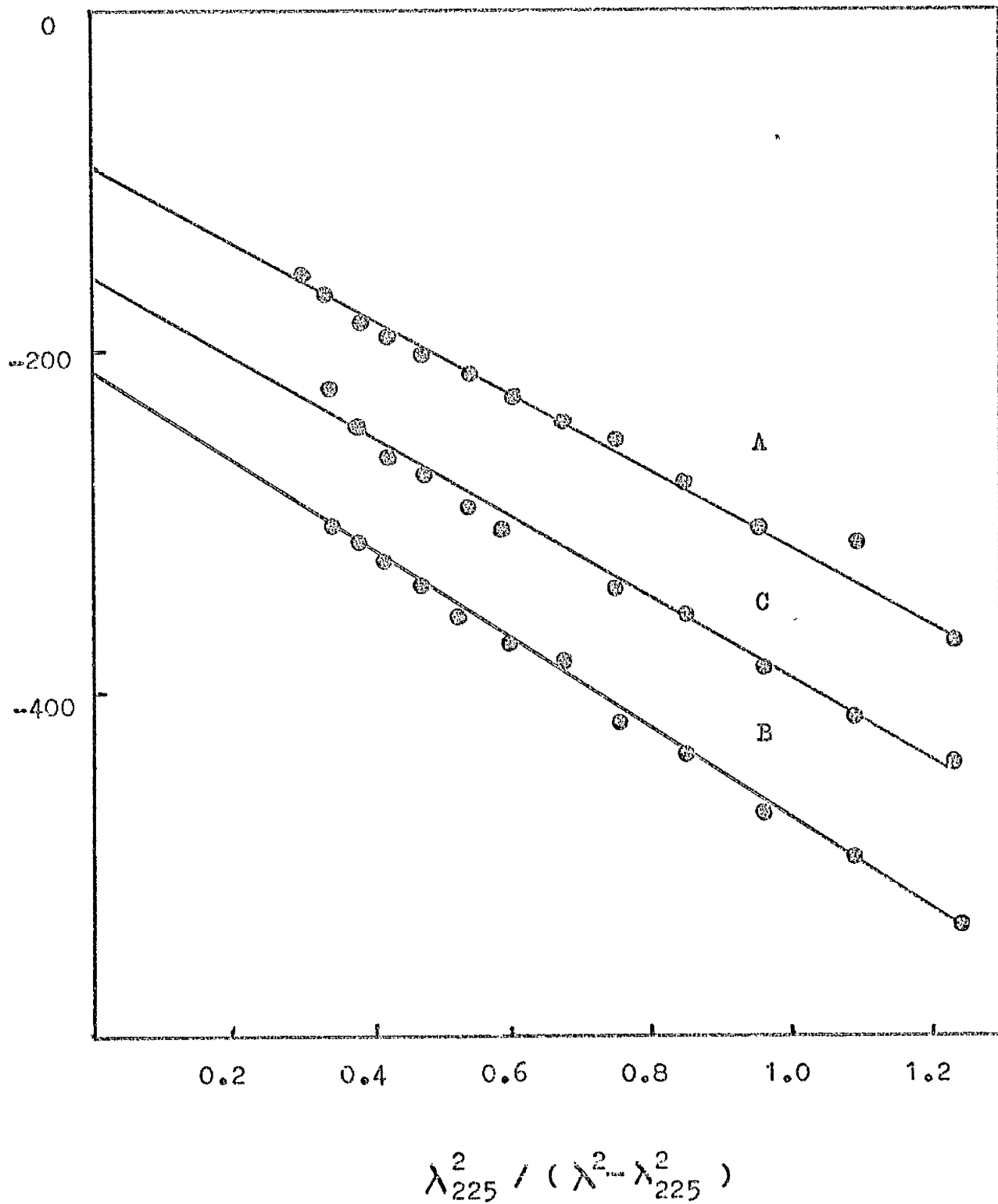


FIG. 3.38

FIGURE 3.38 SCHECHTER-BLOUT PLOT OF THE ORD OF RNA POLYMERASE
IN BUFFER A \pm 0.3M AND 0.5M-KCl

Schechter-Blout analyses of RNA polymerase holoenzyme in
buffer A \pm 0.3M-KCl and buffer A \pm 0.5M-KCl based on their
optical rotatory dispersion over the wavelength range
294nm to 530nm.

○—○ RNA polymerase in buffer A \pm 0.3M-KCl.

○—○ RNA polymerase in buffer A \pm 0.5M-KCl.

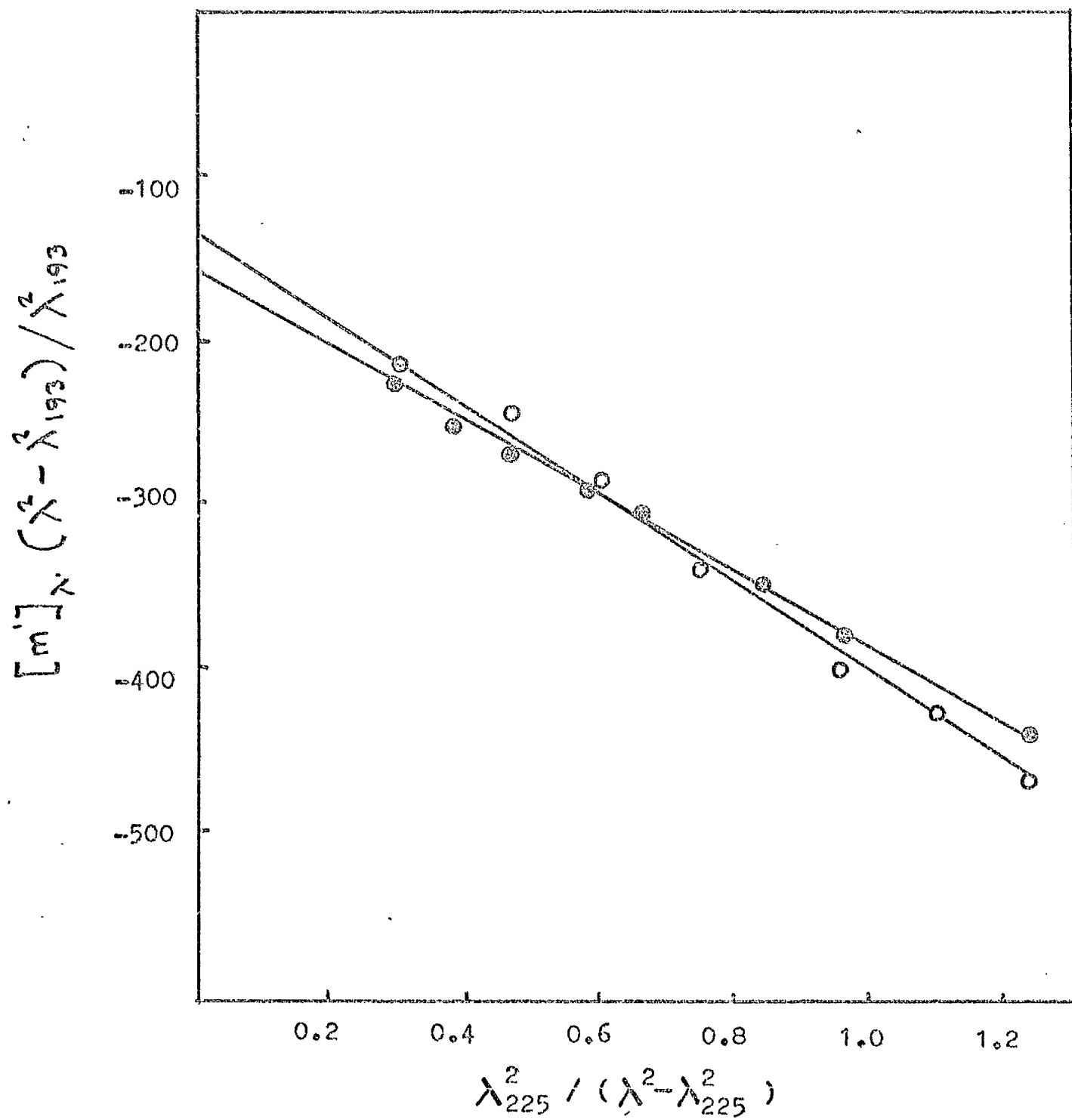


FIG. 3.39

FIGURE 3.39 THE EFFECT OF IONIC STRENGTH ON b_o

The effect of ionic strength on the b_o term of the Moffitt-Yang analyses (section 2.2.9.4) of RNA polymerase in buffers of varying KCl and NH_4Cl concentrations.

- RNA polymerase in buffer A with varying concentrations of KCl (see sections 3.8.6.2 and 3.8.7.2).
- RNA polymerase in 0.01M-trisHCl, pH 7.9, 0.50M-KCl (section 3.8.6.2).
- RNA polymerase in TMA buffer (0.022M- NH_4Cl) (section 3.8.6.2)
- RNA polymerase in TMA buffer (0.14M- NH_4Cl).

PER CENT HELIX

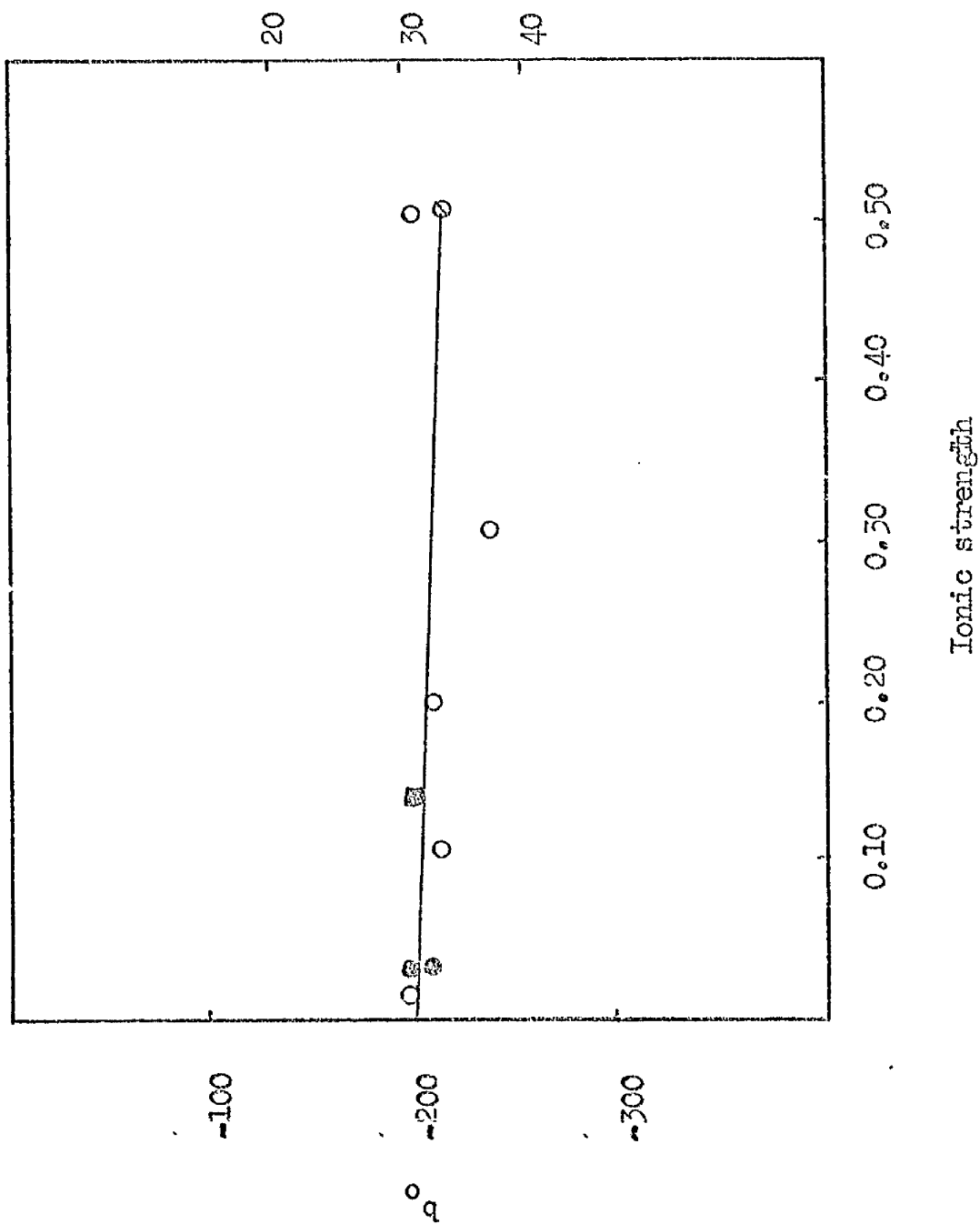


FIG. 3.40

FIGURE 3.40 THE EFFECT OF IONIC STRENGTH ON a_0

The effect of the ionic strength on the a_0 term of the Moffitt-Yang analyses of native RNA polymerase in buffers with varying KCl and NH_4Cl concentrations.

