DEVELOPMENT OF PROCEDURES

FOR DNA SEQUENCING OF THE ATTACHMENT SITE

OF LAMBDA BACTERIOPHAGE

BY

MARGARET A. HADDOW

A Thesis

submitted to

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TO MY FAMILY

ABSTRACT

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In order to sequence the attachment site of lambda virus two different protocols were tried. The first approach to sequencing λ att was to form heteroduplexes of λc_1 and λgal strains which would produce a single stranded loop encompassing the phage attachment region. This area would be subjected to digestion by S1 nuclease and the cleavage product isolated. The DNA fragment would then be reannealed to the appropriate $\lambda c_1 L$ strand to provide the necessary template for DNA polymerase I repair. The repair product would be analysed to establish the sequence. The second approach to sequencing the attachment site involved circularizing the heteroduplex product. The circular DNA was then treated with S1 nuclease, and the linear product labelled with polynucleotide kinase.

The growth of phage strains was essential to all work attempted. The λcIS7 strain was readily grown in lysogenic form and is harvested from the bacterial host after induction.

Growth and plating conditions for the phage were optimized. The plating efficiency was dependant on the host, the use of Difco yeast extract, the multiplicity of infection and the preadsorption of the phage to the host. Following these precautions yields of 10^{13}-10^{15} phage/ml could be obtained for λc_1 phage.

The low yield of λgal strains was not resolved by these procedures. Initial work was done on λgal which was used to infect a susceptible host. A second strain was also studied. This strain was used in a similar manner to λcIS7. The use of higher Mg^{++} ion concentration seemed to improve the yield somewhat. The use of pancreatic DNase and chloroform resolved problems with phage loss.

Despite these modifications to the harvesting procedure, the yield was only 10^{11} phage/ml.

To overcome the problem of growing λgal phage, experiments were developed to obtain a lysogen. Several procedures were tried which were unsuccessful. In the course of these experiments it was determined that the addition of 0.8% bile salts would differentiate between λc_1 and λgal.

The purification of S1 nuclease was studied. The assay procedure was dependant on the substrate size and structure. Experimental work with alkaline gradients demonstrated that a DEAE column purified the nuclease sufficiently without reducing the yield excessively.

The specificity of the enzyme was apparently associated with physiological conditions rather than purification conditions. Enzyme studies on specificity were related to the substrate used and the concentration involved. Using denatured DNA, the optimum substrate concentration was 160 μg/ml. In the presence of low concentrations of native DNA (less than 15 μg/ μl S1) some cleavage did occur. This cleavage could be overcome by the addition of small amounts of denatured carrier DNA.

The separated strands of DNA required for annealing were subject to degradation after a period of six weeks.

The isolated strands could be annealed to produce heteroduplexes. After analysis of the heteroduplexes on alkaline sucrose gradients, the pH used in incubating the DNA was determined to be critical.

The heteroduplex DNA and nuclease product had to be recovered from the gradients. It was found that the yield of DNA was low.

Pretreatment of the polyallomer tubes with a mixture of DNA-EDTA-PPi or the use of higher concentrations of sample alleviated the problem somewhat. The denaturation conditions prior to loading the gradients could also be modified to produce clearer peaks.

It was necessary to determine the precise cleavage site of the S1. Initial experiments with various templates indicated that the S1 produced butt ended cleavage.

The final aspect studied was the repair reaction as catalysed by DNA polymerase I. The isolated S1 cleaved fragments annealed to a $\lambda c_1 L$ strand were repaired and the repair product isolated. The 3' end was analysed and the preliminary indication was an adenylate residue. Further work was not done with this system.

Using the second approach to sequencing involved circularizing the heteroduplex product. The procedure to form circles was studied. In general the yield was 10 -30% of the sample treated. Using air to concentrate the DNA and a longer milder ligase treatment gave the best results.

Alkaline gradients were used to analyse the circular product. The peaks for the circles were very low and the profile indicated a mixed linear and circular population. Consequently agarose gels were used to isolate and analyse the circles. Ethidium bromide was used to visualize the DNA samples. Conditions were modified until the circular and linear DNA could be resolved.

Using disc gels on a preparative basis, the bands containing circular DNA were cut out and eluted. The recovery from the eluted sample was very low, about 20%. Further work was not done due to the poor yields.

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ABBREVIATIONS:

MOI	Multiplicity of infection i.e., No. of phage/bacteria.
λc_1	$\lambda c_1 857 Sam7$
λgal	$\lambda gal8c_1857$
λgal S7	$\lambda gal8c_1857Sam7$
DNA	Deoxyribonucleic acid
λ	Lambda virus
NaOH	Sodium hydroxide
$MgSO_4$	Magnesium sulphate
Mg^{++}	Magnesium divalent ion
$ZnSO_4$	Zinc sulphate
Zn^{++}	Zinc divalent ion
CsCl	Cesium chloride
HCl	Hydrochloric acid
tris	tris(hydroxymethyl)aminomethane
EDTA	Ethylene diamine tetra acetate disodium salt
SM	Diluent buffer
SM-Mg	Diluent buffer with high magnesium concentration
^oC	Degrees Centrigrade
poly UG	Copolymer of uracil and guanine
S1	S1 nuclease (EC 3.1.4.21)
kinase	polynucleotide kinase (EC 2.7.1.78)
pol 1	DNA dependant DNA polymerase 1
panc. DNase	Pancreatic DNase

ABBREVIATIONS:

LB	Luria Broth
gal	Galactose utilizing mutant
phage	Bacteriophage
McConkey	Selective and differential medium for specific sugars such as lactose or galactose
$CHCl_3$	Chloroform
CSH	Cold Spring Harbor

. . . "little did I know what lay afore me" . . .

abridged...from David Copperfield

ch. 40 by Charles Dickens

HISTORICAL

In approaching the problem of sequencing the λ attachment site, two areas should be discussed. First, a discussion of how lambda virus lysogenizes its host (in particular the role of the attachment site) and second, a discussion of the DNA sequencing methods available for study of DNA fragments.

A I Introduction

The bacteriophage λ has been the object of intensive study due to, among other reasons, its ability to lysogenize its host, Escherichia coli.

Lysogeny involves the decision not to lyse the host cell as determined by environmental influences [1,2] and the genetic incorporation of the virus genome into the host DNA. The phenomenon of lysogeny has been the subject of many excellent reviews.[3,4,5,6,7]

Campbell[2] proposed a model for lysogeny on which the linear phage DNA first formed a circle by joining ends. The incorporation of the phage into the bacteria involved insertion of the circular λ phage into the circular bacterial chromosome by crossing-over at a specific site. This reciprocal recombination event could account for the difference between vegetative and prophage maps, as well as the appearance of transducing phage.[2]

II Control of Recombination

Recombination in phage is under the control of various systems. Generalized recombination can occur throughout the phage genome. Signer and Weil[8] studied the phage recombination system called *red* through the use of point and deletion mutants. They determined that the *red* system was linked to vegetative phage reproduction and that it can be replaced by the bacterial recombination system *rec*. The majority of recombination seemed to be a product of the *red* rather than the *rec* system. Further work demonstrated that the *red* and *rec* systems were largely independent of each other.

Complementation studies and deletion mutants allowed the *red* system to be analyzed further. Two genes were mapped, *red* α and *red* β which produce respectively λ exonuclease and β protein.[10] The β protein interacts with λ exonuclease to increase its affinity for DNA. Recombination is envisioned to involve some sort of breakage and reunion model, not necessarily reciprocal.[11]

Some workers suggested the role of λ exonuclease in recombination may involve exposure of single stranded regions, which subsequently form a heteroduplex. This is followed by cleavage of redundant areas leaving a nick which is sealed by polynucleotide ligase.[12]

Another gene, γ, has also been implicated with the *red* system. Sakaki determined that the γ gene product is a protein which inhibits the host *recBC* DNase. The inhibition by γ is specific to the host *rec* system. Since the γ gene

lacks nuclease or ATPase activity, it is believed to have a regulatory role.[13] Other research indicated the γ protein facilitates the survival of irradiated lysogens. It is postulated that the protein inhibits only detrimental activity of the red β gene, thus allowing greater survival.[14]

III Integration and Excision as Site Specific Recombination

Site specific recombination involves the int system.[15,16] Mapping studies showed that integration as proposed by Campbell's model[2] occurred at a specific site, designated att.[15]

Gingery and Echols[17] studied integration deficient mutants and suggested an enzymatic role for the int gene, enhancing the structural recombination occurring at the att site. Their work was confirmed[16,18] and the results therefore support Campbell's model[2] where phage-directed site-specific recombination was postulated. Although the relationship between the generalized (red) and specific (int) recombination systems was unclear,[18] workers determined them to be independent.[15,16]

Although the nature of attachment was unclear, Echols, Gingery and Moore[15] suggested that if the int enzyme is involved in recognition of phage and bacterial DNA, there could be two sites on the protein for recognition of DNA.

In working with transducing phage, which have altered attachment sites, Guerrini[19] found that some integration and excision exchanges were not as efficient as others. He proposed that the attachment sites in bacteria were not identical to the phage attachment sites. As a result, he suggested

that int was a bifunctional protein allowing different recombinations depending on whether it was operating in an integration or excision mode. To account for the difference, the excision mode was considered to have a restricted set of exchanges possible during recombination.

The phenomena of integration and excision were complicated by the discovery of another gene, called xis.[20] Guarneros and Echols studied those phage which were unable to excise from their host after a brief heat induction. They determined that int and xis were two different genes. They complemented each other, while none of the xis mutants would complement each other. From deletion mutant studies, the xis site was mapped between int and exo (of the red system). The xis gene appeared to be functionally distinct from the int gene during the excision process. The authors suggested that xis was only involved in excision and that it could produce a protein which would restrict recombination.

In further studies, Echols[21] compared the recombination frequencies of λ with the transducing phages, λbio and λgal. He determined that the xis gene was required in addition to the int gene for excision, while only the int gene was necessary for integration to occur. The data also showed that xis was specific for those end normally present in the integrated state (Figure 1).

Both genes int and xis were found to be site specific.[21,22] This specificity of the int gene in recombination caused some workers to consider that int may be involved in recognizing the

site as well as facilitating the recombination[23].

Echols[21] suggested various modes of action for int and int complexed to xis. Free int product would favour integration of phage into the lysogenic state. Once integrated, the repressor would keep the levels of both gene products low. However, when induced, the formation of both int and xis would allow the formation of a complex and thus favour excision[23].

In order to explain the phenomenon of insertion and excision, Dove[24] proposed that the integrated state of the prophage was inherently more stable than the excised state. He suggested that the int gene product is a catalyst for both reactions, while the xis gene provides the necessary energy. The lysogenic state of λ is thus stabilized by a favourable energy situation. The xis gene product could provide energy by complexing with the int gene product[24].

The excision was determined to be reciprocal, regenerating the bacterial attachment sites. Also the excision process required a pair of attachment sites at the extremities to be effective[25].

The int protein has been purified[26,27] and the purity checked using SDS polyacrylamide gels. Ausubel[27] confirmed the identity of his extract by using different amber mutants in combination with permissive and nonpermissive hosts. He found the peak of the int protein would disappear under non-permissive conditions and reappear when a favourable host was used.

In experiments on the binding of the purified int protein, both Nash and Ausubel found only non-specific binding. Nash[26] suggested that the specific or essential factors were lost during the purification, or alternatively that the specific binding by int was masked by another protein found in the extract.

Further work[28] with an in vitro system and partially purified cell extracts defined some of the requirements for integrative recombination. It was found that spermidine, Mg^{++}ion and ATP were absolute requirements for the reaction.

Later studies[29] demonstrated that the int gene product did not regulate integration. The int gene product was determined to be a component of the recombination reaction in vivo and in vitro. Using a direct DNA binding assay, other workers determined that the int protein was involved in recognition of DNA sequences at the attachment site[30].

In studies on the excision process, Freifelder and Levine determined that lambda requires some transcription before excision can occur[52]. Gottesman and Gottesman[31] found no need for RNA or protein synthesis, using an in vitro system. There was an absolute requirement for ATP and a partial requirement for Mg^{++}ion and spermidine. Other studies by Kotewicz et al[30] suggest that the xis gene product may help mediate recognition of certain DNA sequences.

Thus the roles of int and xis in site-specific recombination are much closer to being understood.

IV Control of Lysogenic and Lytic Growth

The physical integration of the virus into the host combined with

its stable maintenance as prophage constitutes lysogeny. The decision to enter a lysogenic as opposed to a lytic cycle involves a choice and is under regulatory control. These controls have been the subject of intensive study.

When a cell is infected by λ, the phage genome circularizes and the cro and N genes are transcribed. This is known as immediate early transcription[1]. The N gene activates early gene transcription, which includes genes for replication (O and P), recombination (int) and repression (C_{II} and C_{III}). At this point the decision to enter the hytic or lysogenic cycle is made. If the lytic cycle is followed, transcription of the Q gene turns on late gene synthesis, including head and tail genes. If the lysogenic cycle is chosen, repression is established by means of the C_I gene product, known as the repressor. The achievement of repression and subsequent integration constitutes lysogeny[1] (Figure 2).

i. Effect of C_I

Steinberg and Ptashne[32], using highly purified repressor from the C_I gene and from binding studies, determined that it bound specifically to two operators, O_L and O_R, thus preventing the transcription of all other phage genes. Since different concentrations of repressor were required for binding, it was thought that the operators from the left and right differed in their affinity for repressor. The repression was shown to be a direct interaction between operator and repressor[32].

In further work with the λ repressor, Maniatis and Ptashne[33] isolated six different lengths of DNA fragments, after nuclease digestion, depending on the repressor:operator ratios. The experimental

procedure involved binding various amounts of repressor to the operator region of λ DNA, followed by nuclease digestion. The protected operator fragments were then isolated and studied. The authors concluded that the repressor in the form of either a dimer or tetramer binds preferentially to the operator region. Then additional monomer or dimer units are added sequentially in the leftward direction for O_L and rightward direction for O_R. The binding to the O_L was tighter than the binding to O_R, indicating some difference between the operators. In addition the DNA sequences of the O_R and O_L fragments have been shown to differ[33] (Figure 2b).

ii. Effect of C_{II} and C_{III}

a. Control of C_I

The repressor itself was also found to be under regulatory control[34]. Evidence from radioimmune assays showed that the repressor was produced at a linear rate after a short lag. Once the maximal level was reached, the amount of repressor was maintained but not increased. The different rates of repressor synthesis and the different binding led to the idea of the two pathways being involved in repressor control. The two promotors are called prm, promotor for repressor maintenance, and pre, promotor for repressor establishment.

Reichardt and Kaiser determined that pre is controlled by the C_{II} and C_{III} gene products, while prm is controlled by the repressor itself. The pre was mapped at the structural gene Cy[34]. However, studies with a λsar mutant which suppressed Cy mutants indicated another promotor, designated pro, was involved[35]. Since pro and pre

are functionally identical, it was not clear whether the pro promotor was simply a different map site or an alternate promotor[36]. The prm was mapped beside the righthand operator O_R where the repressor binds[34].