- ① RNA polymerase in buffer A with the KCl concentration varying from 0.01M to 0.50M (sections 3.8.6.2 and 3.8.7.2).
- ② RNA polymerase in 0.01M trisHCl, pH 7.9, 0.50M-KCl (section 3.8.6.2).
- ③ RNA polymerase in TMA buffer (section 3.8.6.2).

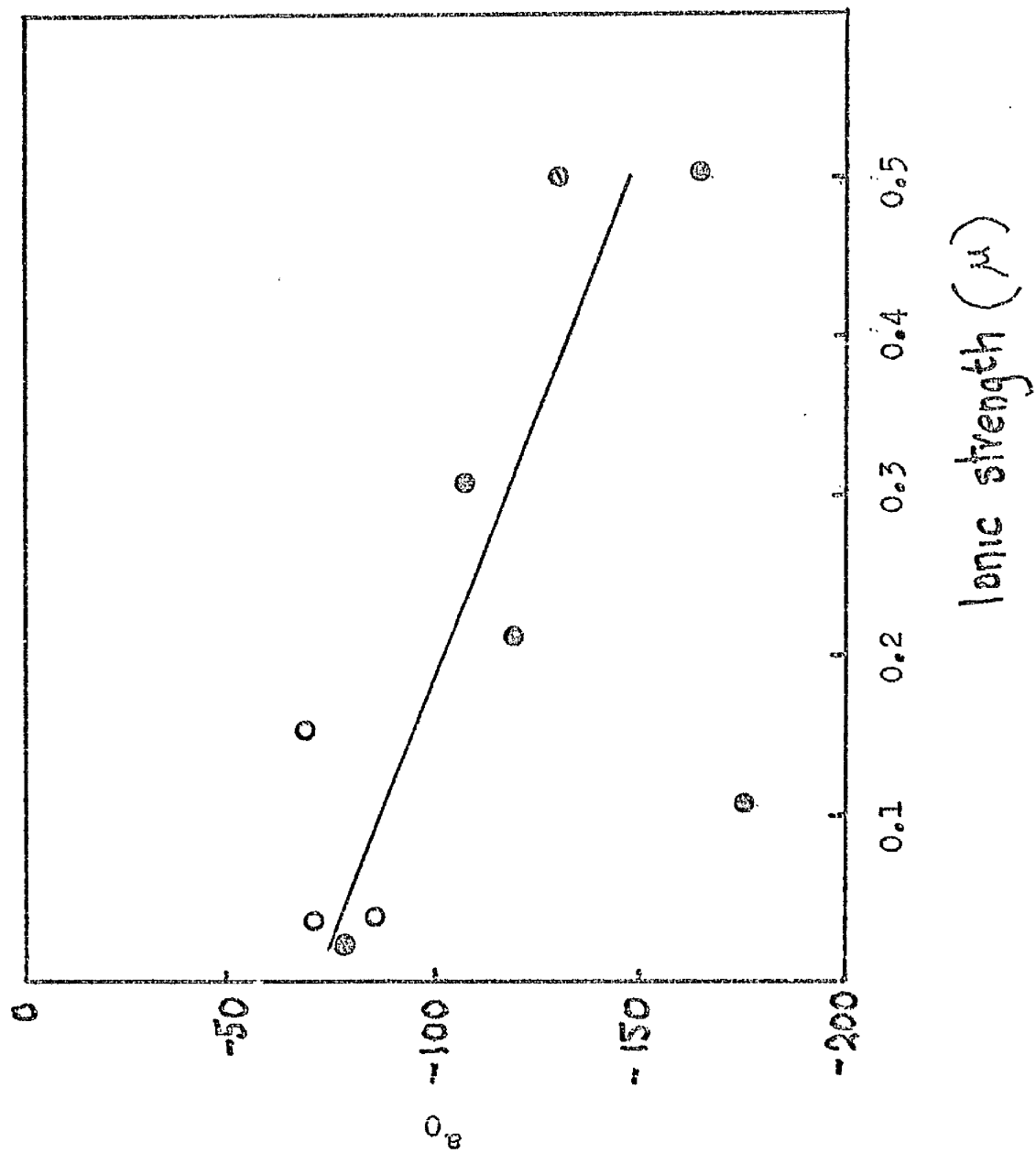


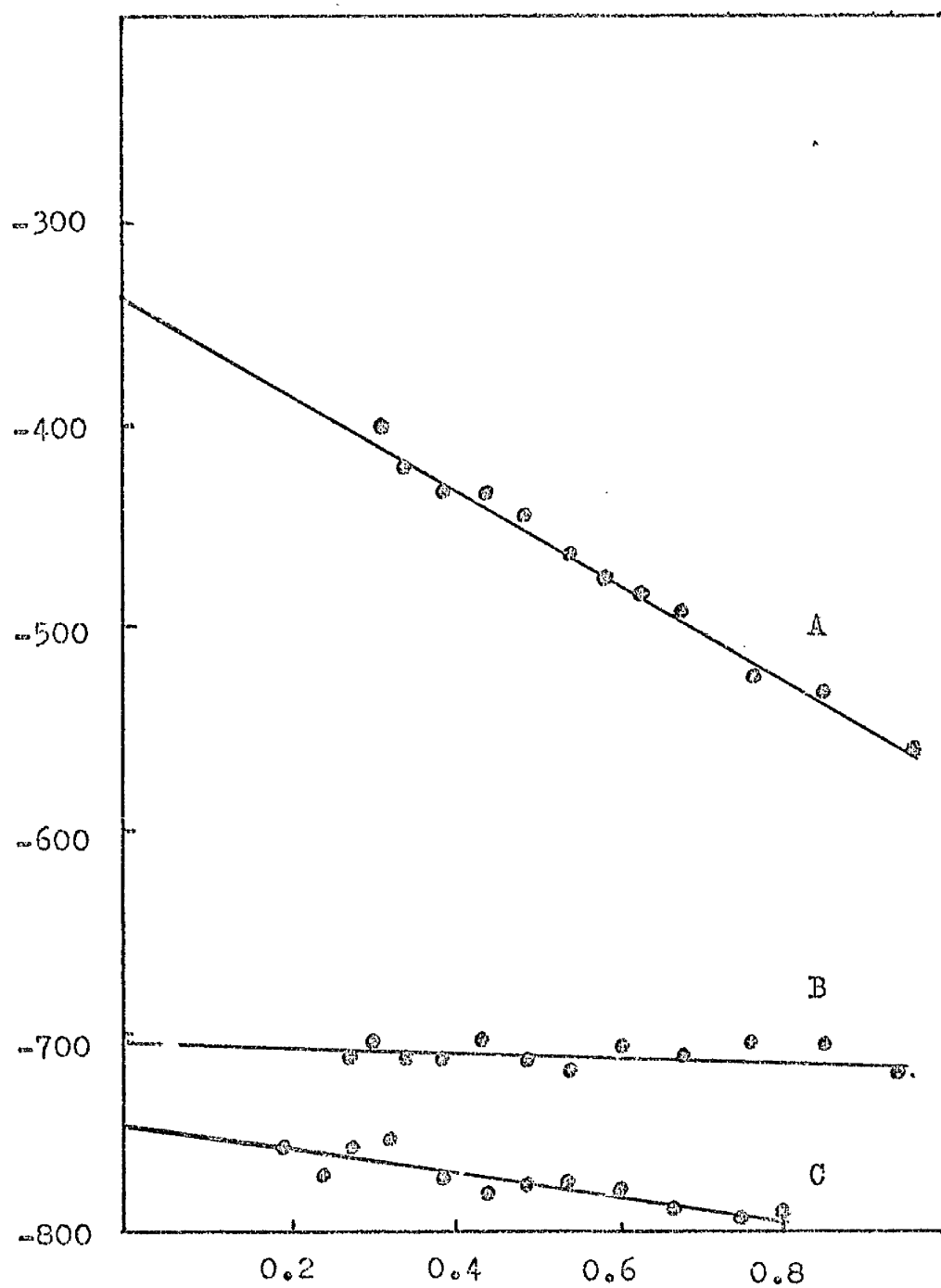
FIG. 3.41

FIGURE 3.41 MOFFITT-YANG PLOTS OF THE ORD OF RNA POLYMERASE
IN DENATURING SOLVENTS

The Schechter-Blout plots were performed as described in
section 2.2.9.4.

- A RNA polymerase in 1% (w/v) SDS, 1% (v/v) 2-mercapto-
ethanol, 0.1M-sodium phosphate, pH 7.1.
- B RNA polymerase in 6M-guanidine hydrochloride, 1% (v/v)
2-mercaptoethanol.
- C RNA polymerase in 8M-urea, 1% (v/v) 2-mercaptoethanol.

$$[M]_{\lambda} (\lambda^2 - \lambda_0^2) / \lambda_0^2$$



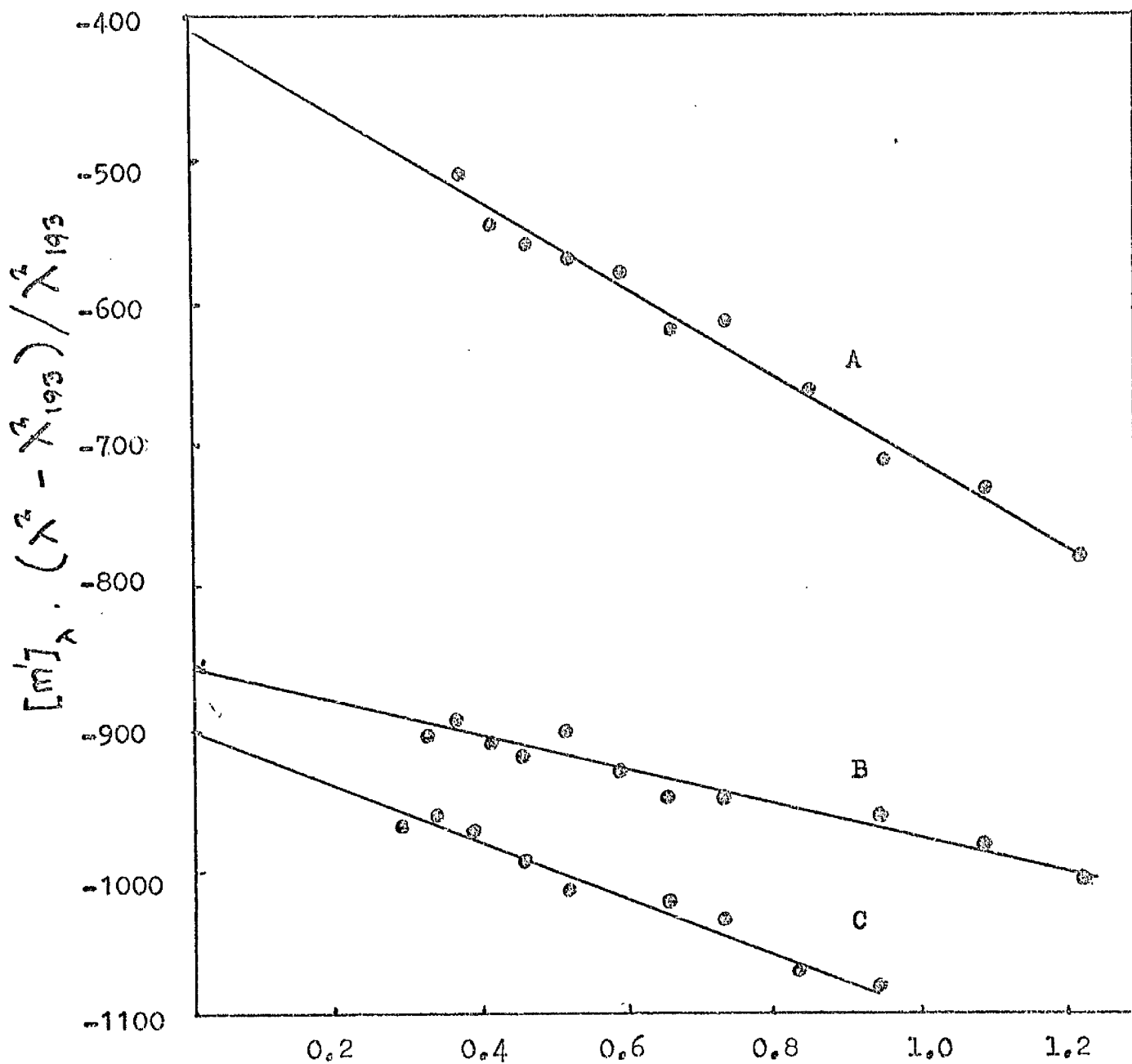
$$\lambda_0^2 / (\lambda^2 - \lambda_0^2)$$

FIG. 3.42

FIGURE 3.42 SCHECHTER-BLOUT PLOT OF THE ORD OF RNA POLYMERASE
IN DENATURING SOLVENTS

The analyses were performed as described in section 2.2.9.4.

- A RNA polymerase in a solution containing 1% (w/v) SDS,
1% (v/v) 2-mercaptoethanol, 0.10M-sodium phosphate, pH 7.1.
- B RNA polymerase in 6M-guanidine hydrochloride, 1% (v/v)
2-mercaptoethanol.
- C RNA polymerase in 8M-urea, 1% (v/v) 2-mercaptoethanol.



$$\lambda_{225}^2 / (\lambda^2 - \lambda_{225}^2)$$

FIG. 3.43

FIGURE 3.43 A_{193} VERSUS A_{225}

1. Several preparations of E.coli MRE 600 RNA polymerase in buffers of varying ionic strength (sections 3.8.6.2 and 3.8.7.2).
2. RNA polymerase in 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 0.10M-sodium phosphate pH 7.1.
3. RNA polymerase in 8M-urea, 1% (v/v) 2-mercaptoethanol
4. RNA polymerase in 6M-guanidine hydrochloride, 1% (v/v) 2-mercaptoethanol.
5. Poly-L-glutamic acid at pH 7 (100% random coil) (Blout et al., 1967).
6. Poly-L-glutamic acid at pH 4 (100% helix) (Blout et al., 1967).

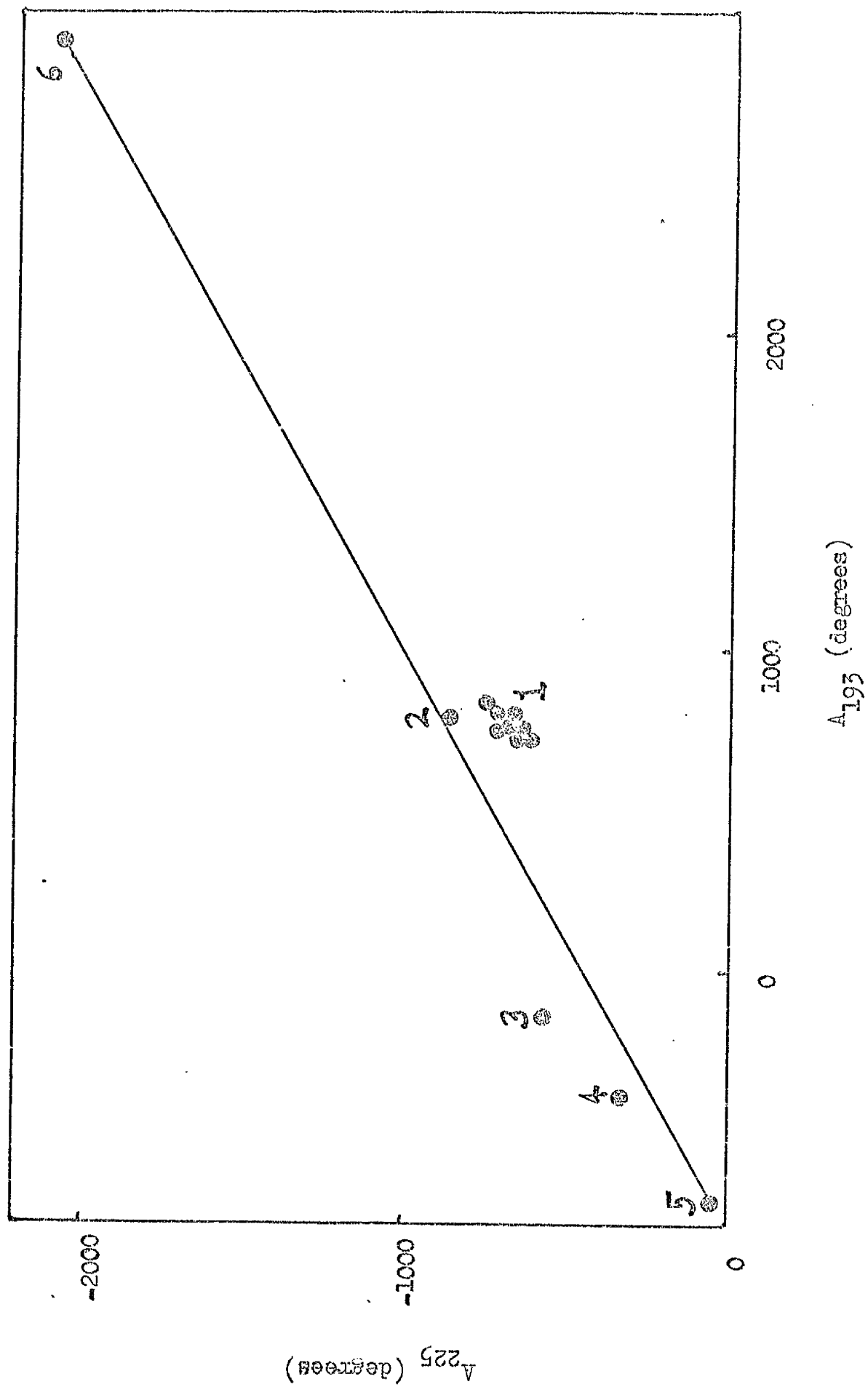


FIG. 3.44

FIGURE 3.44 OPTICAL ROTATORY DISPERSION OF CALF THYMUS DNA

The specific rotation of calf thymus DNA in buffer containing 0.10M-tris HCl, pH 7.9, and 0.10M-KCl, at both 20°C and 45°C is identical and similar to that published (Samejima and Yang, 1965).

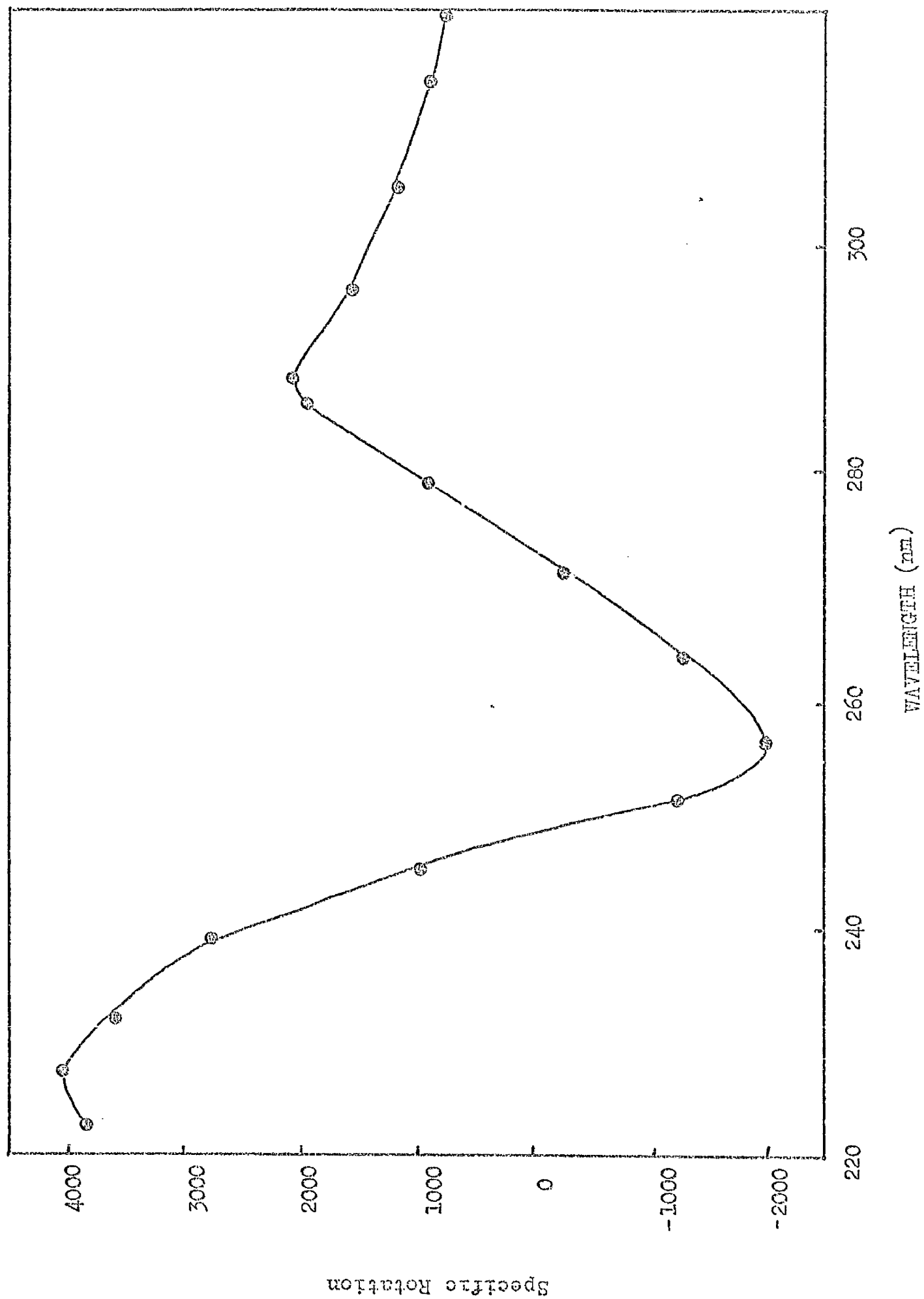


FIG. 3.45

FIGURE 3.45 EFFECT OF TEMPERATURE ON THE ULTRA-VIOLET,
OPTICAL ROTATORY DISPERSION OF RNA POLYMERASE

Optical rotatory dispersion of RNA polymerase
holoenzyme in 0.10M-tris HCl, pH 7.9, 0.10M-KCl
prepared by the Burgess (1969a) procedure as
described in section 2.2.3 at both 20°C and 45°C
The optical rotatory dispersion is identical
at both temperatures.

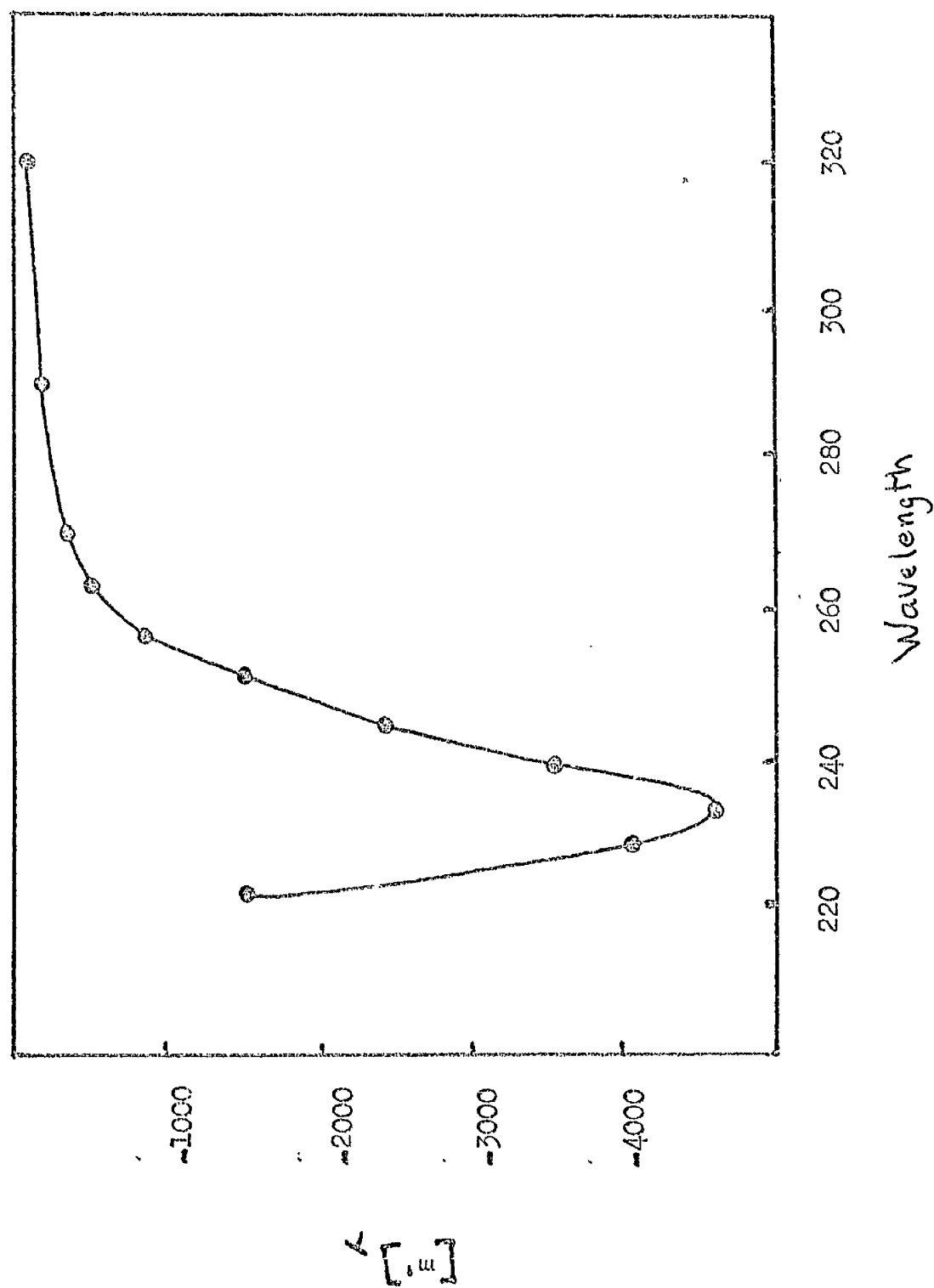


FIG. 3.46

FIGURE 3.46 OPTICAL ROTATORY DISPERSION OF AN
DNA-RNA POLYMERASE COMPLEX

This figure shows the optical rotatory dispersion of RNA polymerase holoenzyme (45 μ g/ml) and calf thymus DNA (30 μ g/ml) in series (e—o) in 1cm path length cell compartments in a tandem cell, and after mixing to form a DNA-RNA polymerase complex (o—o). The temperature was 20°C. The change in specific rotation around 232nm suggests that RNA polymerase holoenzyme has undergone a change in conformation on binding to DNA. There is no apparent change in the conformation of the DNA.