Echols and Green[37] confirmed the idea of diffusible products from C_{II} and C_{III} binding at pre, thus promoting the C_I repressor synthesis. It was found that the C_{II} gene product was stringently required, which led Reichardt to propose that C_{II} could bind polymerase or DNA. He suggested the function of C_{III} was to stabilize C_{II} or to modify a host protein[38].

The self-regulatory role of C_I, exerting positive control over its own synthesis through the prm site, in addition to negative control over other λ genes was also confirmed[39,40].

b. Control of int

The isolation of a mutant, int-c, which constitutively produced int gene product, led to the postulation of another promotor p_I[41]. Normal transcription begins from the leftward promotor p_L, generating int gene product. In fact some evidence was found that the p_I promotor was located within the xis gene[42].

Further studies indicated that C_I and int are regulated coordinately. It was proposed that the C_{II} and C_{III} gene products activated p_I as well as pre[43]. Other workers confirmed the positive regulation of int by C_{II} and C_{III}[30,44]. This control over int and not xis allows for a further differentiation between lysis and lysogeny[44,45].

Thus C_{II} and C_{III} gene products act bifunctionally as regulators with positive control of C_I and int negative control of late genes[45] (Figure 2c).

iii. Effect of the N gene

The integration process requires both the int gene product and DNA replication. In order to satisy these requirements, the early genes must be turned on immediately after infection. However, to maintain the lysogenic state, they must be turned off once that integration has taken place.

The N gene which turns on transcription of delayed early genes as well as the Q gene is another control point in the integrative process. Dottin and Pearson[46] assayed N gene activity using amber mutants, and found that the N protein acted as an antiterminator factor. Further, the C_I repressor was found to exert negative control over the N gene product. Using an endolysin assay of N gene product, Greenblatt[47] found that cro inhibited N gene function. He also suggested an antiterminator role for N.

Additional studies[48] indicated that the N gene was specific to the immunity region. Due to this specificity, the authors postulated that the recognition regions of N gene were distinct from the sites of action. They considered the N gene product to bind to RNA polymerase and the resulting complex formed then moved to the action site[48]. Other workers also suggested that the N gene product modified RNA polymerase[49]. Wood and Konrad suggested that the function of the N gene was to increase the frequency of RNA chain initiation. Although the mechanics of N gene action are unknown, its positive regulation over C_{II} and C_{III} genes is undisputed[38,50].

iv. Effect of Cro

A complicating factor in the control over the lysogenic state is the cro gene. The cro gene has been linked to various functions including antirepression (opposing the establishment of repression) and turnoff of recombinational genes as transcription enters the late gene stage. Genetic studies have shown that antirepressor and turnoff functions were always affected by the same mutation. Echols et al[51] determined that the failure to establish repression was the result of a lower rate of C_I synthesis. Also, the decreased rate of C_I synthesis was linked to repression of the C_{II} and C_{III} genes. The authors suggested two alternatives. Either the cro gene turned off the delayed early genes as the cell entered the late stage in the lytic cycle, or it prevented the transcription of delayed early genes in the lysogenic cycle[51].

Reichardt found that cro gene product prevents transcription of the C_{II} and C_{III} operons. By reducing the N protein synthesis, the N activation of C_{II} and C_{III} was also stopped. Since the C_{II} and C_{III} are unstable, they decay[38].

Other studies found that cro activates late genes[45], and apparently has an essential function in the late state of DNA replication[52]. There is a problem in the timing of the phenomenon. How can the lytic cycle proceed further? Various explanations have been offered including: cro mediated "repression" requires higher levels of cro than of C_I to be effective[51], or that C_{II} and C_{III} gene products have altered sensitivity to cro[38,51].

There is an additional function of the cro gene: repression of the self activation of the C_I gene at prm[40]. In general, cro can be

viewed as a regulator over the lytic pathway, conserving energy by turning off the lytic genes, once they are no longer required. Secondly, the antagonism toward the lysogenic pathway allows the phage to opt out if necessary.

Comparing the cro and C_I gene products, one sees them to be very similar. Both repress C_{II} - P and C_{III} - int genes (delayed early) as well as the N gene. From mutation studies the lefthand binding sites for cro and C_I appear to be identical[51,53]. The righthand binding sites show only partial overlap, however, mutations will remove C_I binding without affecting cro binding[51]. The development of two repressors allows the phage to use cro for the transient repression necessary for the lytic pathway and C_I for the more permanent lysogenic pathway[52]. (Figure 2e)

v. Effect of cAMP

It has been suggested that the decision between a lytic or lysogenic cycle may be linked to the energy level of the cell. Since cAMP levels are known to reflect the energy status of the cell, that is, a high level of cAMP indicates a poor energy situation within the cell, a connection between the cAMP and lysogeny has been suggested[54,55].

Jordan, Green and Echols[56] argue against a regulatory role for cAMP. Studies on the effect of cAMP after infection showed no significant difference with or without the addition of cAMP. They conclude cAMP does not have a major role in lambda regulation, although a threshold level may be required for certain genes[56].

A more indirect role for cAMP in the establishment of lysogeny has been suggested. Belfort and Wulff studied the role of C_{III} gene and host catabolite repression in relation to lysogeny. They isolated

a mutant which was released from control by either C_{III} or cAMP. This "relaxed" mutant, called hfl, established a high frequency of lysogenization. They postulated that C_{III} and/or cAMP was involved in the lysogenic pathway[57].

V. Models of Lysogeny

Apart from the intricate pathways involved in regulating the decision between lysis and lysogeny, and in maintaining the chosen state, the physical accomplishment should be considered. Lysogeny involves both repression of the lytic cycle and integration into the host genome[17]. Consequently the mechanics of site specific recombination, integration and excision should be discussed.

The idea that site specific recombination promoted by int and xis gene products involves breakage and reunion has been suggested by various authors[23,58]. One of the models presented suggested that the breakage and reunion occur at a precise crossover point, in both integration and excision. Dove considered the integrated state of the prophage was inherently more stable than the excised state. In regard to stabilization energy, he concluded there is no common core or homology in the att site[24].

An alternative model postulates cleavage and recombination in the att site in a similar manner to the formation of cohesive ends[59,60]. In considering site specific recombination Signer, Weil and Kimball[59] suggested that the attachment site contained recognition regions on either side of a homologous core. The model postulates recombination occurred by staggered single nicks at either end of a common nucleotide sequence, followed by rejoining and repair[59]. This recombination can

be compared to the analogous cutting of the cohesive ends by ter[61]. Shulman and Gottesman also suggested a staggered cut in site specific recombination. They suggested that the int and xis genes may be responsible for the cleavage[60]. Although a staggered model for site specific recombination is appealing, no firm conclusions can be drawn until more is known about the nature of the attachment site.

In studying induction, in particular derepression prior to excision, the levels of C_I repressor were found to decrease. The data supported the idea that induction was the result of the repressor being released from the immunity region. The authors observed a requirement for protein synthesis to reduce the levels of repressor, when the lysogen was chemically induced. When heat induction was used there was no requirement for protein synthesis, indicating that different pathways of induction may be possible[62].

Roberts and Roberts[63] studied the effect of induction on the lambda repressor and determined that induction involved the irreversible cleavage of the repressor to a molecule of 14,000 MW instead of 27,000 MW. In discussing the phenomenon of derepression they suggested two lines of thought. First, the repressor and a cofactor formed a complex which became susceptible to proteolytic cleavage and/or lost affinity for the repressor. Second, the repressor molecule was cleaved directly, and this cleavage constituted derepression[63]. With the repressor inactivated, the excision can proceed.

VI Structure of att

The attachment site is a unique region of DNA as it is the focus for recombination to integrate or excise the phage[17]. Before discussing the attachment site in detail, the terminology that has developed should be explained. The attachment site is generally considered as a crossover point which has a recognition region on either side. In bacterial DNA, the attachment site is written as B.B', and in phage DNA as P.P'. Thus, when integrated, the lefthand site will be B.P' and the righthand will be P.B'. A transducing phage such as λgal will only contain the lefthand site B.P', while λbio has a P.B' attachment site. Finally, some deletion mutants have lost some of the attachment site, replacing it with DNA from other parts of the genome[7]. Deletions are represented as Δ, thus λb_2 is represented as Δ.P' [23].

From various genetic studies with deletion mutants, Signer, Weil and Kimball[59] suggested that attachment sites are not identical in bacteria and phage. Genetic crosses demonstrate that there are critical regions (B and P) on either side of the crossover point. These recognition regions do not act independently as reciprocal combinations of the attachment site have different efficiencies in recombination. The int gene product seems to recognize the entire attachment site as a unit and respond to it accordingly. The authors suggest a common core region at the crossover point in both phage and bacteria[59].

Using a transducing mutant, λdg, the efficiency of integration and excision was studied. A helper phage was necessary for efficient recombination. The authors suggested that the attachment regions were not homologous as the defect was determined to be structural, not functional. Since the λdg integration involved B.P' x B.B' while the

normal wild type involves P.P' x B.B', non homologous regions seemed to enhance recombination[64].

Guerrini studied the genetic exchanges involved when various transducing phage were used. Of all the exchanges he tested, only B to P interchanges were efficient. Thus, he suggested only P.P' x B.B' or B.P' x B.P' were possible. He postulated that the attachment site elements were not identical. Again, the idea that the int gene modulates the efficiency of recombination was presented[19].

Parkinson and Huskey[65] isolated several int⁻ mutants by heat or pyrophosphate inactivation. Survival of phage was strongly correlated with smaller DNA content. The mutants were then tested to determine if any modification in the attachment had occurred[66].

In studies with these deletion mutants, Parkinson wanted to test the previously presented idea that attachment sites were not identical and consist of two recognition elements, REL and RER (recognition element left or right) which influence the efficiency of integration. Also, the crossover point, XOP could be a specific internucleotide position or a common core of homology[59,66].

It was possible to classify the deletion mutants in regard to their attachment site by genetic tests. Parkinson found P.P', .P', B.P', P. ', and . ' exhibited different functions after crossing thus supporting the model of two recognition sites surrounding the crossover area. He suggested that integrative recombination is a function of the orientation of REL and RER.

In considering the processes of integration and excision, the relationship between integration frequencies and a specific attachment

requirement is not clear. In excision, the xis gene product seems to require a P._' x _.P' cross to be efficient. The complexity of the integrative process could be due to external factors such as C_1 mediated repression, or the tendency for 'inviable' recombinants such as Δ.Δ ' to segregate out of the recombination process and thereby create unbalanced recombination frequencies[66]. There is no simple pattern to explain the int gene recognition of the att site[23,66].

In order to determine the physical size of the attachment area, Davis and Parkinson[67] used heteroduplex technique. This technique involved isolating strands of DNA and annealing a mixture of single stranded molecules together to form heteroduplexes. Using deletion and substitution mutants to form these heteroduplexes it was possible to visualize and measure regions of homology.

The authors found all deletion mutants and transducing phage mapped at one site, the XOP, at 0.574 fractional length of the genome. In looking at homology between attP and attB, the authors could not find any evidence in favor. With controls, it was possible to detect homology between as few as 100 base pairs. Since the cohesive ends containing twelve base pairs were stable under mounting conditions, Davis and Parkinson argued that the stability and sensitivity of the heteroduplex procedure should not have affected the ability to detect regions of homology. They concluded the crossover point involved 10 - 20 base pairs if any homology at all is present.

The discovery that lambda can be integrated in secondary sites in the bacterial chromosome suggested some degree of homology between the sites existed. Shimada, Weisberg and Gottesman[68] were able to

accomplish the integration after deletion of the bacterial B.B' site. The process was int dependent and the integration only occurred at specific sites. The authors believe the secondary sites are B.Δ' or Δ.Δ'.[68] Studies on secondary site integrations demonstrated the reversibility of the phenomenon. These mutations were linked to the int-xis system, and as a result, the authors have suggested a common core nucleotide sequence is involved[69,70].

In further work, the authors found that transductants from secondary sites seemed to have a normal core homology but that the recognition elements varied[71]. Some point mutations were isolated in the common core regions. These mutants were unable to undergo int promoted recombination which suggested that the common core has a unique sequence[72].

In order to resolve the controversy over the nature of site specific recombination, whether by cleavage and repair at a specific junction[67] or a staggered cut similar to the ter enzyme, which generates cohesive ends,[60,67] knowledge of the nucleotide sequence of the attachment site would be beneficial.

Having considered the phenomenon of lysogeny and in particular the function of the attachment site, the methods available to sequence this DNA fragment should be discussed. DNA sequencing is inherently more difficult than RNA sequencing due to the large size of the molecule. Consequently, the methodology can be divided into general areas; first, analysis of specific oligonucleotide fragments and second, the isolation of specific fragments to be studied. The methods involved in DNA sequencing have been reviewed in detail[73,74,75].

B I Sequence Analysis of Oligonucleotides

i. Nearest Neighbour Analysis

One of the first methods used to analyse oligonucleotides involved enzymatic degradation of partially labelled nucleotides. Using micrococcal nuclease the nucleotides would be cleaved at the 5' phosphate producing 3'- fragments, which would be degraded to 3'-nucleoside monophosphates (dNMP's) by spleen phosphodiesterase. If only one base was labelled, for example dGTP, then the resulting nucleotides with a labelled phosphate contained the base next to the guanine residue[76]. This method has been used to elucidate or corroborate DNA sequences[77].

ii. Chemical Degradation

Chemical degradation of oligonucleotides to pyrimidine or purine tracts has been very useful in sequencing[78,79]. Treatment with diphenylamine and formic acid caused hydrolysis of purine bases which left clusters of pyrimidine bases to be analysed[80]. Purine tracts were formed when treated with hydrazine under alkaline conditions. The products were fractionated on DEAE columns, and identified by chromatography[81].

iii. Mobility Methods

Chromatographic and electrophoretic methods have been developed[82,83,84]. Sanger developed a two dimensional ionophoretic method. Oligonucleotide digests upon electrophoresis on cellulose acetate at pH 3.5 were separated on the basis of charge. The samples were then transferred to DEAE paper and electrophoresed in a second direction. The mobility was related to charge and ion exchange properties[82].

A different system was developed by Murray and Murray using the initial cellulose acetate step but substituting polyethyenemine (PEI) cellulose chromatography on thin layer plates in the second direction. The use of PEI allowed for better fractionation[83].

A powerful technique developed by Brownlee and Sanger was homochromatography. The use of a mixture of oligonucleotides created by controlled alkaline hydrolysis of RNA as a component of the solvent, caused the displacement of the labelled DNA digest on the DEAE plate according to its size. When combined with cellulose acetate ionophoresis, a fingerprint of the sequence was obtained and the sequence could be read directly from an autoradiograph of the DEAE plate[84].

iv. RNA Methods

Another approach to analysis of DNA was more indirect, using promotors or synthetic primers to generate RNA segments by transcription[85]. RNA can be readily sequenced with the aid of highly specific nucleases.

v. Other Methods

An extremely valuable technique has been developed to label the 5'-ends of DNA. Polynucleotide kinase from T4 phage catalysed the exchange of a p^{32} -ATP with the 5'-termini[86]. After labelling the 5'-end, the oligonucleotide can be digested partially or completely and the results analysed[87,88].