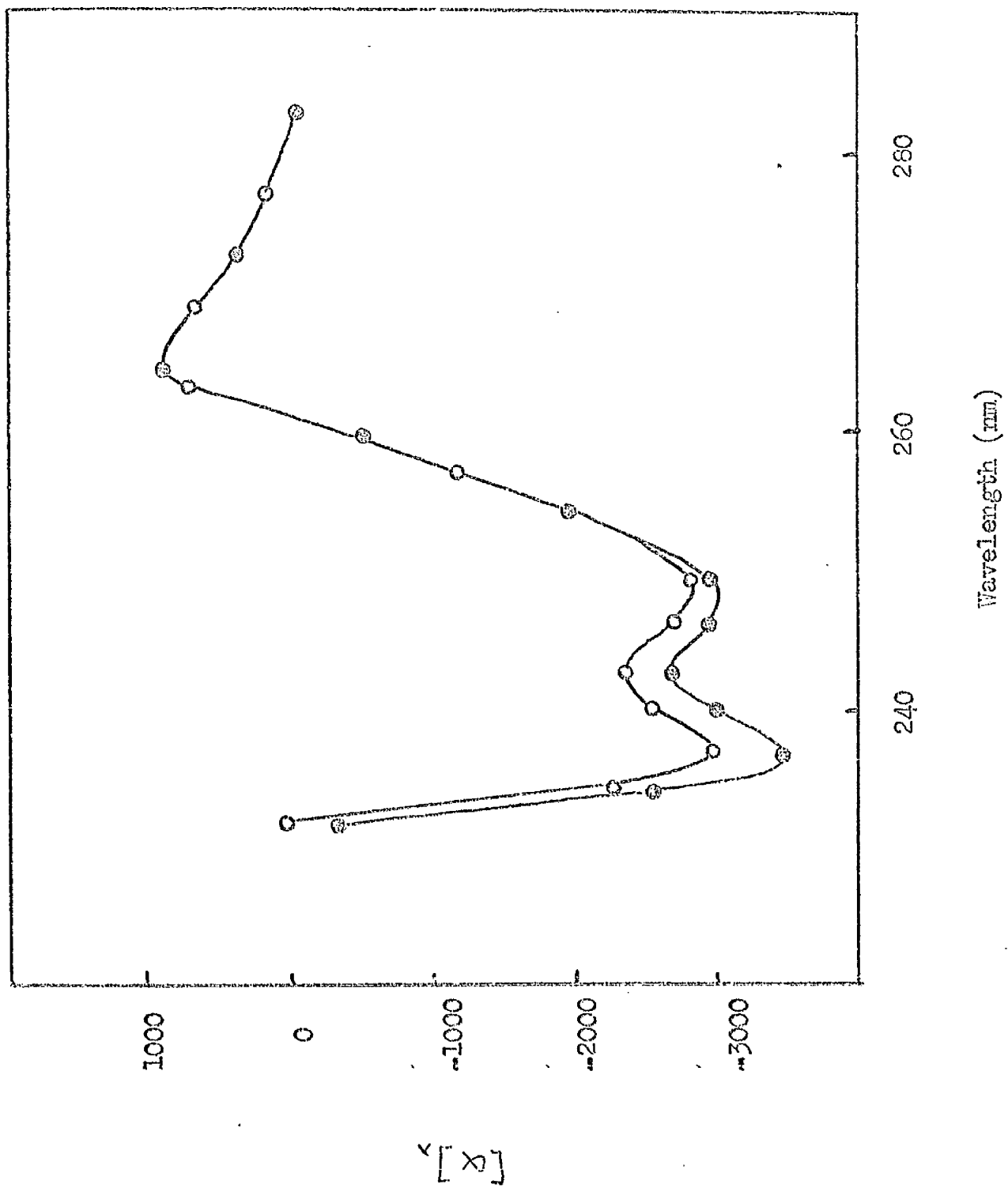
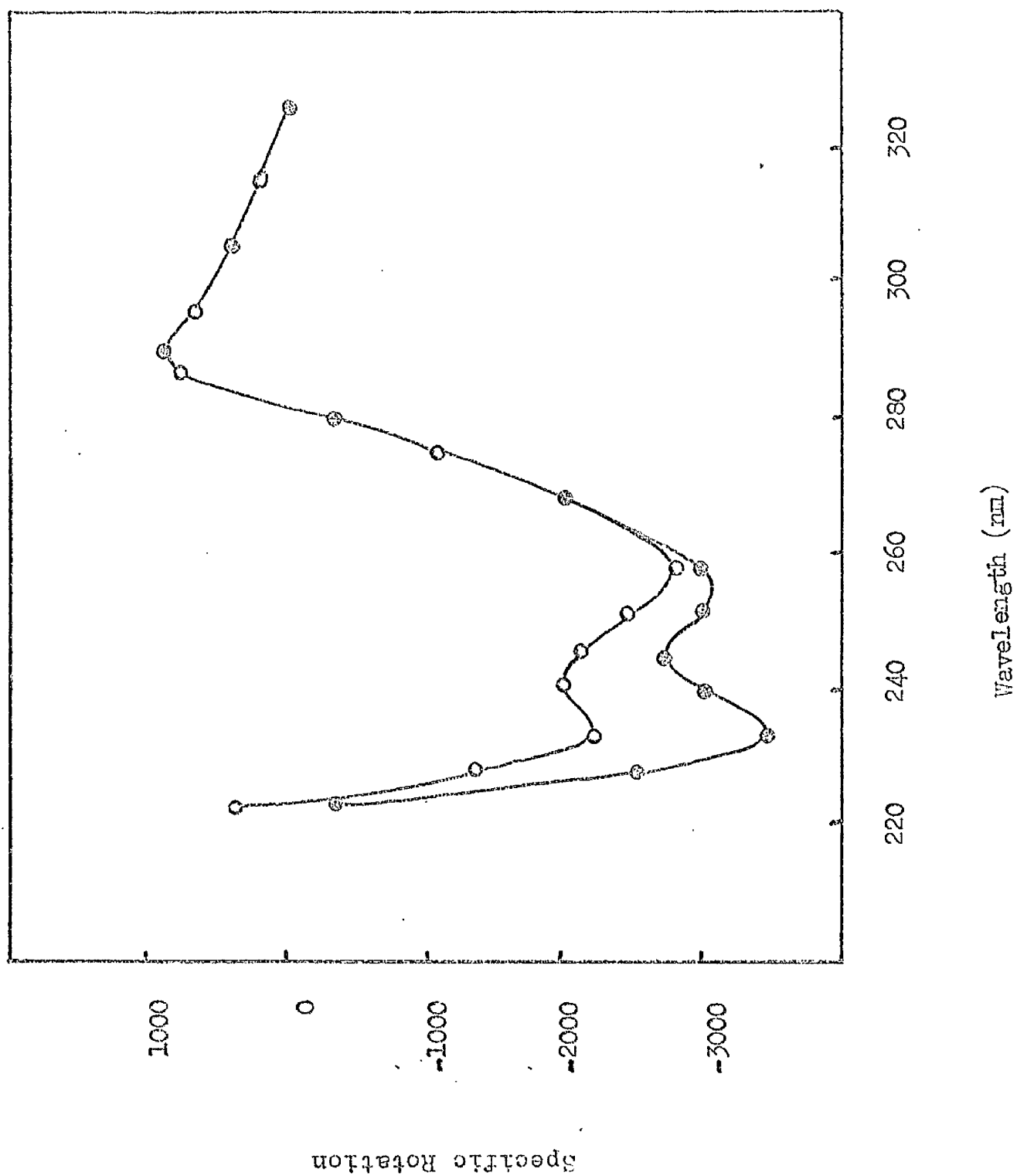


FIG. 3.47

FIGURE 3.47 OPTICAL ROTATORY DISPERSION OF AN RNA POLMERASE-DNA COMPLEX AT 45°C

○——○ The optical rotatory dispersion of RNA polymerase holoenzyme and calf thymus DNA in series at both 20°C and 45°C.

○——○ The optical rotatory dispersion of the RNA polymerase-DNA complex at 45°C. There has been a further decrease in the helix content of the RNA polymerase holoenzyme on heating to 45°C compared to the helix content of the enzyme in the complex at 20°C.



SECTION 4

DISCUSSION

4.1.

DISCUSSION OF RESULTS

4.1.1.

INTRODUCTION

Although DNA-dependent RNA polymerase, one of the two major enzymes of nucleic acid synthesis (the other being DNA-dependent DNA polymerase) was first detected by Weiss and Gladstone in 1959 in rat liver nuclei, it was not until 1962 that Chamberlin and Berg first partially purified the native enzyme from the common colon bacterium *Escherichia coli* and thus were able to commence a detailed study of its physical and chemical properties. The bulk of the work on the study of the properties of RNA polymerase has been performed on the *E. coli* enzyme.

It was not until 1966 that Zillig et al. first attempted an investigation into the possible subunit structure and physical properties of RNA polymerase. Originally it was believed that the enzyme consisted of two major subunits each of which had an S-value of 13 and together made a protein species of 23S (Zillig et al., 1966). Richardson (1966a) was the first to physically characterise the properties of a highly purified RNA polymerase species. He found that the 13S form of the molecule^t had a molecular weight of around 440,000 daltons and that the 23S form of the enzyme was a dimer of the 13S species with a molecular weight of 880,000 daltons. The 23S species was present in buffers of low ionic strength and the 13S in buffers of high ionic strength. This dimerisation process was completely reversible and was

dependent on the ionic strength of the solution (Richardson, 1966a). The problem still remained, which form of the enzyme was the active species? Stevens et al. (1966) showed that in experiments where RNA polymerase was bound to low molecular weight nucleic acid species, the enzyme existed primarily as the 13S monomer. This is now known to be the active form of the enzyme in vitro.

Early electron microscopy appeared to show that RNA polymerase consisted of six identical subunits in the form of a rosette with a hole in the middle. The dimensions of the hole were such that it was suggested the double-stranded DNA could pass through the centre (Zillig et al., 1966). More recent research has shown that these rosettes are either artefacts or impurities and not true RNA polymerase molecules themselves (Lubin, 1969). That RNA polymerase was composed of several smaller protein subunits was known for some time as several other protein species sedimenting at less than 13S had been observed in the analytical ultracentrifuge, especially in aged or denatured enzyme preparations (Zillig et al., 1966), but the exact nature of these subunits was not known. It was not until 1969 that the fine subunit structure of *E. coli* RNA polymerase was first determined. Berg et al. (1969) reported that a protein subunit could be dissociated from RNA polymerase which was actively transcribing RNA from a DNA template. This subunit had a molecular weight of around 80,000 daltons. A similar subunit, termed γ , was detected by Krakow (1969) when *A. vinelandii* RNA polymerase combined with native and synthetic polyribonucleotides. Burgess (1969) meanwhile had advanced the purification of *E. coli* RNA polymerase by using phospho-

cellulose chromatography as a purification step which showed that native RNA polymerase probably consisted of two main sizes of protein subunits. The technique of SDS-polyacrylamide gel electrophoresis, first used by Shapiro et al. (1969) to determine protein molecular weights was applied to the study of RNA polymerase and proved invaluable in determining the molecular weights of each of the subunits of the enzyme and their stoichiometry in the intact RNA polymerase molecule. Burgess et al. (1969) first published the subunit composition and the molecular weights of each of the subunits of RNA polymerase. RNA polymerase holoenzyme was shown to consist of four major subunits with the stoichiometry $\beta'\beta\alpha_2\sigma$, and the core enzyme i.e. RNA polymerase lacking σ , the subunit which can be removed from the holoenzyme by phosphocellulose chromatography, had the subunit composition $\beta'\beta\alpha_2$.