An analogous procedure using terminal deoxynucleotidyl transferase to add adenylate residues to DNA allowed the 3'-end of DNA fragments to be labelled.[89]

These methods for sequencing oligonucleotides are frequently used in series with oligonucleotide being subjected to partial enzymatic digestion, terminal labelling and homochromatography. The results obtained are often corroborated or extended by labelling the opposite end. Since this work was terminated, an elegant and highly useful sequencing method involving chemical degradation of terminally labelled fragments has been introduced by Maxam and Gilbert[90].

II Sequence Analysis of Large Molecules

Apart from the detailed analysis of oligonucleotides, specific methods are required to isolate particular fragments of DNA.

i. Repair

One of the first methods employed was DNA repair by DNA dependent DNA polymerase I. Using labelled nucleotides a specific sequence could be repaired and the product isolated[91]. In addition, partial repair with one or two nucleotides could provide direct information on the sequence[92]. This method of repair has been used for different studies[93], including sequencing of the lambda cohesive ends[91].

ii. Use of Primers and Ribosubstitutes

In order to extend the use of polymerase I repair, DNA can be used. A chemically synthesized fragment can be annealed to a strand of DNA and then repaired. This technique[94] is useful for sequencing region beyond the structural portion of certain genes or for specific genes[96]. If the DNA sequence cannot be determined, it may be surmised from the amino acid sequence of certain proteins[97] where these can be isolated.

Another improvement to the repair technique was the use of ribonucleotides as substitutes. Once a ribonucleotide was incorporated, it could be used as a handle to isolate the repaired segment or to fragment the segment as ribonucleotides are sensitive to alkaline hydrolysis[98].

iii. Binding Techniques

In order to choose a specific fragment of DNA, functional binding procedures have been used. Using ribosomes or RNA polymerase to bind to DNA, the DNA molecule can then be exposed to nuclease cleavage. The protected fragment is then isolated and the masking protein removed, allowing analysis of the oligonucleotides[99,100].

The lefthand operator of λ was sequenced in this manner. Using λ repressor to bind to the operator, Maniatis and Ptashne were able to determine the sequence[101]. Although binding techniques are very effective, they are limited to areas of DNA which bind protein molecules.

iv. Restriction Enzymes

The isolation and characterization of a special class of nuclease has provided another approach to sequencing. These nucleases known as restriction enzymes cleave DNA as specific sequences. A large molecule of DNA can be fragmented and the segments ordered according to size and location. Further digestion with other restriction enzymes will ultimately produce a managable oligonucleotide[102]. Some of the many restriction enzymes are HindIII,[103] EcoRI[104] and HpaI[105] isolated respectively from Hemophilus influenzae, Escherichia coli, and Hemophilus parainfluenzae.

v. Gels

The use of polyacrylamide and agarose gels has allowed fractionation and isolation of several nucleic acid fragments[106]. These gels give good resolution and are capable of accepting large molecules.

III Conclusion

After infection lambda has a choice between a lytic or lysogenic cycle. If chosen, the process of lysogeny involves repression of the lytic cycle and incorporation into the host genome. The integration system can be regarded as an intricate mechanism which focuses on the attachment site. Sequencing methods have been developed which can be applied to the problem of sequencing the attachment site.

Figure 1.

Campbell's model for lysogeny from Gottesman & Weisberg (23).

bacterial DNA

phage DNA

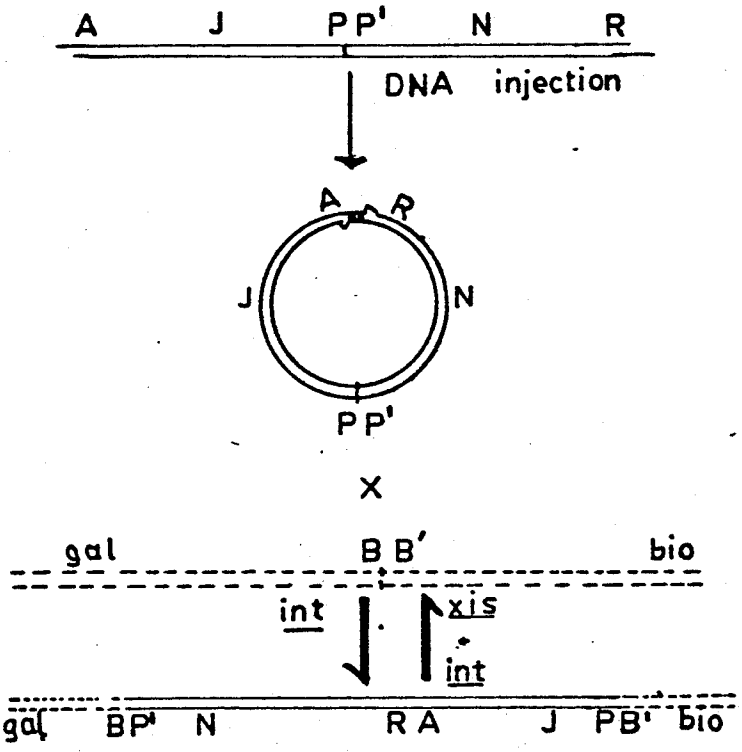

A J P P' N R
DNA injection
A R
J N
P P'
X
gal B B' bio
int
xis
int
gal BP' N R A J PB' bio

Figure 2.

Representation of control mechanisms for virus.

See text for references.

⊖ negative control

⊕ positive control

transcription

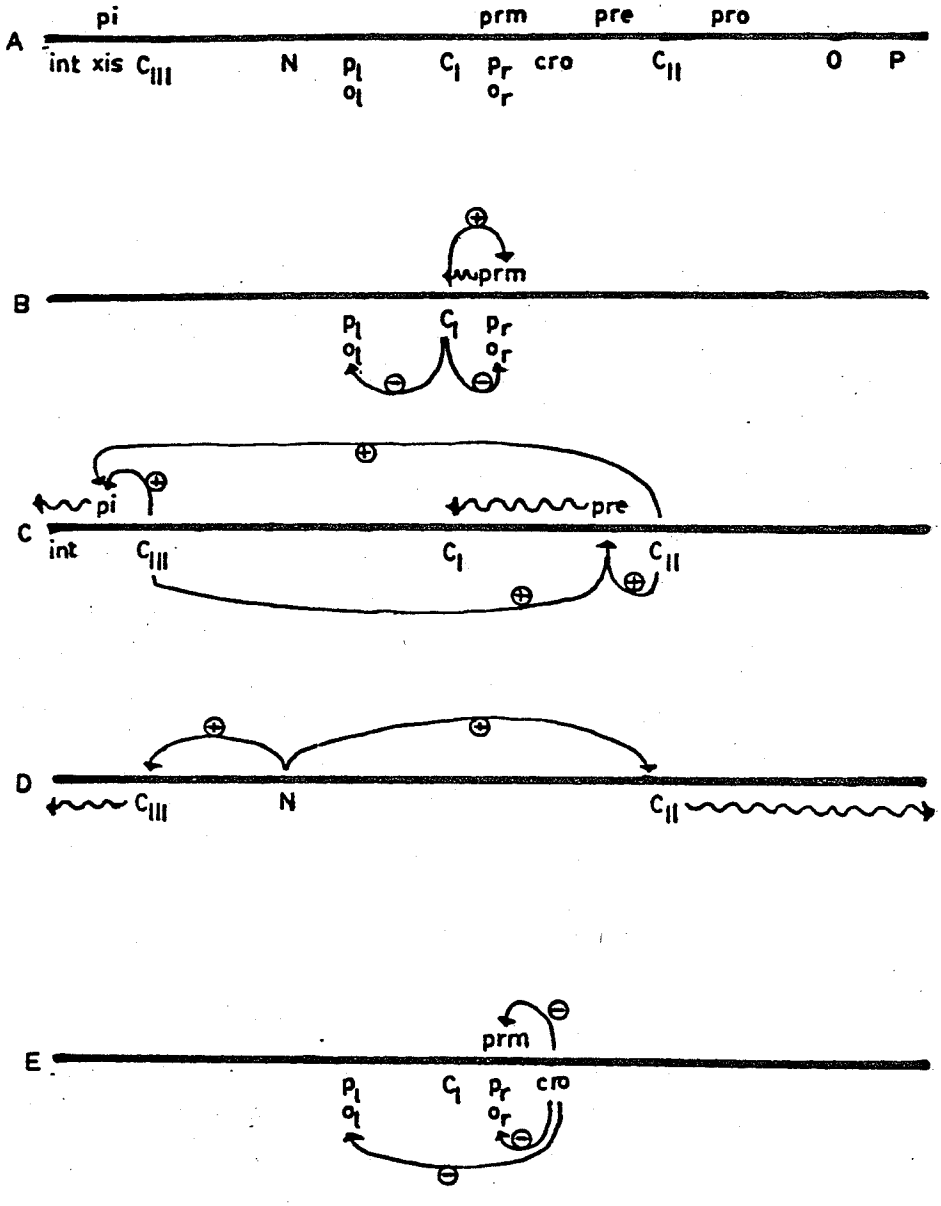
A -lambda control genes

B -effect of C_1

C effect of C_{11} and C_{111}

D effect of N

E effect of cro


A
pi
prm
pre
pro
int xis C_{III}
N
p_l o_l
C_I
p_r o_r
cro
C_{II}
O
P
B
prm
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C_{II}
D
C_{III}
N
C_{II}
E
prm
p_l o_l
C_I
p_r o_r
cro

MATERIALS AND METHODS

MATERIALS

I ENZYMES

The following enzymes were purchased from Sigma Chemical Co.:

- micrococcocal nuclease
- spleen phosphodiesterase
- pancreatic DNase

The following enzymes were gifts prepared according to the reference cited.

- DNA dependant DNA polymerase I - Dr. H. G. Khorana[107]
- DNA ligase - Dr. H. G. Khorana[108]
- polynucleotide kinase - Dr. H. van de Sande[109]

S1 nuclease

The S1 nuclease was prepared according to Vogt,[110] with the following modifications. The purification was carried out up to and including the DEAE column. At that point, the enzyme was dialysed against buffer A (20 mM sodium acetate pH 4.6, 0.1 mM $ZnSO_4$, 50 mM NaCl) with 50% glycerol. The nuclease was stored at -20° C.

The assay of S1 was as follows. The assay buffer contained 30 mM sodium acetate pH4.6, 50 mM NaCl, 1 mM $ZnSo_4$ and 5% glycerol. A standard reaction involved 48 µg of DNA in assay buffer to give a final volume of 300 µl. Incubations were carried out at 45° C for 15 minutes followed by chilling on ice. The reaction was stopped by the addition of 1 ml of 10% perchloric acid (final concentration 6.7%). The samples were centrifuged at 1500xg for 30 minutes. The supernatent was removed and the amount of cleavage determined spectrophotometrically at 260 nm.

S1 cleavage conditions were as follows. The DNA sample was made 100 mM in sodium acetate buffer pH 4.6, and the final pH adjusted to pH 5.0 with 1.0 M acetic acid. The solution was then made 1 mM in $ZnSO_4$ and the purified S1 added. The samples were incubated at 37° C for 15 minutes and then the enzyme action was stopped by the addition of sodium hydroxide (180 mM) and EDTA (25 mM).

II CHEMICALS

The following chemicals were purchased from Difco Co.

- agarose
- tryptone
- yeast extract

The following chemicals were purchased from Sigma Chemical Co.

- dithiothreitol (DTT)
- ethidium bromide
- poly (UG)
- salmon sperm DNA
- nucleotides (dATP, dCTP, dGTP, TTP)

The DE52 cellulose was purchased from Whatman Co.

The SP-C25 Sephadex was purchased from Pharmacia.

The polyethylene glycol (flake 6000) and the phenol were from Fisher Chemical Co.

The P^{32} was purchased as phosphoric acid from New England Nuclear.

The CsCl was purchased from Schwartz Mann and subsequently purified by boiling with Norite to remove any OD absorbing material. The solution was then filtered and the CsCl crystallized to give a saturated solution. All other chemicals were reagent grade.

III MEDIA AND BUFFERS

LB medium; per litre: Difco yeast extract 5 g, Difco tryptone 10 g and NaCl 5 g.

Bottom agar; Difco yeast extract 8 g, Difco tryptone 16 g, NaCl 8 g, glucose 1 g, NaOH 1.2 mM, agar 11 g and water (distilled) 1000 ml.

Top agar; Difco tryptone 5 g, glucose 0.5 g, NaCl 4 g, NaOH 1.5 mM, $CaCl_2$ 5 mM, $MgCl_2$ 10 mM, agar 3.5 g and distilled water 500 ml.

Stab agar; nutrient broth 5 g, vitamin free casamino acids 5 g, sucrose 50 g, glucose 0.5 g, agar 11 g and distilled water 500 ml.

SM buffer; 20 mM tris-HCl pH 7.6, 100 mM NaCl, 1 mM $MgSO_4$, 0.01% gelatin (SM-Mg buffer contained 10 mM $MgSO_4$ with all other ingredients identical).

TNE (dialysis buffer I); 10 mM tris-HCl pH 7.6, 100 mM NaCl and 0.5 mM EDTA.

Dialysis buffer II; 10 mM tris-HCl pH 7.6

IV PHAGE AND BACTERIA STRAINS

See Table 1.

TABLE 1

List of all bacterial and phage strains.

TABLE 1

BACTERIAL STRAINS

Lab #		Genetics	Source
7	QD5003	su^+	lab strain
31	YMC	permissive for Sam7	M.Gottesman
32	215/1	non-permissive	M.Gottesman
5	CA274	lac^- trp^- su^-(for ϕ80)	lab strain
6	CA274(ϕ80$suIII^+$)	ϕ80suIII lysogen	lab strain
8	M5107	F^- $galE^-galK^-$ $T_1{}^r$ $T_5{}^r$ str^r($\lambda c_1$857)	lab strain
14	LS476-1	F^- $lac_{z659am}{}^-$ $lac_y{}^-trp^-str^s$supF	lab strain
15	LS476-2	as LS476-1 but str^r	lab strain
23	M50	($\lambda c_1$857Sam7) lysogen $T_1{}^r$	lab strain
29	PL2	HFrH $galE^-$	CSH strain
30	SA706	HFrH $galT^-$	CSH strain
33	L1	(λ gal8$c_1$857) lysogen PL2 host	this work
	4207	su^-	CSH strain
	CSH57b	su^- leaky	CSH strain

PHAGE STRAINS

	Genetics	Source
λc_1	$\lambda c_1$857Sam7	lab strain[111,112]
λ gal1	λ gal8$c_1$857	lab strain[113]
λ gal1S7	λ gal8$c_1$857Sam7	M.Gottesman

METHODS

GROWTH OF BACTERIA

All bacteria strains were generally grown in LB medium. In addition an enriched version of LB called bottom agar, was used for plating. When a phage lysis was set up, a soft agar overlay was used to facilitate phage dispersion and removal of the lysate from the plates. For long term storage a high sucrose agar was used, called stab agar. All media used in this work were sterilized in an autoclave at 15 psi for 20 minutes.

In addition to the standard media, two other media were used. Commercial McConkey medium was used to check any lactose mutants. The McConkey medium was also prepared in the lab, according to the standard formula, with the replacement of 10g galactose/litre for the lactose usually present.

Minimal media were used to allow most nutritional requirements to be checked. The medium was prepared in two steps. One flask (A) would contain 11 g agar/litre in 500 ml distilled water. The second flask (B) would contain 500 ml of double strength salt solution. The salt solution contained the following components in grams per litre: potassium phosphate monobasic 4.5, potassium phosphate dibasic 10.5, and ammonium sulphate 1. After autoclaving flasks A and B were mixed, then supplemented with sugars, vitamins and amino acids as required. For sugars, 10 ml of a 20% stock solution were added (final concentration 0.2%). Also, $MgCl_2$ was added (final concentration of 3 mM) as were vitamins at 5 mg/ml. When amino acids were required, 200 μl of a 1.0% stock were spread over the surface of a basic minimal medium plate.

Generally, all bacterial cultures were maintained in stabs. At three month intervals, strains were grown up, isolated and checked. Once all the genetic properties were confirmed, a colony was selected and grown in LB. This culture was used to inoculate the stab bottles. Working cultures were taken from the stabs, streaked out and grown in LB for everyday use.

GROWTH OF PHAGE

i. Phage stocks

Two buffers were used for all work with phage. One buffer, designated SM, was used for dilution or reconstitution of phage in particular λc_1 phage. SM buffer was used in a modified form SM-Mg, for work with λgal phage.

Stock solutions of phage were developed from a single plaque. A well isolated plaque was picked off a phage titre plate and blown into 1 ml of SM (SM-Mg) buffer. A small lysis was set up using 0.3 ml QD5003 as host and 0.1 ml of the phage solution. The phage were allowed to preabsorb at 42° C for 20 minutes. Top agar was added (1.5 ml) and the tube contents were swirled and plated. The plates were incubated overnight at 37° C.

The lysis plates were scraped and washed with 2 ml of SM (SM-Mg) buffer. Chloroform was added to the lysate and the solution was spun in the Sorvall GS-3 rotor for 10 minutes at 6,000 rpm (6089 x g). The supernatent containing the phage was titred and the phage used as a stock to generate more phage.

ii. Large scale growth

λ phage were grown in two ways: lysogenically and lytically. The λc_1 phage ($\lambda c_1 857$Sam7) were in lysogenic form with a heat inducible repressor. The M50 host was inoculated with a 2% inoculum of a stock culture, into 500 ml of LB media. The cultures were grown overnight with aeration at 32° C. An additional 500 ml of LB was prewarmed to 50° C. The overnight cultures were added to the prewarmed media and swirled. The culture was allowed to stand for 20 minutes at 42° C, then grown for three hours at 37° C with shaking. If the temperature for induction was not correct, low levels of phage would result. After the three hours of growth, the infected bacterial cells were harvested by centrifugation. The cells were spun down in a R2-CB Sorvall GS-3 rotor at 6000 rpm (6089 x g) for 10 minutes. The pellet was kept and resuspended in SM buffer (20 ml/1 of original media). Good induction was indicated by gummy cells. After the cells were resuspended, 2 - 3 ml of chloroform were added as well as 10 μl of pancreatic DNase at 10 μg/ μl (final concentration 1 μg/ml). The solution was shaken at 37° C for 30 minutes and then recentrifuged as above. The supernatant which contained the phage lysate was kept and the bacterial debris discarded. The phage lysate was stored at 4° C until further purification was required.

The λ gal phage were grown lytically except for λgalS7 which was grown in a similar manner to λc_1. The QD5003 host was inoculated at 1% in a litre of LB media. The cultures were grown with aeration at 37° C until a titre of 5×10^8 cells/ml was reached. (Generally three hours). The lambda gal phage were added to the culture at a MOI of 2 to 3. The cells were grown for three hours until lysis

occurred. Ten ml of chloroform were added to each flask to ensure phage release. The suspension was then spun at low speed in the Sorvall CS-3 rotor for 10 minutes at 6089 x g.

The phage lysate was treated with NaCl (32 g/l) and polyethylene glycol (135 g/l) and stirred overnight at 4° C. This treatment caused the phage to precipitate. The phage were removed by a low speed spin as before. The pellets containing the phage were resuspended in 15 ml SM-Mg buffer per litre. The suspension was treated with 1-2 ml chloroform and pancreatic DNase as for λc_1. The suspension was spun at low speed again and the pellet washed with SM-Mg to ensure no phage loss. The phage lysate was stored at 4° C until further purification was required.

iii. Further Purification of Phage

Phage suspensions were purified and concentrated in the following manner. The lysate was centrifuged at high speed in the Beckman L3-50 (or L or L2-65b) ultracentrifuge using the ti60 rotor at 35K for 30 minutes (87,000 x g). The pellets were resuspended in 1-2 ml of SM (SM-Mg) buffer. The suspension was spun in a Sorvall SS34 rotor at 10,000 rpm (12,100 x g) for 10 minutes to remove any debris. The precipitate was washed and respun. The supernatants were pooled and mixed in a 1:1 ratio with saturated CsCl. The phage:CsCl solution was centrifuged to equilibrium in the Beckman SW41 rotor, at 25K (76,400 x g) for 16 hours. The phage band was removed from the side of the nitrocellulose tube by syringe. The concentrated purified phage were then stored at 4° C until needed.

STRAND SEPARATION

The isolated strands of DNA were obtained by the method of Hradneca and Szybalski[114] modified in the following manner. One or two ml of the CsCl banded phage stock were dialysed against dialysis buffer I or II. The concentration of the dialysed phage was determined spectrophotometrically at 260 nm. The conversion factor was 1 OD equaled 40 μg DNA. The phage were made 15 mM in NaOH and the DNA denatured by incubation at 37^{b} C for 10 minutes. The solution was neutralized to pH 7.0 by the addition of potassium phosphate monobasic (25 mM) and poly(UG). The poly(UG) was added to give a final ratio 0.75 μg poly(UG): 1.0 μg DNA. The solution was diluted to the appropriate concentration for ultracentrifugation. Separated strands were diluted to 1.2 ml phage/tube to which 3.8 ml of saturated CsCl was added and gently mixed. The DNA was centrifuged in the Beckman ti50 rotor at 40K (106,500 x g) for 44 to 48 hours. After centrifugation fractions were collected dropwise by puncturing the bottom of the polyallomer tubes. The DNA content was determined spectrophotometrically at 260 nm. The isolated strand peaks were pooled and stored at 4^{o} C until further use.

ANNEALING PROCEDURES

The strands of DNA were annealed[67] in the following manner. Purified strands were dialysed overnight against one litre of dialysis buffer. Equimolar amounts of each strand were mixed and made 100 mM in NaOH followed by incubation at 37^{o} C for 10 minutes to denature any secondary structure. The solution was then neutralized with 100mM sodium glycinate pH 9.2. The pH was checked to be pH 9.5 and

adjusted if necessary with 1 N HCl. The mixture was then annealed for 4 hours by incubation at 65°C followed by slow cooling overnight.

GRADIENTS

Alkaline sucrose gradients were used to analyse and isolate different DNA complexes. The 5 to 20% w/v sucrose gradients were made in 1.0M NaCl and 200 mM NaOH. To isolate S1 cleaved fragments, the gradients were run in the Beckman SW50.1 rotor at 50K (234,000 x g) for 2 hours for small scale preparations and in SW41 for 3 hours at 40K (195,700 x g) for large scale preparations. To isolate circles the gradients were run for 75 minutes at 38K (68,249 x g).

Neutral sucrose gradients were used to isolate native DNA after phenol extraction. The 5-20% sucrose gradients were prepared above a 500 µl pad of 60% sucrose. The buffer used was 10 mM tris-HCl pH 7.6, 200 mM sodium chloride and 1 mM EDTA. The gradients were run in a Beckman SW 50.1 rotor for 4 hours at 45K (189,000 x g). The intact λ DNA was isolated from the 60% sucrose solution.

Gradients were collected dropwise after puncturing the bottom of the tubes with a syringe. Generally 25 fractions were collected and read spectrophotometrically at 260 nm. All gradient profiles are read from bottom to top.

REPAIR CONDITIONS

Repair with DNA polymerase I was carried out according to Khorana[115]. Analysis of 3' ends was according to Englund[116]. Analysis of 5' ends was according to Weiss, and Richardson[117].

CIRCLES

The circles were prepared according to a modification of Paoletti's procedure[118]. The first step was to form hydrogen bonded circles from the annealed heteroduplex DNA. The annealed DNA was diluted to 10 μg/ml with circle buffer. The circle buffer contained 10 mM tris-HCl pH 7.6, 5 mM EDTA and 800 mM NaCl. The solution was incubated for 15 minutes at 75°C followed by 2 hours at 55°C. The DNA solution was then allowed to slow cool overnight.

The hydrogen bonded circles were concentrated by evaporation with air filtered through glass wool. When the sample was sufficiently concentrated, it was dialysed against 10 mM tris-HCl pH 7.6 and 1 mM $MgCl_2$.

The DNA sample was then treated with ligase to seal the end annealed circles. The circles were treated with DTT (10 mM) and ATP (0.066 mM) and ligase. The solution was incubated at 55°C for 2 hours. The circle preparation was again concentrated by air, as above. The circles were analysed by alkaline gradients or agarose gels to determine the quality of the preparation.

AGAROSE GELS

Agarose disc gels were used to analyse and isolate DNA circles. The agarose (0.6%) was dissolved in 100 mM potassium phosphate pH 6.9. The gels were formed in glass tubes 0.5 cm x 10 cm. Due to the slippery nature of agarose, nylon mesh was placed over the end of the tubes. The gels were loaded with samples (up to 100 μl or 4 μg DNA) which were mixed with an equal volume of 20% sucrose, 0.001% bromphenol blue. The running buffer was 100 mM potassium phosphate pH 6.9. Gels were run at 15 ma/tube until the dye

marker reached the bottom of the tubes.

The gels were removed and stained with 0.5 μg/ml ethidium bromide. The bands were visualized with a UV lamp and if warranted, photographed.

RESULTS

RESULTS

Introduction

In order to sequence the attachment site of lambda virus, two different protocols were tried. The first approach to sequencing att was to form heteroduplexes of λc_1 and λgal strands which would produce a single stranded loop encompassing the phage attachment region. This area would be subjected to digestion by S1 nuclease and the cleavage product isolated. The DNA fragment would then be reannealed to the appropriate $\lambda c_1 L$ strands to provide the necessary template for DNA polymerase I repair. The repair product would be analysed to determine the sequence.

The second approach to sequencing the attachment site involved circularizing the heteroduplex product. The circular DNA w as then treated with S1 nuclease and the linear product labelled with polynucleotide kinase.

PHAGE PLATING PROCEDURES

The growth and yield of bacteriophage could be influenced by many factors. The phage were generally plated on LB plates, using a serial dilution of the phage stock together with sufficient bacteria to form a lawn on the plate. When the λgal phage was titred, the values increased from 10^{11} to 10^{14} phage per ml if fresh, moist plates were used.

It was also found that preabsorption of the phage to the bacteria helped. To do this, the bacteria phage mixture was allowed to incubate at 42° C for 15 minutes. This was followed by addition of the top agar (1.5 ml) and plating.

The use of log cultures of bacteria also influenced the titre favorably. As a precaution, a control plate of host bacteria was run. This allowed any phage contamination to be readily observed. In addition, the condition of the lawn would indicate how healthy the original lawn was.

The titre was also affected by the host used. Although virus infects Escherichia coli K strains,[119] the plating efficiency showed a large variance. To determine how significant the effect was, a $\lambda c_1 S7$ phage stock was plated versus five different suppressor strains. The titres obtained ranged from 8×10^8 phage/ml to 3×10^{10} phage/ml. The strain with the best efficiency, QD5003, was chosen as a standard host (Table 2).

PHAGE GROWTH CONDITIONS

The growth conditions of the phage would radically affect the yield. Several factors were investigated to improve the generally poor titre values obtained.

One factor was the composition of the growth medium. The standard for the growth of the phage is LB broth (as outlined in Material and Methods). One of the components of the medium is yeast extract (Difco) at a 0.5% concentration. This brand was determined to be essential to good phage yields. The substitution of Difco brand yeast extract resulted in an improvement from a low titre of 10^7 phage/ml to 10^9 phage/ml. In addition, it was noted that the plaques were larger and clearer when the Difco brand was used. For all subsequent phage preparations this requirement was followed.

The multiplicity of infection (MOI), that is, the number of phage per bacteria was also crucial to the growth and yield of phage[120]. The bacteria would lyse from without, producing cleared plates but extremely low titres, when a high MOI value was used.

In general, the optimal MOI for a λ infection was about 2 to 3. One complication in determining the MOI was the plating efficiency of the phage. Even using QD5003, which has been shown to be the most favorable host (Table 2), did not ensure absolute efficiency. To correlate the MOI values obtained experimentally with the correct physiological value, a series of small scale lysis flasks were set up. The flasks contained 60 ml of medium and cultures were grown until they reached log phase, 5×10^8 bacteria/ml. Each flask was grown in the standard manner; the cells lysed with chloroform after 3 hours,

and the lysates kept. These lysates were titred and from the results in Table 3, the MOI of 0.5 was the best. Further work showed that a MOI of 0.2 to 0.25 would also give good lysis. In conclusion, the phage were plating with only 10 to 25% efficiency.

By considering the MOI during the scaling up of a virus stock from a plaque to a large lysate, the system would give maximum yields. However, since the infection process contained so many possibilities for error, another λgal mutant was selected. This λgal mutant (λgal18$c_1$857S<u>am</u>7) is genetically similar to the $\lambda C_1$857S<u>am</u>7 phage already used, in that it contained an amber mutation in the lysis gene <u>S</u>, which prevented the phage from being released from the host. Thus, the progeny could accumulate over the growth period, and be released upon the addition of chloroform. This procedure was already used for λc_1S7 phage, and generally gave high yields. The most convenient mode of growing these phage was to have the phage as lysogens, but the new mutant, as obtained from M. Gottesman, was not in this state.

Initially, as the phage stocks were developed, the λgalS7 phage were titred at 10^{10} phage/ml with the permissive host, YMC. The levels of phage after lysis were in the range of 10^6 to 10^8 phage/ml. The growth of the host was also poor. After checking the host and obtaining good growth, the phage were checked under a variety of conditions. It was found that the addition of Mg^{++} ion in the form of $MgSO_4$ enhanced the titre values. As a result, the SM

buffer was modified to give a final concentration of 10 mM $MgSO_4$, rather than the standard 1 mM. Maltose has also been suggested as a growth requirement for the host[121] so that virus receptors would be developed. Addition of 0.2% maltose to the medium failed to have a significant effect. The length of incubation of the infected cells was also examined, but there was no improvement even when long incubation times were used. One factor which did have a favorable effect was chloroform. When chloroform was added to the lysate, followed by vortexing of the lysate suspension and removal of the cell debris, the phage yields improved.