Recently the subunit structure of RNA polymerase from other bacteria has been determined (see table 1.4) and they have been found to be very similar to the E. coli enzyme. RNA polymerase molecules have now been found which consist of only one polypeptide chain. Chamberlin et al. (1970) have purified T7-bacteriophage-coded RNA polymerase which has a molecular weight of 107,000 daltons and Kuntzell and Schafer (1971) have isolated the mitochondrial RNA polymerase from Neurospora crassa and have shown that it consists of only one polypeptide chain of molecular weight 64,000 daltons.

It would appear, therefore, that the bacterial DNA-dependent RNA polymerase because of its large size and complexity relative to the other more simple i.e. smaller enzymes which perform the same basic function,

RNA transcription on a DNA template, is probably subject to more sophisticated and complex control mechanisms and could probably be more correctly defined as an enzyme complex. There is no doubt that the mammalian DNA-dependent RNA polymerase(s) will be exceedingly more sophisticated and complex, both in structure and function, than the bacterial enzyme.

4.1.2.

THE SUBUNIT COMPOSITION OF RNA POLYMERASE

DNA-dependent RNA polymerase can now be routinely obtained from *E. coli* in a fairly pure form by several different procedures (Babinet, 1967; Burgess et al., 1969; Burgess, 1969a; Berg and Chamberlin, 1970; Zillig et al., 1970c). There is now some evidence that both the purification procedure and the strain of *E. coli* dictate not only the subunit composition of the enzyme but also the stoichiometry of some of these subunits.

Most procedures for the preparation of RNA polymerase utilise ammonium sulphate fractionation and DEAE-cellulose chromatography as early steps in the purification procedure (Burgess et al., 1969; Burgess, 1969a; Berg and Chamberlin, 1970; Zillig et al., 1970c). The main differences in these published purification procedures occur only at later stages in the preparation of the enzyme.

Preparation of RNA polymerase using phosphocellulose chromatography as a further purification step leads to an RNA polymerase molecule which consists of the protein subunit β' , β and α ; the stoichiometry of these subunits is $\beta'\beta\alpha_2$. Enzyme of this subunit composition has been termed

the core enzyme by Burgess et al. (1969) as they believe that no further subunits may be removed without inactivating the enzyme. Core enzyme shows only little activity as an RNA polymerising enzyme and has been shown to be active only on calf thymus DNA and poly dAT as templates (Burgess et al., 1969a; Chamberlin and Berg, 1970). Core enzyme has very little activity on native E. coli or E. coli bacteriophage DNAs as templates.

RNA polymerase purified by procedures which do not utilise phosphocellulose chromatography but instead use both low and high ionic strength sucrose or glycerol gradients as preparative steps shows not only the same three subunits as the core enzyme β' , β and α but a fourth σ (σ is the subunit which is removed from the core enzyme by phosphocellulose chromatography). (Burgess, 1969a; Berg and Chamberlin, 1970; Zillig, et al., 1970c). The stoichiometry of the subunits in this RNA polymerase species has been found to be $\beta'\beta\alpha_2\sigma$ (Travers and Burgess, 1969) although the yield of σ in relation to the amount of core enzyme ($\beta'\beta\alpha_2$) appears to vary in RNA polymerase molecules purified from different strains of E. coli, although there is the possibility that the fine differences in the various purification procedures may be affecting the σ content of the enzyme. $\beta'\beta\alpha_2\sigma$ is known as RNA polymerase holoenzyme (Burgess et al., 1969). In addition to the subunits mentioned above another low molecular weight subunit (9,000 daltons) termed ω (Burgess et al., 1969) can be detected in some preparations of both core and holoenzyme, however as the number of ω molecules present per core or holoenzyme can vary from zero to two (Berg and Chamberlin, 1970) and so far no function has been allocated to it, it would appear there-

fore that it is an impurity of the enzyme preparation. A minor protein component τ is a contaminant of some RNA polymerase preparations (Burgess et al., 1969). The molecular weight of this component is around 100,000 daltons, the molecular weight of *E. coli* polynucleotide phosphorylase; the presence of this impurity, detected by SDS-polyacrylamide gel electrophoresis, in the RNA polymerase preparation of Babinet (1967) probably accounts for the polynucleotide phosphorylase activity associated with his enzyme (Hirschbein et al., 1969).

RNA polymerase purified by procedures which also utilise ammonium sulphate fractionation and DEAE-cellulose chromatography as early purification steps but which use two agarose gel filtration chromatography columns in both high and low ionic strength buffers (section 2.2.3) to separate RNA polymerase from impurities of different molecular size, instead of sucrose and glycerol gradients (Burgess, 1969a; Berg and Chamberlin, 1970; Zillig et al., 1970c), yields a holoenzyme species which is similar to that of Burgess et al. (1969) but has an additional protein component or subunit which was termed θ . From the results in section 3.1.4.2. it would appear that RNA polymerase, purified by the modified Burgess procedure (section 2.2.3), consists of a mixture of two distinct enzyme species - $\beta'\beta\alpha_2\sigma$ and $\beta'\beta\alpha_2\theta$ (see section 3.2.2.2.3).

SDS-polyacrylamide gel analysis of RNA polymerase purified from *E. coli* A19 (Hirschbein et al., 1969) shows a similar subunit composition as the *E. coli* MRE 600 enzyme purified by the modified Burgess (1969a)

procedure (section 2.2.3), i.e. θ was present. Both these strains of *E. coli* are RNase 1⁻ strains and the possibility existed that the presence of θ as an RNA polymerase component was in some way due to this fact. To test this hypothesis RNA polymerase was purified from *E. coli* B (also obtained from MRE Porton) by the procedure in section 2.2.3. On SDS-polyacrylamide gel analysis of the purified *E. coli* B enzyme, the θ subunit was found to be present as well as the β' , β , α and σ subunits i.e. the subunit compositions of *E. coli* B and *E. coli* MRE 600 enzyme were identical when prepared by the same procedure. The subunit composition of *E. coli* B RNA polymerase, prepared by a procedure which utilised glycerol gradient centrifugation in both high and low ionic strength buffers, has already been published (Bautz et al., 1970) and shown to consist of β' , β , α and σ subunits only.

A partial purification of *E. coli* MRE 600 RNA polymerase was performed substituting a low ionic strength buffer glycerol gradient ultracentrifugation step in place of the Biogel A5M gel filtration step in the purification procedure outlined in section 2.2.3, again the component θ was found to be present on analysis of the holoenzyme by SDS-polyacrylamide gel electrophoresis. However more recently in this laboratory, *E. coli* MRE 600 RNA polymerase when prepared by ultracentrifugation in both high and low ionic strength buffer glycerol gradients shows only the subunits β' , β , α and σ , θ sedimenting ahead of the holoenzyme as a very high molecular weight aggregate (P.J. Roach and E. Robertson, personal communication).

E. coli RNA polymerase can now be obtained commercially from Miles

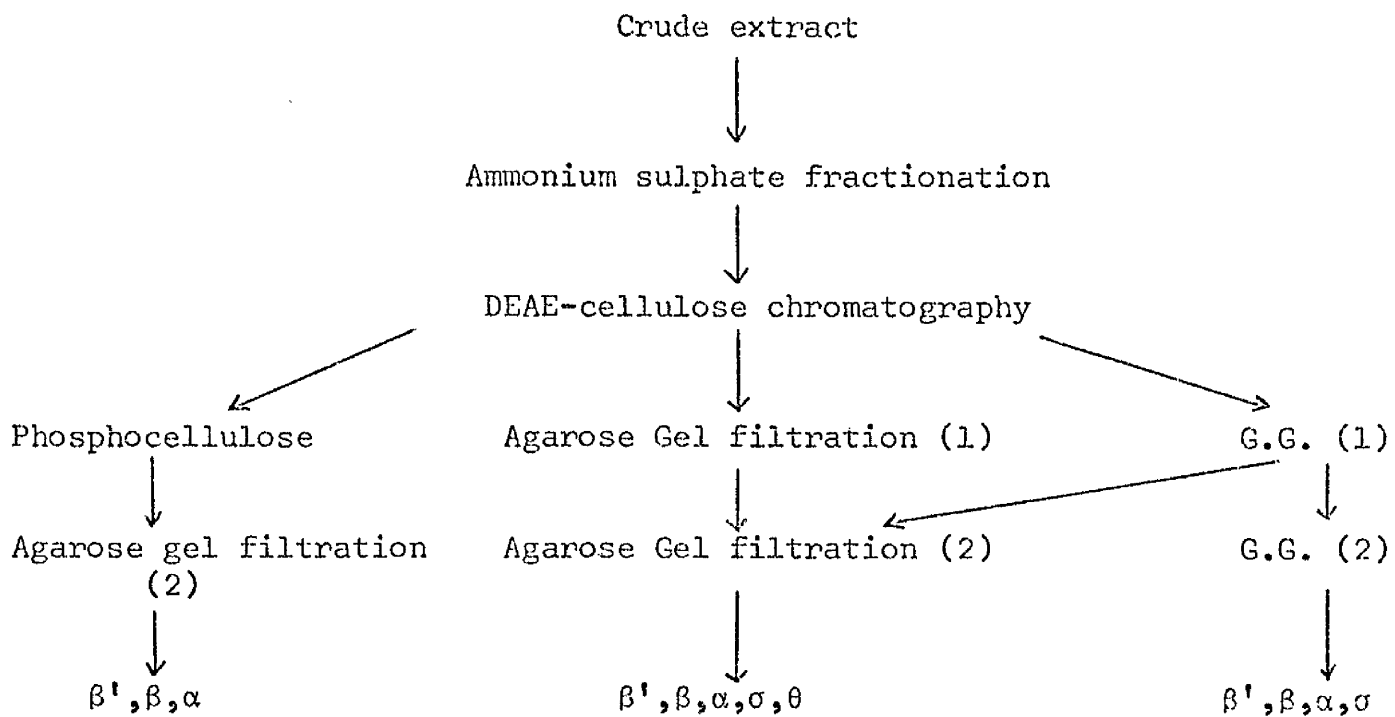
Seravac. On analysis of the commercial enzyme by SDS-polyacrylamide gel electrophoresis the component θ was found to be present. Therefore it has now been almost conclusively proved that it is the method of preparation of E. coli RNA polymerase which dictates the number and stoichiometry of the subunits present in the enzyme. Figure 4.1 summarises the purification procedures which lead to the different subunit compositions of RNA polymerase. To obtain core enzyme ($\beta'\beta\alpha_2$) phosphocellulose chromatography is a necessary purification step; enzyme of subunit composition $\beta'\beta\alpha_2\sigma$ requires glycerol gradient ultracentrifugation in both high and low ionic strength buffers; purification of RNA polymerase using agarose gel filtration yields enzyme which contains the subunits $\beta',\beta,\alpha,\sigma$ and θ . The use of the phosphocellulose chromatography as a necessary step for the preparation of core enzyme can be substituted by hydroxyapatite chromatography as it too can remove σ (and θ) from the core enzyme molecule in a manner analagous to phosphocellulose (section 3.3.4). It would appear, therefore, as both phosphocellulose and hydroxyapatite used charged phosphate groups to displace σ (and θ) from the core enzyme and to bind the core enzyme, that phosphate groups play an important role in the release of σ from a DNA-RNA polymerase-RNA complex actively synthesising RNA.

4.1.3.

θ AS A COMPONENT OF RNA POLYMERASE

All preparation of RNA polymerase purified from E. coli MRE 600 by the modified Burgess (1969a) procedure (section 2.2.3), when analysed by SDS-

FIGURE 4.1. PURIFICATION PROCEDURES FOR THE PREPARATION
OF RNA POLYMERASE OF DIFFERENT SUBUNIT
COMPOSITIONS



polyacrylamide gel electrophoresis showed the presence of the protein component θ . This polypeptide appears to be fairly strongly bound to the RNA polymerase molecules and seems unlikely to be a protein impurity completely unrelated to the enzyme which is an artefact of the purification procedure. There are several reasons for this hypothesis -

(1) The RNA polymerase purification procedure: the enzyme purification method, which involves ammonium sulphate fractionation, stepwise DEAE-cellulose chromatography and two agarose gel filtration steps, the first of which selects only high molecular weight protein species of molecular weight around 900,000 daltons (RNA polymerase dimer) and the other only proteins of about 450,000 daltons in molecular weight (RNA polymerase monomer) almost eliminates the possibility that aggregates of θ (molecular weight 80,000 daltons) coincidentally co-purifying with the RNA polymerase molecules.