Finally, in case an external factor was affecting the system, λ c_1 phage were substituted for λgal S7. The λc_1 phage were titred at 10^{10} phage/ml, much higher than the 10^6 phage/ml value for λ gal S7. The problem therefore seemed to be with the λgal S7 phage.

The λgal S7 phage were difficult to use, but the phage titre could be improved through the proper use of Mg^{++}, chloroform and the MOI. Since the small scale lysis conditions on the plates had been improved, the large scale lysis using the nonpermissive host was studied, only to find that the yields were still low. Consequently, the host was tested and determined to be "leaky". That is, phage was not retained within the cell, but was released into the medium. Since only bacterial cells were harvested, it was understandable that the yield was reduced.

To circumvent the problem of the leaky nonpermissive host, the method of harvesting was altered to the polyethylene glycol procedure outlined in Methods. Since a permissive host could now be used,

QD5003 was chosen as it was a trouble free strain. However, the λ gal S7 phage itself still posed some problems.

As a final check on the phage purification system, the purification procedure was tested. Aliquots were removed at each step and titred to determine whether there was a physical reason for the low phage yields. Each sample was dialysed and then titred in the standard manner. Allowing for natural loss during the fractionation, there were two critical areas where serious losses occurred. The problem areas were the low speed spins done to remove bacteria and debris. The first case was after the polyethylene glycol precipitated phage were resuspended and then centrifuged. Although the phage may have been associated with the bacteria and precipitated as a result, it was more likely that the phage were still affected by the presence of polyethylene glycol and sodium choride. The second case of phage loss was after the phage were resuspended and centrifuged at low speed following the ultracentrifugation step. In this case, it was likely that the phage were associated with some form of bacterial debris. To overcome these problems, two changes were made. First the pellets from the low speed spins were washed with buffer and then recentrifuged. In this way, any residual chemicals were removed. Second, the lysate was treated with pancreatic DNase to break down the bacterial DNA and thus reduce any possible bacteria:phage associations.

Despite these improvements, the phage yield for λgal S7 was consistently lower than for $\lambda c_1 S7$. The real problem with λgal S7 was found when some suppressor strains were being routinely checked.

From the initial group of six strains, two warranted further study, CSH57b and 4207. Each bacterial strain was plated against three phage mutants, $\lambda c_1 857 \underline{Sam}7$, $\lambda gal 8c_1 857 \underline{Sam}7$ and $\lambda gal 8c_1 857$ (Table 4). As a control, a known suppressor strain QD5003 was also tested. It was found that 4207 was essentially suppressor free, but still supported λ gal S7 growth. Consequently, λ gal S7 did not have the expected amber mutation. As a result, the original λ gal mutant ($\lambda gal 8c_1 857$) was used for all further work.

In addition to problems with plating, growth and phage identity, there were some difficulties with extraneous phage contamination. Initially, the source of contamination could not be identified. All reagents were checked and the plaques formed were studied. In early work, both large (2 mm) and small (.5 mm) plaques appeared on titre plates of λ gal. However, if each type of plaque was isolated and then replated, both large and small plaques would appear again. Thus, there seemed to be heterogeneity in plaque size for λ gal phage. When the "contaminated" plates were studied, each type of plaque was isolated and then replated. It was found that two different types of phage were present, that is, phage contamination.

To overcome the problem of phage contamination, the laminar flow hood was used during the scraping of the lysis plates. In addition, screw cap Pyrex centrifuge bottles were autoclaved and used to centrifuge the lysate. The unidentified phage contamination occurred occasionally, perhaps from an aerosol in the lab. To circumvent

any additional problems, a phage control was run for any lysis experiments. The phage stock being tested was diluted to a lower concentration and plated so that plaque appearance could be checked.

After considering all the above information, it was possible to get the phage titres for λgal into the range of 10^{11} phage/ml but no higher. Since λgal phage were in short supply, this severely limited the preparation of heteroduplex DNA in subsequent experiments.

TABLE 2

Effect of the bacterial host on plating efficiency of λ c_1 phage. Bacteria (0.3 ml log culture) and phage (0.1 ml diluted in SM buffer) were mixed and incubated at 40° C for 15 minutes to allow the phage to preabsorb. Top agar was added (1.5 ml) and the entire sample plated on LB medium. The plates were incubated at 37° C overnight, then counted.

TABLE 2

bacterial host	phage titre (plaques/ml)
CA274 (ϕ80 psuIII)	1.3×10^{10}
CA274	1.8×10^{10}
14	2×10^{9}
15	8×10^{8}
QD5003	3×10^{10}

TABLE 3

Effect of the multiplicity of infection (MOI) on the yield of λ gal phage. Sixty ml flasks of LB broth were inoculated with QD5003. The cultures were infected with the appropriate MOI after the bacteria reached log stage (5×10^8 bacteria/ml). After lysis was evident, the phage lysates were collected and titred in the standard manner.

TABLE 3

MOI	Phage titre (plaques/ml)
2.0	3×10^9
1.0	3.5×10^9
0.5	4.9×10^9
0.1	7.7×10^8

TABLE 4

Test of suppressor strains with various lambda mutants. Each bacterial strain was plated against each phage using the standard procedure for titres. Of the two test bacteria, strain 4207 is suppressor free. It should be noted that λ gal8c$_1$857Sam7 phage formed plaques with this host.

TABLE 4

bacterial strain	4207(su?)	CSH57b (su?)	QD5003 (su+)
phage mutant			
$\lambda c_1 857Sam7$	-	plaques	plaques
$\lambda gal8c_1 857Sam7$	plaques	plaques	plaques
$\lambda gal8c_1 857$	plaques	plaques	plaques

PHAGE LYSOGENS

Due to the difficulties encountered in the preparation of purified phage, several attempts were made to isolate a lysogen and correspondingly an unleaky non-suppressor host.

Initially, an enrichment technique was used to try to isolate a λgal lysis-minus mutant. To accomplish this, strain CA274 was infected in the usual manner with λgal phage. After a standard period of incubation (three hours), the unbroken cells were harvested by centrifugation, then lysed with $CHCl_3$. The lysate was purified in the standard manner for phage purification up to and including the high speed ultracentrifugation step. This purified sample was used to infect the host CA274 and the entire cycle was repeated. However, no lysis-minus phage were found, even after seven consecutive cycles.

In the next attempt to find a lysogen, a mixed infection of $\lambda c_1 857 \underline{Sam}7$ and $\lambda gal8c_1857$ was tried. The object was to select recombinants which showed $\lambda gal8c_1857\underline{Sam}7$ responses. The permissive host was infected with equal amounts of both phage. The cells were harvested and the lysate kept. After high speed ultracentrifugation, the purified phage were plated to select any recombinants. Two procedures were used: a mixed host plating, (permissive QD5003 and nonpermissive CA274) which would differentiate any phage with an amber mutation, and an overlay method where the nonpermissive strain was added, after the phage were plated with the permissive strain. In either case, the phage with an amber mutation would be able to plate only with the permissive host, thus the plaques would be turbid.

In testing the genetics of the lysate, contradictory results were obtained which stopped further work. For example, when checking its susceptibility to infection, the host QD5003 was resistant to the lysate, while other suppressors were sensitive. However, the strain QD5003 was lysed when a confluent lysis plate was prepared.

Some work was also done on locating a strain which would be selective and plate only λgal, thus allowing differentiation of mixed infections. Initially, it was found that the medium used seemed to affect the plaque forming ability. Using PL2, a suppressor-minus strain, λc_1 phage were able to plate on LB medium, but not McConkey medium. The λgal phage could plate on either medium. An experiment was devised where different components of the media, LB and McConkey, were tested to determine the effect of plating. The presence of bile salts at a concentration of 0.8% caused the difference in plating. By adding bile salts to LB medium, it was possible to differentiate between λc_1 and λgal phage. Unfortunately, this fact could not be exploited in the present work.

After receiving a sample of $\lambda gal8c_1857\underline{Sam}7$ phage (λgal87) some attempts were made to lysogenize the phage using PL2 (gal^-) as a host. Using indicator plates of McConkey-gal or gal minimal agar, together with spotting of phage or dilutions of a bacteria:phage mixture, some tentative gal^+ lysogens were found. These gal^+ colonies could either be revertants or lysogens. To check them, master plates were prepared, then replica plated for incubation at 32° C and 44° C. It was expected that λgal lysogens would not grow at 44° C since they contain the temperature sensitive mutation in the

c_1 gene. All of the potential colonies exhibited some growth at 44° C, but the growth at 44° C was reduced from that at 32° C. Eight colonies were selected and tested by cross-streaking into λc_1 phage. Two resistant colonies were found and designated L1 and L5. A final test was made by growing up the two strains and isolating the lysates after heat induction. L1 produced a lysate with a titre of 9×10^7 phage/ml, while L5 did not produce phage. The work with L1 (strain # 33) was taken to the next step and a large scale growth was attempted. The yield of phage proved to be low in several attempts, apparently due to partial lysis of the host. Furthermore, only a portion of the phage population was gal^+. Thus, although strain # 33 (L1) was lysogenic, it still presented problems.

Two different approaches were tried in the next attempt to isolate a λgal $lysis^-$ lysogen. First, bacterial strains were determined to be gal^- (PL2, # 30, # 19) and then they were infected with λgal phage ($\lambda gal 8 c_1 857$). All gal^+ cells were to be selected for further work. Unfortunately, no gal^+ colonies were found. Secondly, M5107, the $\lambda c_1 857 \underline{Sam}7$ host (gal^-) was treated with heat and UV light to cure the bacteria. The survivors were checked for sensitivity to phage and if sensitive, they were infected with λgalS7 phage. The bacteria:phage mixture was diluted in SM buffer and plated on differential and selective media. Both UV and heat treated samples had several intermediate gal colonies. These colonies had dark centres favoring gal^+ and white edges indicating gal^-. One colony was found which was totally dark, i.e. gal^+. All suspect colonies

were picked off, gridded onto master plates and then replica plated. The replica plates were incubated at 32°C and 44°C to check for any phage. One gal$^+$ colony was chosen from the master plates which was found to be resistant to lambda infection as expected. However, when the bacteria were grown and induced, no phage were produced. No further work was done on this isolate.

Another attempt was made to isolate a λgal lysis$^-$ lysogen using λgal S7 as the phage and several different hosts. Each bacterial strain was infected and the bacteria:phage mixture diluted and plated. Eleven hundred colonies from hosts # 25, # 30 and # 32 were checked for lysogens, with no positive results, although initial indications had been favorable.

Yet another approach, using the method outlined by Hoffman[122] with starved cells infected and subsequently plated on bromthymol blue agar was attempted. The colonies were examined, but there was no difference found among the cells plated. Therefore, no lysogens could be selected.

Finally an attempt was made again to cure strain # 8 which was the host for $\lambda c_1 857 \underline{Sam7}$. A repeated cycle of heat induction followed by isolation of the survivors was carried out. The survivor strain, designated 844, was checked for genetic characteristics and found to be gal$^-$ and lambda sensitive. The subsequent protocol required a mixed infection of $\lambda gal8c_1857$ (λgal) and $\lambda c_1 857\underline{Sam7}$ (λc_1) in equal proportions. After one round of lysis, the progeny would be allowed to preabsorb to the host before plating. Any progeny with the characteristics of $\lambda gal8c_1857\underline{Sam7}$ would be red on McConkey agar. The

parental λgal should lyse the host and the parental λc_1 should respond as gal$^-$. Any possible colonies were checked for phage by incubating at 32°C and 44°C. Although the experiment was repeated four times, modifying the MOI and the incubation time prior to the first lysis, no lysogens were found. The presence of uninfected gal$^-$ survivors tended to obscure the desirable colonies as well.

In conclusion, all of the attempts to obtain a lysogen were partially or completely unsuccessful. Consequently, the growth of λgal phage presented an ongoing and continual problem.

METHOD I

S1 NUCLEASE ASSAY

In assaying S1 nuclease according to the modified procedure of Vogt,[110] (as outlined in Methods), high background values made it difficult to determine the enzyme activity. All components of the assay were tested and it was found that the method of preparation of the single stranded DNA substrate was the critical factor. Optimum conditions for DNA preparations were then determined.

The native salmon sperm DNA solution was made 200 mM in NaOH and incubated at 37°C for 10 minutes, followed by neutralization with sodium glycinate buffer (final concentration 100 mM) to pH 9.5. Unless the single stranded DNA was prepared in this gentle way, shearing would occur and the DNA fragments would not precipitate on addition of 10% perchloric acid during the nuclease assay.

It was also found that the addition of 19 μg carrier double stranded DNA improved the assay by producing a clear supernatant.

S1 NUCLEASE PURIFICATION

The S1 nuclease was purified as described by Vogt[110]. The purification scheme as outlined in Table 5 is characteristic of the enzyme preparation. It should be noted that the SP-C25 Sephadex chromatography resulted in the loss of over 87% of the enzyme activity. Consequently, the fractions IV and V were checked to determine absolute specificity for single stranded DNA.

DNA, purified by phenol extraction and neutral sucrose gradients, was made 40 mM in sodium acetate pH 4.5, and 1 mM in $ZnSO_4$. Aliquots of the DNA were treated with the DEAE and SP-C25 Sephadex nuclease fractions IV and V. After a standard incubation of 37°C for 15 minutes, the DNA samples were denatured with NaOH and centrifuged through alkaline sucrose gradients. As can be seen from Figure 3, both the DEAE fraction IV as well as the SP-C25 Sephadex fraction V were specific for single stranded DNA. As a result, all subsequent preparations of S1 were purified only to fraction IV, thereby avoiding the loss of the enzyme on SP-C25 Sephadex. In addition, a final check was made on each new enzyme preparation, using native DNA and alkaline gradients as outlined above.

In the course of preparing S1 nuclease, it was found that the DEAE cellulose used as the final purification step had to be DE52. When columns were prepared with DEAE cellulose from Sigma or DE23

From Whatman, the enzyme activity was lost entirely, or only a constant background level was found.

S1 NUCLEASE PHYSIOLOGICAL CONDITIONS

It was determined that the physiological conditions affected the activity and in particular the specificity of S1 nuclease. Although there has been some controversy over the single strand specificity of S1[123,124], the substrate concentration was determined to be the critical factor. The effect of substrate concentration on the level of enzyme activity is shown in Figure 4. It can be seen that the enzyme activity increases linearly until 48 μg/assay is reached. At higher levels of substrate, the activity levels off. Thus, a concentration of 48 μg/300 μl (160 μg/ml) was the optimum value used for S1 nuclease assays. A value of 0.1 μg/ml was found by reference[124].

The effect of long term incubation at pH 5.0 on DNA treated with S1 was checked, using alkaline sucrose gradients and both ^{32}P-labelled and unlabelled DNA. It was found that longer incubation led to the degradation of the DNA whether or not the S1 nuclease was present (Figure 5). Thus, the S1 nuclease caused more cleavage of the DNA as it was degraded during longer incubations.

In choosing the pH, two factors had to be considered: the pH optimum of the S1 nuclease and the possibility of depurination of the DNA under acid conditions. When native DNA was incubated for

periods at pH5 or pH7, it was found that at pH 5 a small amount of degradation occurred (Figure 6). Consequently, pH values of 4.5 to 5.0 were used for all S1 treatments but only for short incubation periods, as at higher pH values the nuclease lost 50% of its activity.

In addition to requiring a substrate concentration of 160 μg/ml for optimal activity, it was found that low concentrations of native DNA led to attack on double stranded DNA. In Figure 7, it is shown that decreasing DNA concentrations result in increasing amounts of random double stranded cleavage. This experiment was repeated with the same results using samples with 1.8 to 15 μg of native DNA. It was found that at DNA levels below 15 μg, native DNA would be degraded by S1 nuclease (Figure 8).

Denatured DNA was broken down as expected by S1 treatment (Figure 9).

In addition, the presence of carrier denatured DNA would prevent cleavage of low concentrations of the native DNA, presumably by binding the enzyme (Figure 10).

TABLE 5

Purification scheme for S1 nuclease. S1 nuclease activity was assayed by acid solubilization of denatured DNA as outlined in Methods. One unit of S1 nuclease hydrolyses 10 μg. DNA in 10 minutes according to the standard procedure.

a. Average value from S1 preparations.

b. Total value for nuclease is lower than value for heat step due to inhibitory factors in crude extract.

TABLE 5

PURIFICATION SCHEME

Fraction		Protein (mg/ml)	Nuclease (U/ml)	Total Nuclease (kU)	Activity (U/mg)	Purification Factor	Yield (%)
I	Crude	5.85	253	50.6[b]	43.2	(1)	(100)
II	Heat	2.51	440	83.6	175	4.05	100
III	Ammonium Sulphate	0.82	367[a]	73.4	447	10.3	88
IV	DEAE Cellulose	0.25	624	25.0	2496	57.7	30
V	Sp-C25 Sephadex	0.25	81	2.8	324	7.5	3

FIGURE 3

Test of S1 nuclease fractions to determine if specific for single stranded DNA. DNA was used as the substrate for S1. The DNA was brought to pH 4.5 by the addition of sodium acetate (final concentration 40 mM), and made 1 mM in $ZnSO_4$. Aliquots were then treated in the standard manner for S1 reactions. The untreated DNA (○) was compared to the samples treated with the SP-Sephadex fraction (◑) and the DEAE fraction. (●) Gradients were run for 2 hours in the Beckman SW50.1 at 50K. The bottom of the gradient is on the left. Note that the DEAE fraction does not contain any contaminating double stranded DNA nuclease.

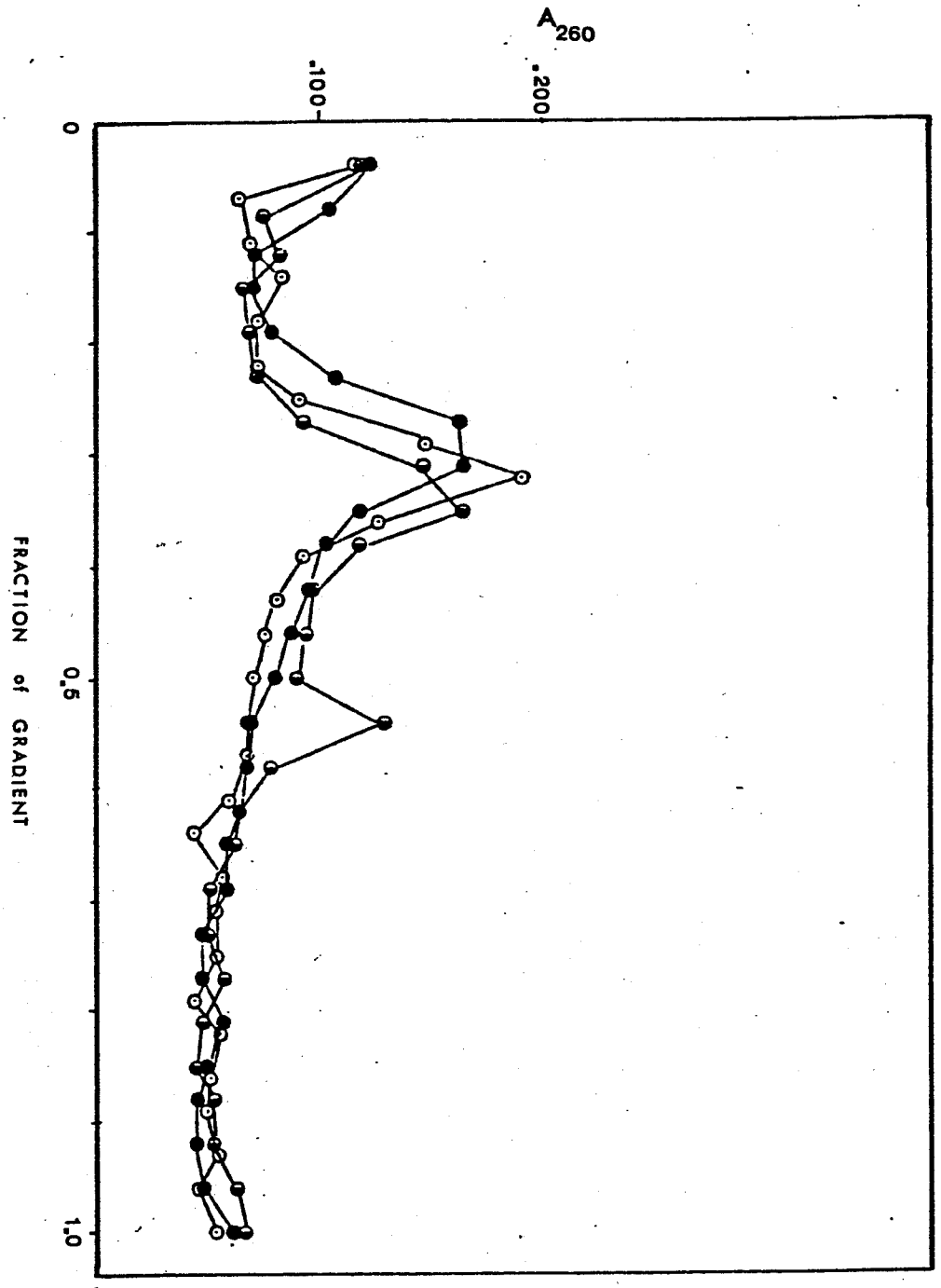

A_{260}
.100
.200
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 4

Effect of substrate concentration on S1 activity. Using crude S1 extract, a series of standard assays were set up with increasing amounts of DNA from 1.8 μg to 92 μg/assay.

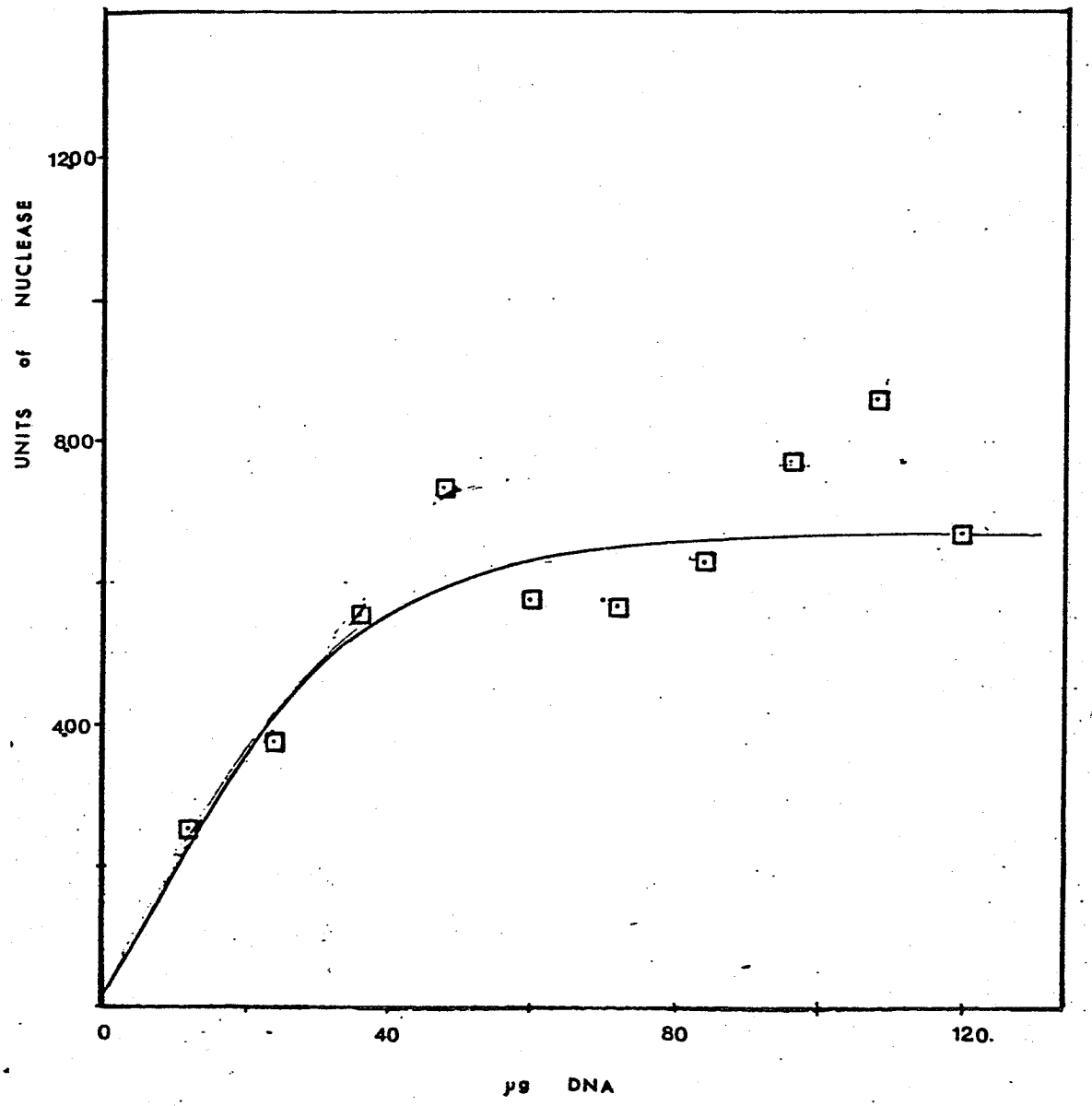

UNITS of NUCLEASE
1200
800
400
0
40
80
120.
µg DNA

FIGURE 5

Effect of long incubation on S1 activity using a labelled substrate. λ DNA labelled with ^{32}P and cold λDNA were treated with S1 for long periods. The assay conditions were 100 mM sodium acetate pH5.0, 10 mM $ZnSO_4$ and units of S1 nuclease. Untreated DNA (O) was compared to S1 treated DNA (◑). The incubation periods were A - 45 minutes, B - 90 minutes, and C - 180 minutes.

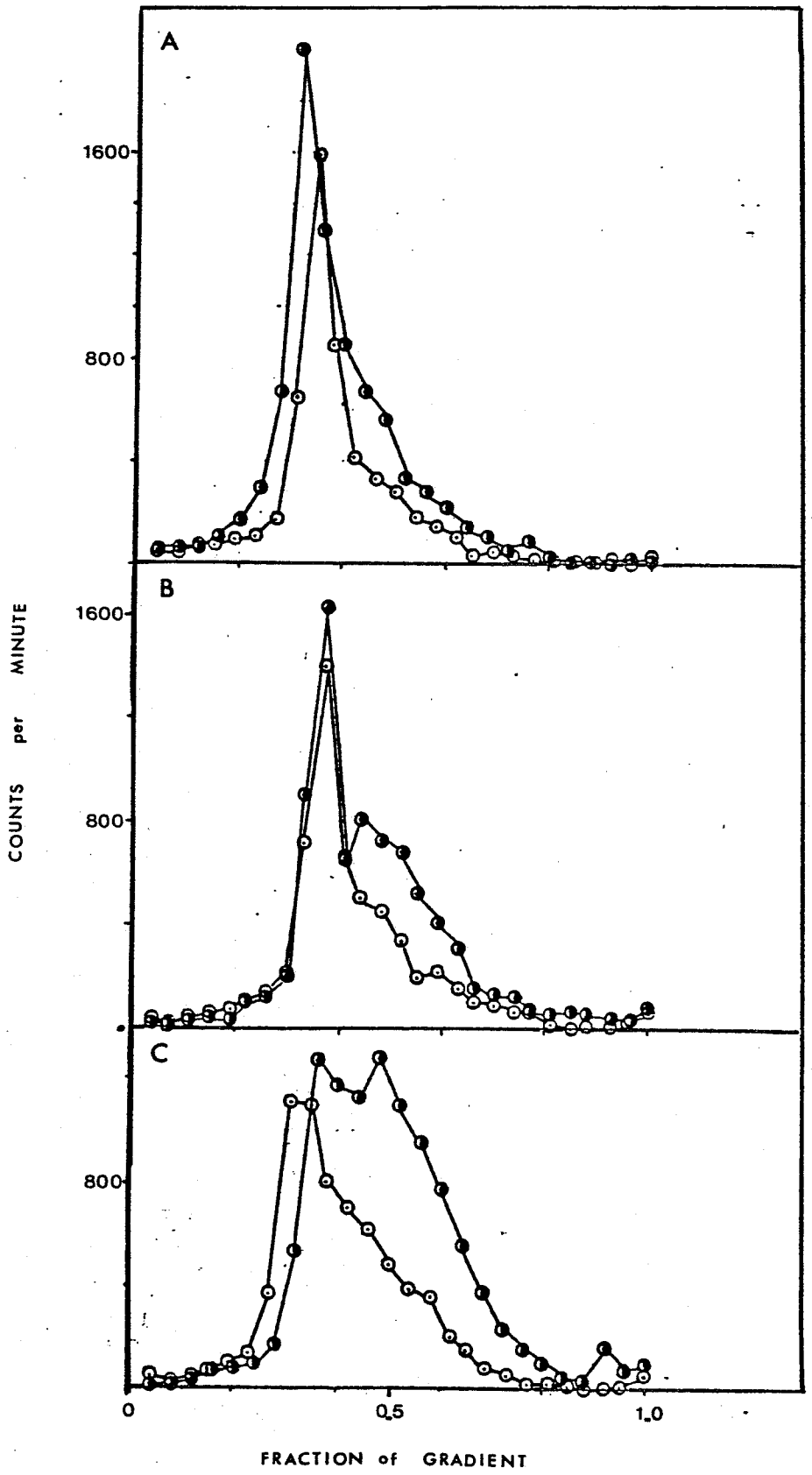

A
1600
800
B
1600
800
C
800
COUNTS per MINUTE
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 6

Effect of pH on DNA degradation. Labelled λ DNA was used as a marker to determine the effect of pH on the DNA. The DNA was made 10 mM in $ZnSO_4$ and 100 mM in Sodium Acetate buffer pH 5.0 or pH 7.0. Both samples at pH 5.0 (O) and pH 7.0 (□) were incubated for 180 minutes.