(2) In Schlieren photographs (figure 3.6) and UV analytical ultracentrifugation profiles (figure 3.7) of RNA polymerase, shown by SDS-polyacrylamide gel analysis to have θ present as about 15% of the total protein, measured by the intensity of the staining of the enzyme subunits with amido black in the gels, there is no detectable low S-value protein species which one would expect if θ was an impurity and not bound to the other RNA polymerase subunits.

(3) The chromatographic behaviour of θ is very like that of σ when RNA polymerase holoenzyme (β' , β , α , σ and θ subunits) is analysed further by several different chromatographic techniques which show almost unequivocally that θ is related to RNA polymerase or one of its subunits.

(a) Phosphocellulose chromatography: both θ and σ appear in the flowthrough from a column of phosphocellulose to which RNA polymerase holoenzyme has been applied, in almost the same ratio that they are present in the holoenzyme molecule (section 3.3.3; figure 3.16).

(b) Hydroxyapatite chromatography: when RNA polymerase is applied to a column of hydroxyapatite the first protein peak to elute as the sodium phosphate concentration in the elution buffer increases also contains mainly θ and σ (section 3.3.4) in approximately the ratio in which they were present in the holoenzyme (figure 3.18).

(c) DEAE-cellulose chromatography: native RNA polymerase holoenzyme when applied to the column elutes as two distinct enzyme species, the first consists of the subunits β' , β , α , σ and θ with the proportion of θ partly decreased relative to the other subunits; the second peak consists mainly of β' , β , α and θ , σ is absent.

(d) DEAE-sephadex chromatography: this was performed on fraction 6 RNA polymerase holoenzyme. RNA polymerase elutes as three distinct protein peaks on salt gradient elution of a DEAE-sephadex column to which the enzyme had been applied (D. Carl, personal communication). On analysis of each of the peaks by SDS-polyacrylamide gel electrophoresis in this laboratory, the subunits present in the three protein species were determined; (i) β' , β , α and θ , (ii) β' , β' , α , σ and θ , (iii) β' , β , α , σ , θ and trace amounts of τ , an impurity of some RNA polymerase preparations.

(e) An apparently similar subunit has been detected as a component of *E. coli* A19 RNA polymerase (Hirschbein et al., 1969; Chelala et al., 1970) prepared by the procedure of Babinet (1967).

The chromatographic behaviour of θ indicates that this protein component of the enzyme RNA polymerase is very unlikely to be an impurity of the enzyme preparation and there is some evidence that it is very closely linked with the enzyme as it is almost impossible to dissociate θ from $\beta'\beta\alpha_2\sigma$. θ may be very closely related to the σ subunit of RNA polymerase.

4.1.4.

THE ORIGIN OF θ

The observation that RNA polymerase purified from *E. coli* MRE 600 (section 3.2.2.2.1) and *E. coli* A19 (Chelela et al., 1970), both RNase 1⁻ strains of the bacterium, contained θ (or μ as Chelela et al. (1970) report) suggested that perhaps the presence of θ as a component of the holoenzyme preparation was in some way associated with this lack of ribonuclease activity in the bacterium. This hypothesis was subsequently disregarded in the light of the evidence that it was the method of preparation of the RNA polymerase which dictated the subunit composition of the enzyme (section 4.1.2).

A subunit of similar molecular weight can also be detected in some preparations of *A. vinelandii* RNA polymerase, this subunit has been termed epsilon (molecular weight 65,000 daltons), by Krakow et al. (1970). One interesting feature of the *B. subtilis* RNA polymerase is that the σ subunit has a molecular weight of 55,000 daltons, similar to that of θ , c.f. *E. coli* σ of 80,000 to 95,000 daltons, although the other RNA polymerase subunits are very similar to those of the *E. coli* enzyme.

However, *E. coli* MRE 600 θ has been shown to exhibit no σ -like activity with respect to its ability to stimulate RNA synthesis on a T7 DNA template (section 3.3.5).

The report of Zillig et al. (1970a) that when isolated σ protein was denatured in the presence of 6M-urea, pH 11, the subunit partially dissociated into two smaller protein species, μ (60,000 daltons) and ν (15,000 daltons) suggested that perhaps σ was a complex of subunit composition $\mu\nu_2$. We were unable to repeat this experiment with partially purified *E. coli* MRE 600 σ subunit (section 3.3.2) but his result did suggest that θ may well be a breakdown product of σ . In *E. coli* MRE 600 RNA polymerase preparations, a minor subunit of molecular weight 17,500 daltons was sometimes detectable, this could correspond to the ν of Zillig et al. (1970a).

We have also made the observation that on storage of RNA polymerase for long periods of time, i.e. over one year, the activity of RNA polymerase on T7 DNA relative to calf thymus DNA decreases indicating that there is probably some breakdown of the σ subunit of the enzyme, in parallel with this loss of activity on T7 DNA templates, SDS-polyacrylamide gel electrophoresis of the aged enzyme shows that the σ content relative to the θ content of the holoenzyme has slightly decreased suggesting that θ may indeed be a breakdown product of σ .

The inhibitor protein fraction isolated from the Biogel A5M column in the preparation of RNA polymerase has been shown to have some apparently non-specific proteolytic activity (section 3.4.3). The possibility cannot be ruled out, however, that some of this fraction may

be present as a trace impurity in RNA polymerase preparations, and it may play a role in the $\sigma \rightarrow \theta$ breakdown.

RNA POLYMERASE INHIBITOR

Although two major protein factors have been isolated which are known to actively stimulate RNA synthesis by DNA dependent RNA polymerase - σ factor, which is the initiation factor necessary for RNA synthesis on bacteriophage DNA templates (Travers and Burgess, 1969), and M-factor which increases the apparent rate of RNA synthesis even in the presence of saturating amounts of σ (Davison et al., 1969), only one factor has so far been discovered which is able to depress the overall rate of RNA synthesis by RNA polymerase, this is rho (ρ) factor which has been isolated and purified by Roberts (1969). Rho factor has been found to act as a termination factor for RNA synthesis and causes the release of RNA chains from the RNA-DNA-RNA polymerase complex. This leads to the apparent depression of RNA synthesis which is the method of assay for ρ factor (molecular weight, 50,000 daltons). Rho factor exists as a tetramer of molecular weight 200,000 daltons (Roberts, 1969). Rho factor is the only protein factor so far discovered which can cause the apparent or real depression of RNA synthesis by RNA polymerase on a DNA template.

It is not impossible that all or part of the depression of RNA synthesis caused by the inhibitor protein (section 3.4) is due to the presence of ρ factor, but it is extremely unlikely for several reasons - (i) ρ factor is known to be inactive in buffers of ionic strength greater

than 0.15 (Richardson, 1970; Goldberg, 1970). All of the RNA polymerase assays in the presence of the inhibitor protein were performed in buffers of ionic strength greater than 0.15.

(ii) the molecular weight of ρ is 200,000 daltons (Roberts, 1969), the molecular weight of the protein in the peak of the inhibition fraction (see figure 3.22) is $320,000 \pm 50,000$ daltons (section 3.4.3).

Could the apparent depression of RNA synthesis by the inhibitor protein be due to any other enzymes known to affect the activity of RNA polymerase? The presence of ribonuclease is the most likely to cause the apparent depression of RNA synthesis, however the inhibitor protein is most unlikely to be ribonuclease for several reasons -

(i) the inhibitor protein is thermolabile, being completely inactivated by heating to 100°C ,

(ii) the molecular weight of the native inhibitor protein is apparently very large, being in the region of $320,000 \pm 50,000$ daltons,

(iii) there is no detectable RNase activity,

(iv) *E. coli* MFE 600 has no ribonuclease activity (Cammack and Wade, 1968).

RNA polymerase is known to be inhibited by low molecular weight RNA which is believed to block the DNA binding site on the enzyme (Alberts et al., 1968). The A_{280}/A_{260} of 1.4 indicates that the inhibitor protein fraction is less than 1% by weight nucleic acid and it therefore seems unlikely that RNA polymerase is being inhibited in this manner.

The inhibitor protein fraction from the Biogel A5M column (figure 3.22) contains some proteolytic enzyme activity (section 3.4.3) although

the very high molecular weight of this fraction $320,000 \pm 50,000$ daltons is very much larger than known proteases. This proteolytic activity could be causing some if not all of the effects shown by the inhibitor protein. Sigma subunit of RNA polymerase is known to be relatively easily removed from the other four core enzyme subunits $\beta'\beta\alpha_2$, which are known to be strongly associated. Thus if the inhibitor protein was acting as a protease the sigma subunit would be more susceptible to proteolytic attack than the core enzyme subunits. This could account for the effect the inhibitor fraction has on both initiation and transcription. (table 3.9).

The proteolytic activity associated with the inhibitor fraction could mean that there may be trace protease contamination of fraction 5 and fraction 6 RNA polymerase (section 2.2.3). This could be the main cause of the slow inactivation of RNA polymerase on storage.

The questions posed by the apparent partial loss of activity of the inhibitor protein as an initiation inhibitor on storage nor on the significance it may have in the σ to θ conversion cannot be easily explained without further detailed experimentation to clarify matters.

4.3.

PHYSICAL AND CHEMICAL PROPERTIES OF E. COLI MRE 600

RNA POLYMERASE

4.3.1.

AMINO ACID COMPOSITION

The amino acid composition of E. coli MRE 600 RNA polymerase, core enzyme and holoenzyme, of known purity and subunit composition was accurately determined as described in section 3.5.2. and analysed on two different amino acid analysers which gave very similar results, the average of which, for each enzyme species, is shown in table 3.10. The overall results obtained are generally similar to the amino acid analysis of whole E. coli protein (Maitra and Hurwitz, 1967).

As mentioned in section 3.5.2 the amino acid composition of E. coli MRE 600 RNA polymerase core enzyme differs from those already published for other E. coli core enzymes (Burgess, 1969b (E. coli K-12); Nicholson, 1971 (E. coli B)), which also differ quite significantly from each other (table 3.11). These differences could be accounted for in one if not all of several different ways. Firstly as a result of the experimental error in the determination of the amino acid analyses; secondly, there may be trace impurities or small amounts of the other RNA polymerase subunits still present leading to an inaccurate amino acid composition (Nicholson, 1971); thirdly the amino acid composition of the core polymerase isolated from these three strains of E. coli may be different (Richardson, 1969). This would appear to be the least likely reason but it cannot be excluded. In any of these three ways the apparent

discrepancies in the amino acid compositions could be accounted for.

It is very difficult to compare directly the amino acid composition of *E. coli* MRE 600 RNA polymerase holoenzyme with those already published (Maitra and Hurwitz, 1967 (*E. coli* ω); Priess and Zillig, 1967 (*E. coli* K-12)) as the subunit composition of these RNA polymerase species is not known and it has been shown that RNA polymerase holoenzyme can vary in the proportion of σ subunit present depending on the method of preparation and the procedure used (Richardson, 1970; Chamberlin, 1970). However the amino acid composition of the *E. coli* MRE 600 holoenzyme is very similar to that obtained by Priess and Zillig for *E. coli* K-12 holoenzyme.

The partial specific volume of both *E. coli* MRE 600 RNA polymerase species determined from the amino acid composition by the method of Cohn and Edsall (1943) is significantly higher than that already determined by other authors (table 3.12) by the same procedure but the value obtained does not greatly effect the calculation of the molecular weight of the enzyme.

An approximate specific absorbance for *E. coli* MRE 600 RNA polymerase was calculated from the amino acid composition by the method of Beaven and Holiday (1952) in order to estimate enzyme concentrations accurately and quickly for enzyme assays and physical studies. The approximate value of the specific absorbance of the core enzyme of 0.62/mg/ml is very close to the value of 0.63/mg/ml obtained by other methods (section 3.6). This result is similar to *E. coli* B holoenzyme (0.65/mg/ml) (Richardson, 1966a) but different from *E. coli* B core enzyme

(0.541/mg/ml) (Nicholson, 1971) and E. coli K-12 holoenzyme (0.83/mg/ml) (Fuchs et al., 1964).

4.3.2.

ULTRAVIOLET SPECTRUM OF RNA POLYMERASE

The ultraviolet spectrum of E. coli MRE 600 RNA polymerase (core and holoenzyme) was that of a typical protein with a maximum at 275.5nm and a minimum at 251nm, the presence of a significant proportion of phenylalanine in the enzyme was indicated by the marked inflexions in the spectrum between 250 and 270nm. The values of the A_{280}/A_{260} of 1.60 for the holoenzyme and 1.87 for the core enzyme compare favourably with the literature values (table 1.1).

The Warburg and Christian (1941) data relating the amount of nucleic acid contamination of a protein to the A_{280}/A_{260} does not strictly apply to any protein with a different extinction coefficient from yeast enolase which they used to compile their data, therefore a similar, but more accurate relationship was calculated using E. coli RNA polymerase (core) and DNA which enabled the percentage nucleic acid contamination of any enzyme preparation to be determined. The Kalckar (1947) formula for determining protein concentrations spectrophotometrically, even in the presence of low concentrations of DNA, was rewritten to be strictly applicable to E. coli MRE 600 RNA polymerase.

$$\text{protein concentration (mg/ml)} = 2.1348A_{280} - 1.1091A_{260}$$

4.3.3.

ANALYTICAL ULTRACENTRIFUGATION STUDIES

The sedimentation behaviour of *E. coli* MRE 600 RNA polymerase is similar to that of most other *E. coli* RNA enzymes in that it exists as a dimer at low ionic strength and a monomer at high ionic strengths. The S-values obtained for homogeneous preparations of both the core enzyme and holoenzyme are in agreement with those in the literature (table 1.2), except that the value for the holoenzyme is significantly lower than that found by Berg and Chamberlin (1970) in an accurate $S_{20,w}$ determination of *E. coli* B holoenzyme with a full complement of σ subunit. One outstanding feature of the sedimentation velocity studies was that in buffer of high ionic strength ($\mu = 0.51$), where the enzyme exists as a monomer, the sedimentation coefficients of the core enzyme ($\beta'\beta\alpha_2$) and holoenzyme ($\beta'\beta\alpha_2(\sigma,\theta)$) species are virtually identical although the holoenzyme has a significantly higher molecular weight (table 3.13). This would appear to indicate that the conformation of the two species in solution differ quite considerably, the holoenzyme being a more compact aggregate of its component subunits than the core enzyme in order to have the same S-value. This is an observation to which the technique of optical rotatory dispersion could be applied.

One species of RNA polymerase prepared by analytical DEAE-cellulose chromatography of the holoenzyme (section 3.3.3) and shown by SDS-polyacrylamide gel electrophoresis (figure 3.9) to consist of the subunits $\beta'\beta\alpha_2\theta$ (i.e. no σ present), does not appear to be able to form a dimer in low ionic strength buffer unlike both core and holoenzyme.

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In high ionic strength buffer ($\mu = 0.51$) the $S_{20,s}$ was 13.5, but in low ionic strength solution ($\mu = 0.01$) the $S_{20,s}$ was only 16.7, indicating that only a weak interaction of the protomer to form a dimer had occurred. From these observations it would appear that θ subunit interfered with the normal aggregation properties of this RNA polymerase species preventing the association of two such protomers to form a dimer. Thus it is possible that θ binds to the core polymerase at a site on the monomer close to that responsible for dimer formation in low ionic strength solutions.

The molecular weight of RNA polymerase has been published by many authors (table 1.2). Since a freshly prepared sample of both species of *E. coli* MRE 600 RNA polymerase exist as single sedimenting species at high ionic strength it was possible, therefore, to determine their molecular weights by low-speed sedimentation equilibrium. Accordingly both core and holoenzyme were subjected to equilibrium sedimentation in high ionic strength buffer ($\mu = 0.51$). The ionic strength of the solution was such that the effects of preferential hydration of the enzyme were kept to a minimum (Berg and Chamberlin, 1970). Complications unfortunately did arise when the observation was made that on standing or over the prolonged centrifugation time required to reach equilibrium, some breakdown of the enzyme occurred which had to be allowed for in the final molecular weight calculations. This breakdown was probably due to the large molecular weight and subunit composition of the enzyme, and its susceptibility to proteolytic digestion.

A value of 364,000 daltons was obtained for the core enzyme, using a \bar{v} of 0.744 (section 3.5.3.2), correcting for partial dissociation of the enzyme during centrifugation (Berg and Chamberlin, 1970) the molecular weight of the core enzyme became 378,000 daltons ($\pm 10\%$). The molecular weight of core enzyme obtained by the addition of the molecular weight of the component subunits determined by SDS-polyacrylamide gel electrophoresis is $364,000 \pm 16,000$, which is in complete agreement with that from sedimentation equilibrium.

Similarly the sedimentation equilibrium molecular weight of RNA polymerase holoenzyme (shown by SDS-polyacrylamide gel electrophoresis to contain θ and not a full complement of σ subunit) was found to be 431,000 daltons ($\pm 10\%$) which was only slightly lower than that calculated from the subunit composition ($442,000 \pm 18,000$ daltons). The molecular weight of the RNA polymerase holoenzyme dimer in low ionic strength buffer ($\mu = 0.01$) is 886,000 daltons ($\pm 10\%$). The molecular weight obtained for the holoenzyme is lower than that published by some other authors (Richardson, 1966a; Ruet et al., 1970) (table 1.3). Errors can arise in the determination of the holoenzyme molecular weight for two main reasons -

(1) most RNA polymerase holoenzyme preparations do not have a full complement of σ subunit (Richardson, 1970; Zillig et al., 1970b; Berg and Chamberlin, 1970),

(ii) partial dissociation of the σ subunit from the holoenzyme can occur during centrifugation (Berg and Chamberlin, 1970).

The first of these complications in the molecular weight de-

termination can be overcome, but the second would appear not to be readily circumvented. However an accurate holoenzyme molecular weight can be calculated by adding the molecular weight of core enzyme and free σ subunit together.

4.3.4.

THE OPTICAL ROTATORY DISPERSION OF RNA POLYMERASE

The ultraviolet and visible optical rotatory dispersion of native *E. coli* MRE 600 RNA polymerase holoenzyme is that of a typical protein containing both helical and random coil (disordered) secondary structures. There is a trough around 232nm with $[\alpha]_{232} = -4.2^\circ$. The helix content measured by the procedure of Urnes and Doty (1961) based on the Moffitt-Yang b_0 gives higher values of helix content (see section 3.8). The ionic strength of the buffer solution does not greatly affect the percentage helix which varies from 31% ($\mu = 0.02$) to 33% ($\mu = 0.51$). The value obtained for the helix content is similar to that found by Novak and Doty (1971) for *E. coli* core polymerase from both ORD and CD studies. This would appear to suggest that there is very little or no detectable conformational change in the RNA polymerase molecule when σ subunit is dissociated from the holoenzyme. However these results do not agree with those of Nicholson (1971) who has determined the helix content of *E. coli* B core polymerase; he obtains a value of only 13% from the Moffitt-Yang b_0 by the Urnes and Doty (1961) procedure. The value obtained for b_0 in the Moffitt-Yang equation is dependent on the method used to determine the concentration of the protein. Nicholson (1971) has

calculated a value of 0.541/mg/ml for the specific absorbance from the amino acid composition. This is much lower than the value of 0.65/mg/ml determined by Richardson (1966a) for *E. coli* B holoenzyme which was the value assumed by Novak and Doty (1970) in their helix content determination. If a value of 0.65/mg/ml is assumed for the specific absorbance of *E. coli* B core enzyme the revised helix content of the Nicholson (1971) *E. coli* B core enzyme still does not approach the value of 32% for *E. coli* MRE 600 holoenzyme (section 3.8.) or *E. coli* core polymerase (Novak and Doty, 1970). The subunit composition of any *E. coli* RNA polymerase must be known, and the enzyme must be of high specific activity (native enzyme) before rotatory dispersion measurements are performed.

It is possible to compute the approximate helix content of a native protein from its amino acid composition. Goldsack (1969) has found a linear correlation between the Moffitt-Yang b_0 and the per cent helix-forming and non helix-forming amino acids (section 3.5.3.4). The amino acid compositions of RNA polymerase species from several *E. coli* are known and table 3.12 shows the helix content determined from their respective amino acid analyses by the Goldsack (1969) procedure. The values determined for both core and holoenzymes are shown in table 3.12 and suggest that the helix content can lie anywhere between 7 and 51% which does not exclude the rather low value obtained by Nicholson (1971). However the results (table 3.12) suggest that the helix content of RNA polymerase, both forms, lies between 30 and 40%.

It is possible to rationalise the UV ORD of a protein by the intro-

duction of β conformation by the method of Greenfield, Davidson and Fasman (1967) based on the ORD of poly-L-lysine in the α -helix, β -sheet and random coil conformations. It has been found very difficult to analyse the ORD of a native protein in terms of the percentage of each of these three conformations as it does not take into account the intrinsic residue rotations of each of the constituent amino acid residues (Jovin et al., 1969). Therefore no attempt was made to analyse the ORD of native E. coli MRE 600 RNA polymerase by this procedure. Instead it was sufficient to show that conformations other than helix and disordered random coils do exist in RNA polymerase. The procedure used was that of Blout et al. (1967) (section 3.8).

RNA polymerase holoenzyme which has been denatured in a 1% SDS solution shows very little change in helix content and on analysis by the procedure of Blout et al. (1967) (section 3.8) shows only helix and random coil conformations any other structures having been lost. The optical rotatory dispersion of RNA polymerase holoenzyme in 6M-GuHCl, 1% (v/v) 2-mercaptoethanol shows a large decrease in the Moffitt-Yang b_0 and adopts a conformation in solution which is consistent with that of a random coil (Tanford et al., 1967). The value for b_0 and the analysis of the ORD by the procedure of Blout et al. (1967) suggest that the conformation of RNA polymerase holoenzyme in 8M-urea, 1% (v/v) 2-mercaptoethanol is not a complete random coil as might be expected but that some helix conformation still exists.

THE INTERACTION OF E. COLI MRE 600 RNA POLYMERASE WITH DNA

Novak and Doty (1970) have performed the only study, by the related techniques of optical rotatory dispersion and circular dichroism, of the interaction of RNA polymerase with DNA. Using E. coli RNA polymerase, core enzyme, they investigated its interaction with calf thymus DNA. Although there was no detectable conformational change in the enzyme on binding to DNA, thermal ORD studies showed that helix conformation in the enzyme had now become relatively unstable and was now more susceptible, than in the free enzyme, to thermal denaturation as shown by the decrease in the $-\left[m'\right]_{232}$ on heating.

The experiments performed by Novak and Doty (1970) do not stand up to critical analysis on several counts. Firstly the published ORD of the calf thymus DNA used in their experiments is similar to that of partially denatured DNA (Samejima and Yang, 1965) and does not correspond to that of native calf thymus DNA (Samejima and Yang, 1965). Figure 3.44 shows the ORD of native calf thymus DNA used in the experiments with E. coli MRE 600 RNA polymerase. Secondly, their procedure for determining the ORD curve of the binary DNA-RNA polymerase complex and comparing it with an ORD curve derived from the sum of the individual components is not able to show whether any small conformational changes have taken place on the binding of the enzyme to the DNA. Thirdly, using core enzyme which has no initiation factor (σ) most probably does not mirror true in vivo binding, as core enzyme is believed to bind only to nicks, free ends or any other irregularities in the secondary structure.

of DNA (Vogt, 1969; Chamberlin, 1970). To overcome these objections, native calf thymus DNA was used in all the experiments; tandem cells were employed so that the ORD curves of the components of the reaction before and after mixing could be determined and their respective rotatory dispersions measured and compared directly; finally, *E. coli* MRE 600 RNA polymerase holoenzyme of known subunit composition, prepared by the Burgess (1969a) procedure (section 2.2.3) was used in all the experiments.

The results are given in section 3.9. Contrary to the results of Novak and Doty (1970) they show that on binding to calf thymus DNA, *E. coli* MRE 600 holoenzyme undergoes a conformational change. This change is shown by a decrease in the $-\left[\alpha\right]_{232}$ of the DNA-RNA polymerase binary complex compared to that of the individual components in series. There was no detectable change in the ORD of the DNA to which the RNA polymerase was bound, although Kosoganov et al. (1971), using a formaldehyde titration technique, have found that partial unwinding of the DNA helix does occur in the presence of *E. coli* RNA polymerase. Disruption of the secondary structure of DNA can be easily detected by a decrease in the $\left[\alpha\right]_{290\text{nm}}$ for the DNA (Samejima and Yang, 1965), however no such change was observed. Raising the temperature of the binary complex of DNA-RNA polymerase from 20° to 45°C gave results similar to those of Novak and Doty (1970) in that there is a decrease in the $-\left[\alpha\right]_{232}$ at 45°C , i.e. the DNA-bound enzyme has undergone partial thermal disruption of its helix conformation (denaturation). No such effect is observed with free RNA polymerase. This thermal denaturation cannot be reversed on cooling the DNA-RNA polymerase complex.