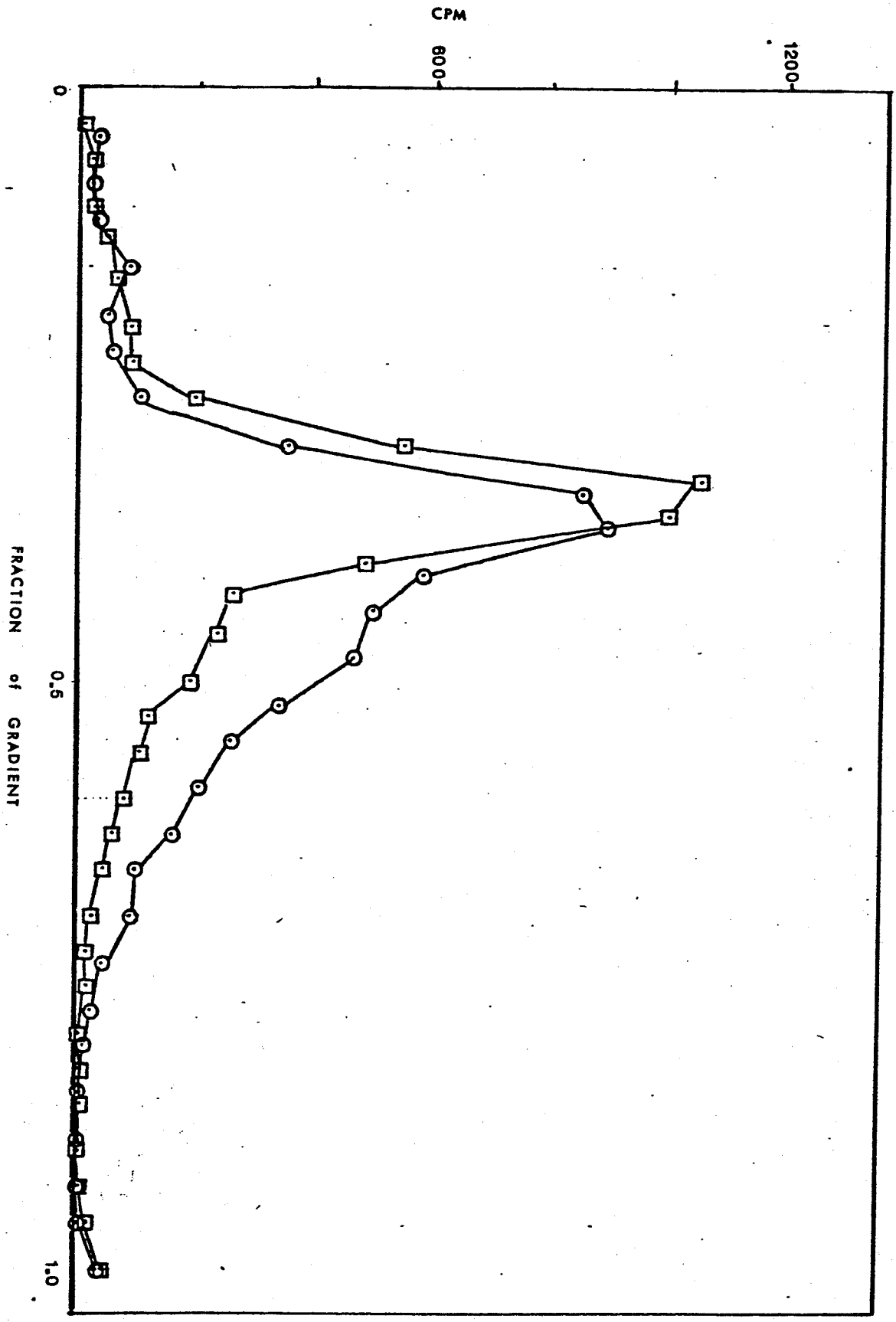

CPM
600
1200
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 7

Effect of substrate concentration on S1 specificity. Labelled λ DNA was used as a marker. An internal control of untreated, unincubated DNA (○), was compared to the incubated DNA control (□). DNA samples of 3 μg (◑), 6 μg (●) and 12 μg (◨) were treated with S1 (5 units per sample). All treated DNA samples were made 100 mM in sodium acetate pH 5.0, 10 mM in $ZnSO_4$.

The untreated DNA was not buffered but simply run on the alkaline sucrose gradients.

All samples were incubated for 3 hours except ◨ which was treated for 2 hours.

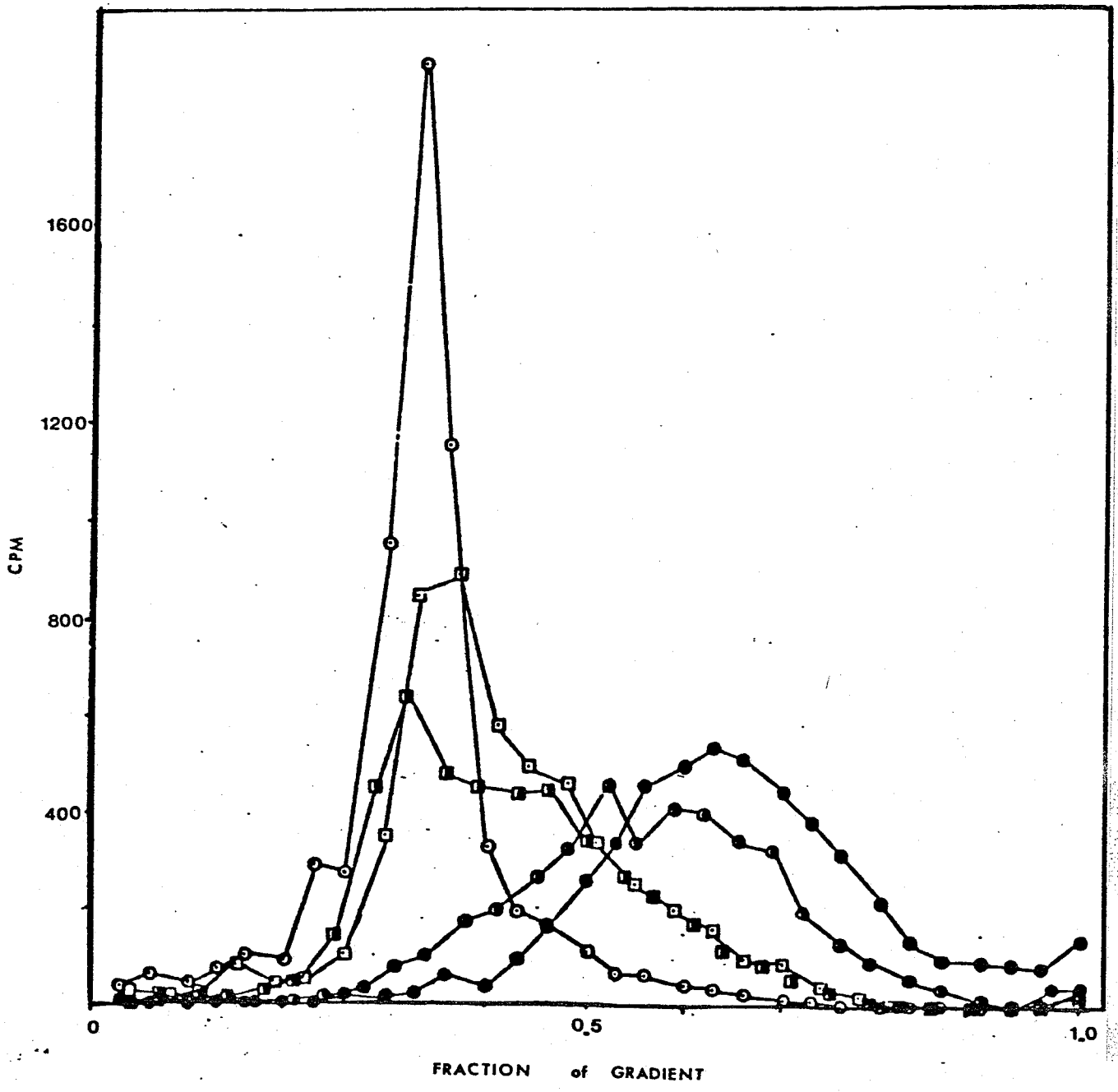

1600
1200
800
400
CPM
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 8

Effect of native DNA on S1 specificity. Labelled λ DNA was used as substrate for S1. The untreated DNA (○) was compared to S1 treated samples of 1.8 μg (◑), 7.4 μg (●) and 14.7 μg (■) of DNA.

The treated samples were 100 mM sodium acetate pH 5.0, 10 mM $ZnSO_4$. All samples were incubated for 90 minutes. Five units of S1 nuclease were added to all enzyme treated samples.

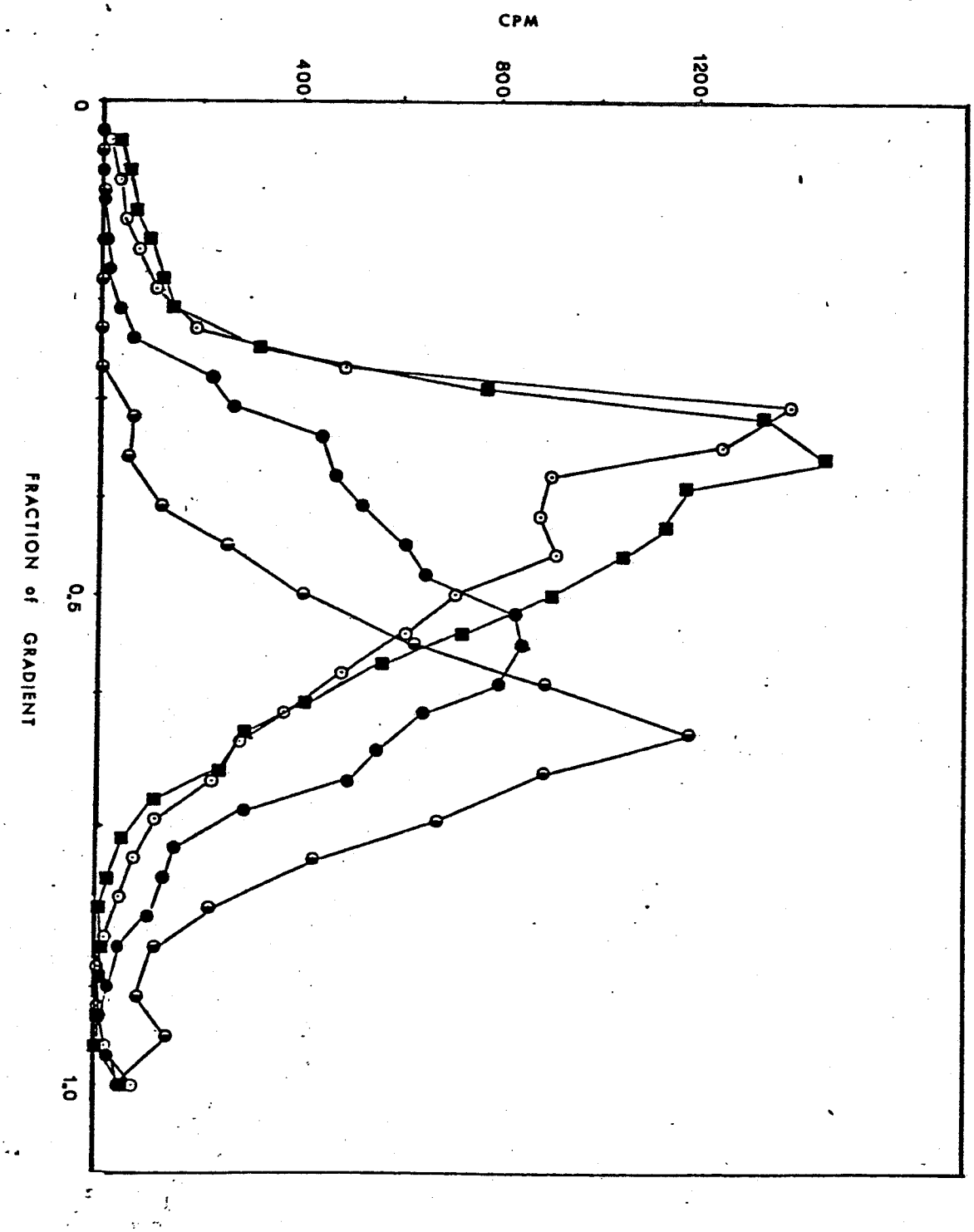

CPM
400
800
1200
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 9

Effect of denatured DNA on S1 specificity. Labelled λ DNA was denatured (as outlined on Page 56) and treated with S1 nuclease. The treated DNA samples were 100 mM sodium acetate pH 5.0, 10 mM $ZnSO_4$. Samples were incubated for 90 minutes. The untreated DNA (○) was compared to S1 treated samples of 2.05 μg (◑), 6.15 μg (●) and 14.35 μg (■).