The ratio of RNA polymerase to DNA in the complex is such that all of the enzyme molecules are DNA-bound (Richardson, 1969), however the number of RNA polymerase molecules most probably exceeds the number of promoter sites on the DNA molecule. This indicates that the interaction between the RNA polymerase and DNA is most probably an electrostatic interaction where positively charged regions of the enzyme (Burgess, 1969b) interact with the DNA (polyanion) and it is this interaction which is causing the small detectable conformational change in the enzyme on binding to DNA. Novak and Doty (1970) have suggested that the strain imposed on the secondary structure of RNA polymerase as shown by its inability to resist thermal disruption of the helical regions when bound to DNA could be due to localised DNA strand separation effected by the enzyme prior to RNA synthesis, as they find that such strain is released after the onset of RNA synthesis. It is not known whether the presence of the substrate nucleoside triphosphate molecules stabilise the conformation of the enzyme and protect it from the effects of heating

If all of the RNA polymerase molecules were bound to true promoter regions and not just indiscriminately along the length of the DNA molecules the effect of heating on the stability of the DNA-RNA polymerase complex may not be as great.

Extensive studies are required using native *E. coli* bacteriophage DNA as the RNA polymerase template. These DNA molecules, e.g. T4 and T7, are known to have a limited number of promoter sites specific for RNA polymerase holoenzyme (Bautz and Bautz, 1970; Chamberlin, 1970), and further experiments along these lines could perhaps elucidate the nature of the *E. coli* RNA polymerase-DNA interaction which is the importa

first step in the transcription process (section 1.4.1.1).

SECTION 5 APPENDIX : ORD COMPUTER PROGRAMS

C-FOCAL,1969

02.10 T "DRUDE EQUATION",!!!
02.20 A "PATH LENGTH (DM) ",D,!
02.30 A "CONCENTRATION (GM/100ML)",C,!
02.40 A "V",B
02.50 A "ALPHA (DEG) ",A,!!
02.60 S $G=(100*A)/(D*C)$
02.70 S $H=10/B$

03.10 T "XD",G
03.20 T " YD", $G*H+2$,!!!
03.30 GOTO 2.4

*

PROGRAM 1 DRUDE EQUATION

Given the constants of the cell path length in decimetres,
PATH LENGTH (DM), and the concentration of the sucrose
solution in gm/100ml, CONCENTRATION (GM/100ML), this program
calculates the two terms $[\alpha]_{\lambda, XD}$, and $\lambda^2 [\alpha]_{\lambda, YD}$, for each
value of the observed rotation in degrees, ALPHA (DEG),
at each wave number ($\times 10^{-3}$), V.

C-FOCAL, 1969

01.20 T "DRUDE EQUATION COEFFICIENTS ",!!!

01.30 S XY=0

01.40 S X2=0

01.50 S X=0

01.60 S Y=0

01.61 S Y2=0

01.70 S K=0

02.10 A "NO OF POINTS",N,!

02.11 A "XD",A

02.20 A "YD",B,!!

03.10 S X=X+A

03.20 S Y=Y+B

03.30 S XY=XY+A*B

03.40 S X2=X2+A²

03.41 S Y2=Y2+B²

03.50 S K=K+1

03.51 I (N-K) 5.1,5.1,2.11

03.60 GOTO 2.1

05.10 S M=(XY-X*Y/N)/(X2-X²/N)

05.20 S C=(X*XY-Y*X2)/(X²-N*X2)

05.21 S D=(X2-(X²)/N)

05.22 S G=FSQT(D)

05.23 S E=(Y2-(Y²)/N)

05.24 S J=FSQT(E)

05.27 S R=(XY-X*Y/N)/(G*J)

05.30 T %6.05,"GRADIENT (L2C)",M,!

05.40 T %6.05,"INTERCEPT (K)",C,!

05.50 T %6.04,"REG COEFF",R,!!!

05.60 GOTO 1.2

*

PROGRAM 2 DRUDE EQUATION COEFFICIENTS

Given as a constant the number of pairs of points,
NO OF POINTS, this program determines the best straight
line by the least squares procedure to the series of
points $[\alpha]_{\lambda}$, XD , $\sum [\alpha]_{\lambda}$, YD , obtained by Program 1.
The GRADIENT (L2C) and INTERCEPT (K) give respectively
the coefficients λ_c^2 and K of the Drude equation. The
regression coefficient, REG COEFF, of the line is
also determined.

C-FOCAL, 1969

01.50 T "SELLMEIER COEFFICIENTS",!!!

02.10 A "LAMBDA(1)",A

02.20 A " REFRACTIVE INDEX(1)",B,!!

02.30 A "LAMBDA(2)",C

02.40 A " REFRACTIVE INDEX(2)",D,!!

03.10 S $K=C^2*(B^2-1)-A^2*(D^2-1)$

03.20 S $L=A^2*C^2*(B^2-D^2)$

03.30 S $M=(B^2-1)*(D^2-1)*(C^2-A^2)$

03.40 T %6.06,"L2V",L/K

03.50 T %6.06," ALPHA",M/K,!!!

03.60 GOTO 1.5

*

PROGRAM 3 SELLMIEIER COEFFICIENTS

This short program calculates the two coefficients λ_v^2 , L2V, and α , ALPHA, of the Sellmeier Equation (section 2.2.9.3) from the refractive indices, REFRACTIVE INDEX(1) and REFRACTIVE INDEX(2), of the solution at the two wavelengths, LAMBDA(1) and LAMBDA(2).

C-FOCAL, 1969

02.20 S J=44944

02.30 T "ORD PROGRAM",!!

03.10 ASK "L2V",K,!

03.20 ASK "ALPHA",L,!

03.30 ASK "MRW",A,!

03.40 ASK "PATH LENGTH (DM) ",B,!

03.50 ASK "PROTEIN CONCENTRATION (GM/100ML) ",C,!!!

03.60 S M=A/B/C

04.10 ASK "V",N,"WAVE NOS"

04.20 ASK " ALPHA",O,"DEGREES"!!

04.25 S P=10000/N

04.30 T %6.06,"LAMBDA",P,"NM"!

04.40 S Q=(L*P+2)/(P+2-K)

04.45 S R=(3*O*M)/(Q+3)

04.50 T "(M) ':",R,!!

05.10 T "XM",J/(P+2-J)

05.20 T " YM",R*(P+2-J)/J,!!

05.30 T "XS",50625/(P+2-50625)

05.40 T " YS",R*(P+2-37249)/37249,!!

05.41 GOTO 4.1

*

PROGRAM 4 ORD PROGRAM

The coefficients of the Sellmeier Equation λ_v^2 , $L2V$, and α , ALPHA, the mean residue weight of the protein, MRW, the path length of the cell in decimetres, PATH LENGTH (DM), and the protein concentration in gm/100ml, PROTEIN CONCENTRATION (GM/100ML), are constants.

The program calculates for each rotation in degrees, ALPHA, at the corresponding wave number ($\times 10^{-3}$), V, the pairs of points $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, XM, and $[m'](\lambda^2 - \lambda_0^2)/\lambda_0^2$ YM, of the Moffitt-Yang analysis of the ORD assuming a λ_0 of 212nm, and the points $\lambda_{225}^2/(\lambda^2 - \lambda_{225}^2)$, XS, and $[m'](\lambda^2 - \lambda_{193}^2)/\lambda_{193}^2$, YS, of the Schechter-Blout analysis of the ORD (see section 2.2.9.4).

C-FOCAL, 1969

01.20 T "MOFFITT-YANG PARAMETERS & HELIX CONTENT",!!

01.30 S XY=0

01.40 S X2=0

01.50 S X=0

01.60 S Y=0

01.61 S Y2=0

01.70 S K=0

02.10 A "N",N,!!

02.11 A "XM",A

02.20 A "YM",B,!!

03.10 S X=X+A

03.20 S Y=Y+B

03.30 S XY=XY+A*B

03.40 S X2=X2+A²

03.41 S Y2=Y2+B²

03.50 S K=K+1

03.51 I (N-K) 5.1,5.1,2.11

03.60 GOTO 2.1

05.10 S $M=(XY-X*Y/N)/(X2-X^2/N)$

05.20 S $C=(X*XY-Y*X2)/(X^2-N*X2)$

05.21 S $D=(X2-(X^2)/N)$

05.22 S $G=FSQT(D)$

05.23 S $E=(Y2-(Y^2)/N)$

05.24 S $J=FSQT(E)$

05.27 S $R=(XY-X*Y/N)/(G*J)$

05.30 T %6.04," GRADIENT",M,!

05.40 T %6.04,"INTERCEPT",C,!

05.50 T %6.04,"REG COEFF",R,!!!

05.60 T "% HELIX(DOTY) ",M/(-6.3)

05.70 T " % HELIX(BLOUT) ",(100-M)/8,!!!

05.80 GOTO 1.2

*

PROGRAM 5 MOFFITT-YANG PARAMETERS & HELIX CONTENT

Given the number of pairs of points, N, as a constant, this program gives a best straight line fit by the least squares method to the series of points $\lambda_o^2/(\lambda^2 - \lambda_o^2)$, XM, and $[m'] (\lambda^2 - \lambda_o^2)/\lambda_o^2$, YM, determined in program 4. The gradient of the line, GRADIENT, gives b_o and the intercept, INTERCEPT, a_o in the Moffitt-Yang Equation. The regression coefficient of the line, REG COEFF, is also determined. The per cent helix content of the protein is calculated by the method of both Urnes and Doty (1961), % HELIX(DOTY), and Schechter, Carver and Blout, % HELIX(BLOUT). See section 2.2.9.5.

C-FOCAL, 1969

```

01.20 T "SCHECHTER-BLOUT PARAMETERS & HELIX CONTENT",!!!
01.30 S XY=0
01.40 S X2=0
01.50 S X=0
01.60 S Y=0
01.61 S Y2=0
01.70 S K=0

02.10 A "N",N,!!
02.11 A "XS",A
02.20 A "YS",B,!!

03.10 S X=X+A
03.20 S Y=Y+B
03.30 S XY=XY+A*B
03.40 S X2=X2+A^2
03.41 S Y2=Y2+B^2
03.50 S K=K+1
03.51 I (N-K) 5.1,5.1,2.11
03.60 GOTO 2.1

05.10 S M=(XY-X*Y/N)/(X2-X^2/N)
05.20 S C=(X*XY-Y*X2)/(X^2-N*X2)
05.21 S D=(X2-(X^2)/N)
05.22 S G=FSQT(D)
05.23 S E=(Y2-(Y^2)/N)
05.24 S J=FSQT(E)
05.27 S R=(XY-X*Y/N)/(G*J)
05.30 T %6.04," GRADIENT",M,!
05.40 T %6.04,"INTERCEPT",C,!
05.50 T %6.04,"REG COEFF",R,!!!
05.60 S K=M/.3591
05.61 S L=C-K*1.3591
05.62 S U=(L+750)/36.5
05.63 S T=(K+60)/(-19.9)
05.64 S P=(L-K+650)/55.8
05.65 T "A193",L
05.66 T " A225",K,!!

06.10 T "H193",U
06.20 T " H225",T
06.30 T " HELIX(MEAN)",P,!!!
06.40 GOTO 1.2
*
```

PROGRAM 6 SHECHTER-BLOUT PARAMETERS & HELIX CONTENT

Given the number of pairs of points, N, as a constant, this program gives a best straight line fit by the least squares procedure to the series of points $\lambda_{225}^2 / (\lambda^2 - \lambda_{225}^2)$, XS, and $[m'] (\lambda^2 - \lambda_{193}^2) / \lambda_{193}^2$, YS, determined in program 4. From the GRADIENT and INTERCEPT of the line the Schechter-Blout coefficients A_{225} , A_{225} , and A_{193} , A_{193} , are calculated (section 2.2.9.4). The regression coefficient, REC COEFF, of the line is also determined. The per cent helix is calculated from the coefficients A_{193} , H193, and A_{225} , H225, (section 2.2.9.5).

C-FOCAL,1969

```
02.10 T "SPECIFIC ROTATION PROGRAM ",!!!  
02.11 A "PATH LENGTH (DM) ",D,!  
02.12 A "CONCENTRATION (GM/100ML) ",C,!!  
02.20 A "WAVE NO.",W,!  
02.30 A "ALPHA (DEG) ",R,!  
02.31 T "LAMBDA",10000/W,!  
02.32 T "SPECIFIC ROTATION",(100*R)/(D*C),!!  
02.33 GOTO 2.2  
*
```

PROGRAM 7 SPECIFIC ROTATION PROGRAM

This program calculates the specific rotation, SPECIFIC ROTATION, (section 2.2.9.3) and the wavelength in nm, LAMBDA, from the rotation in degrees, ALPHA (DEG), and the wave number ($\times 10^{-3}$), WAVE NO., where the path length in decimetres, PATH LENGTH (DM), and the concentration in gm/100ml, CONCENTRATION (GM/100ML), are constant for the given solution.

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