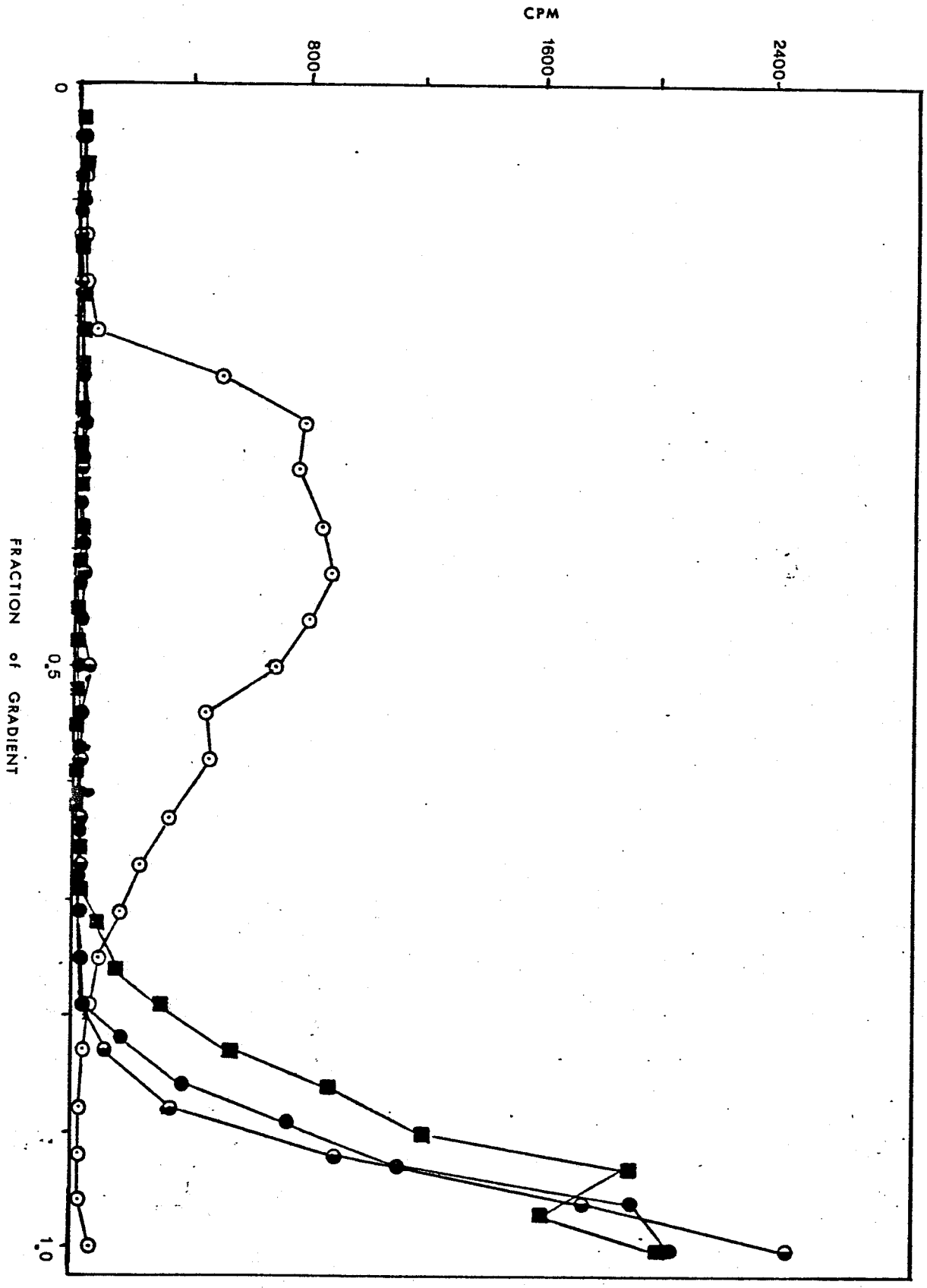
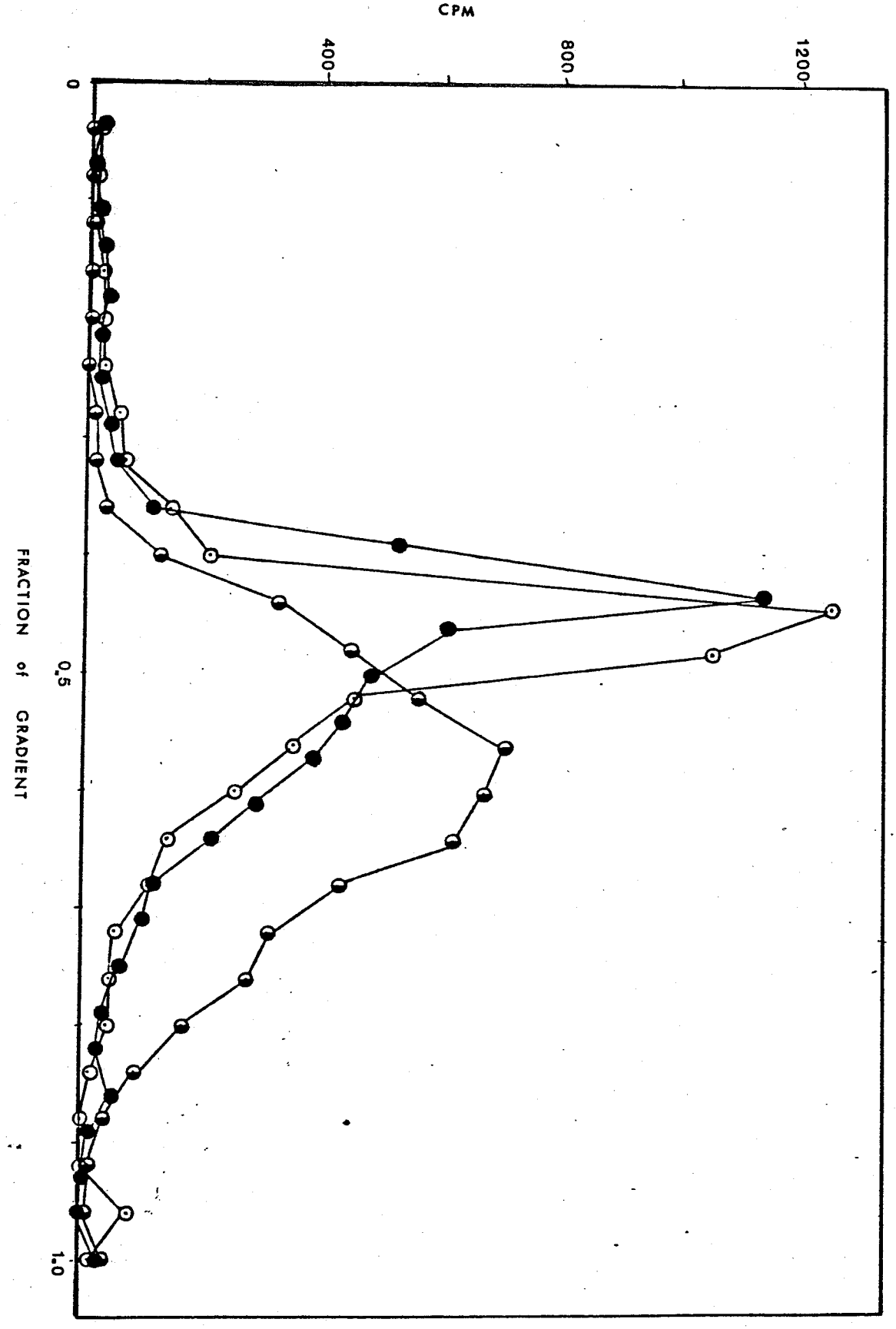

CPM
800
1600
2400
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 10

Effect of carrier single stranded DNA on S1 specificity. Labelled λ DNA was treated with S1 according to the standard reaction (100 mM sodium acetate pH 5.0, 10 mM $ZnSO_4$ 5 units of S1). The samples were incubated for 75 minutes. The untreated native DNA (○) was compared to the native DNA in the presence or absence of carrier DNA.

native DNA - 3 μg (◑)

native DNA - 3 μg (25 μl) with 4.5 μg (50 μl) denatured carrier DNA (●).


CPM
400
800
1200
0
0.5
1.0
FRACTION of GRADIENT

ANNEALING STRANDS

Either purified phage or isolated DNA strands could be annealed to prepare DNA duplexes. Annealing using whole phage as the source of DNA gave a large broad peak on an alkaline sucrose gradient (Figure 11). Annealing with separated strands gave sharper peaks (Figure 12) but the DNA concentration was lower.

Although homoduplexes could be used in initial studies to characterize the annealing procedure, the separated strands were essential to the preparation of heteroduplexes. A serious complication arose when it was found that the prepared strands were degraded during storage. The separated strands had to be used within six weeks of preparation. This is illustrated in Figure 13. Separated strands of various ages were dialysed and run on alkaline sucrose gradients. The only preparation which still had a sharp peak corresponding to DNA strands was 37 days old. Storage of the same strands for 24 days, after overnight dialysis to remove excess CsCl, resulted in faster degradation of the strands. The large tailing to the top of the gradient may have been poly UG although this was not determined. The most likely explanation for this slow degradation was the presence of small amounts of nuclease in the solutions.

In order to prepare the heteroduplexes from the strands, the strands had to be annealed and it was found that the pH used in the annealing had a pronounced effect on the DNA integrity. As shown in Figure 14, the DNA annealed with sodium glycinate, pH 9.2, as the buffer showed less breakdown than the DNA annealed with tris-HCl,

pH 7.6 as the buffer. All subsequent annealings were carried out adding sodium glycinate buffer to the alkaline denatured DNA solution to a concentration of 100 mM and then bringing the pH to 9.5 by the addition of 1N. HCl. Finally, the DNA was incubated in the standard manner to anneal the strands.

CONDITIONS FOR ALKALINE GRADIENTS

Once the annealing procedure was optimized, the next manipulation was cleavage of heteroduplexes by purified S1 nuclease. This was followed by isolation of the cleaved fragments. The simplest procedure to moniter the annealed and S1 treated DNA was to use alkaline sucrose gradients.

While the expected peaks could be isolated from the alkaline sucrose gradients, there was a problem with the loss of DNA. The recovery of the DNA ranged between 10 and 60% and the DNA seemed to be lost on the polyallomer tubes. To overcome this problem different batches of tubes and different treatments of the tubes were tried.

The tubes were pretreated by rinsing the tubes with a high concentration of DNA (approximately 100 OD) and by boiling the tubes with a mixture of DNA, EDTA and pyrophosphate. Residual DNA from the DNA treatments only served to mask the desired phage DNA peak, but the combined DNA-EDTA PP1 treatment did increase the yield of annealed DNA significantly (Figure 15). Greasing the tubes with silicone grease did not affect the gradient profile of the DNA. It was noted that diluting the phage DNA sample tenfold radically affected the profile. Although the yield of DNA was not altered, the concentration of DNA

was decreased from 39 μg/ml to 12 μg/ml (Figure 16).

The effect of DNA concentration was studied in two ways. When gradient profiles were compared for isolated strands, annealed DNA and phage, the most pronounced peak corresponded to the phage. Although the yield of DNA did not differ significantly, the concentration of DNA in the peak was four times greater for phage than for strands or annealed DNA (Figure 17).

Further experiments were tried studying the effect of buffer and Zn^{++}ion on the loss of DNA. Although the background absorbance varied, the difference between the peak and the background (i.e. the height of the peak) was decreased in the presence of Zn^{++}ion. This could be overcome by adding EDTA to chelate the zinc (Figure 18).

Some work was done on the condition of the DNA prior to loading the gradients. In general, the DNA samples were brought to 180 mM sodium hydroxide and incubated at 37°C for 5 minutes to denature the DNA before loading onto the alkaline sucrose gradients.

When DNA was denatured using 50 mM EDTA in addition to the NaOH as described above, the gradient profiles became sharper. Isolated strands were buffered with the addition of 50 mM tris-HCl pH 7.6 and 10 mM $MgCl_2$ and then denatured using either NaOH or EDTA and NaOH (Figure 19). The use of EDTA to chelate any divalent ions present seemed to be essential.

An experiment was designed to optimize the denaturation conditions. The concentration of EDTA was tested as was the length of incubation at 37°C. The best profile was obtained using denaturation conditions of 25 mM EDTA, 180 mM NaOH and 10 minute incubation at 37°C (Figure 20).

Having determined the conditions for alkaline sucrose gradients, the gradients could be used to isolate annealed and S1 treated DNA. A standard profile of such an experiment is shown in Figure 21. The annealed DNA peak is coincident with untreated, separated strands, indicating that no accidental cleavage had occurred during the preparation of the heteroduplex. It can also be seen that the S1 nuclease caused a shift in the DNA peak towards the top of the gradient. Using the method of Abelson and Thomas[125], the smaller DNA formed in the reaction was estimated to be half size as expected.

FIGURE 11

Profile of annealed DNA using phage as the source. Using λ gal phage a homoduplex was prepared according to standard procedure. The untreated phage (○) was compared to the annealed phage treated with S1. (◑)

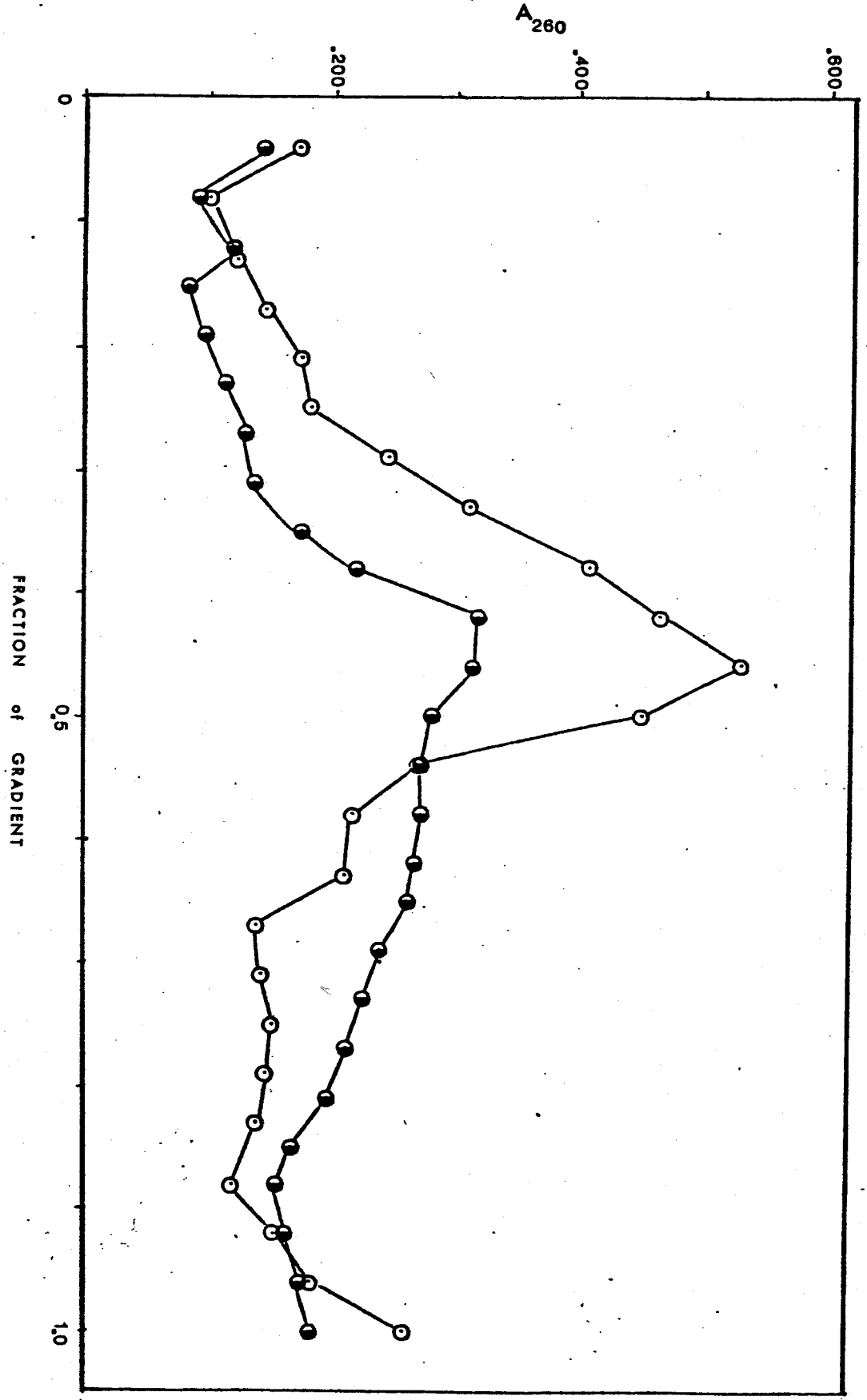

A_{260}
.200
.400
.600
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 12

Profile of annealed DNA using separated strands as the source. Isolated strands were used to prepare a heteroduplex according to the standard annealing procedure. The untreated strands (◔) were compared to the annealed heteroduplex (◑) and the S1 treated annealed DNA. (●)

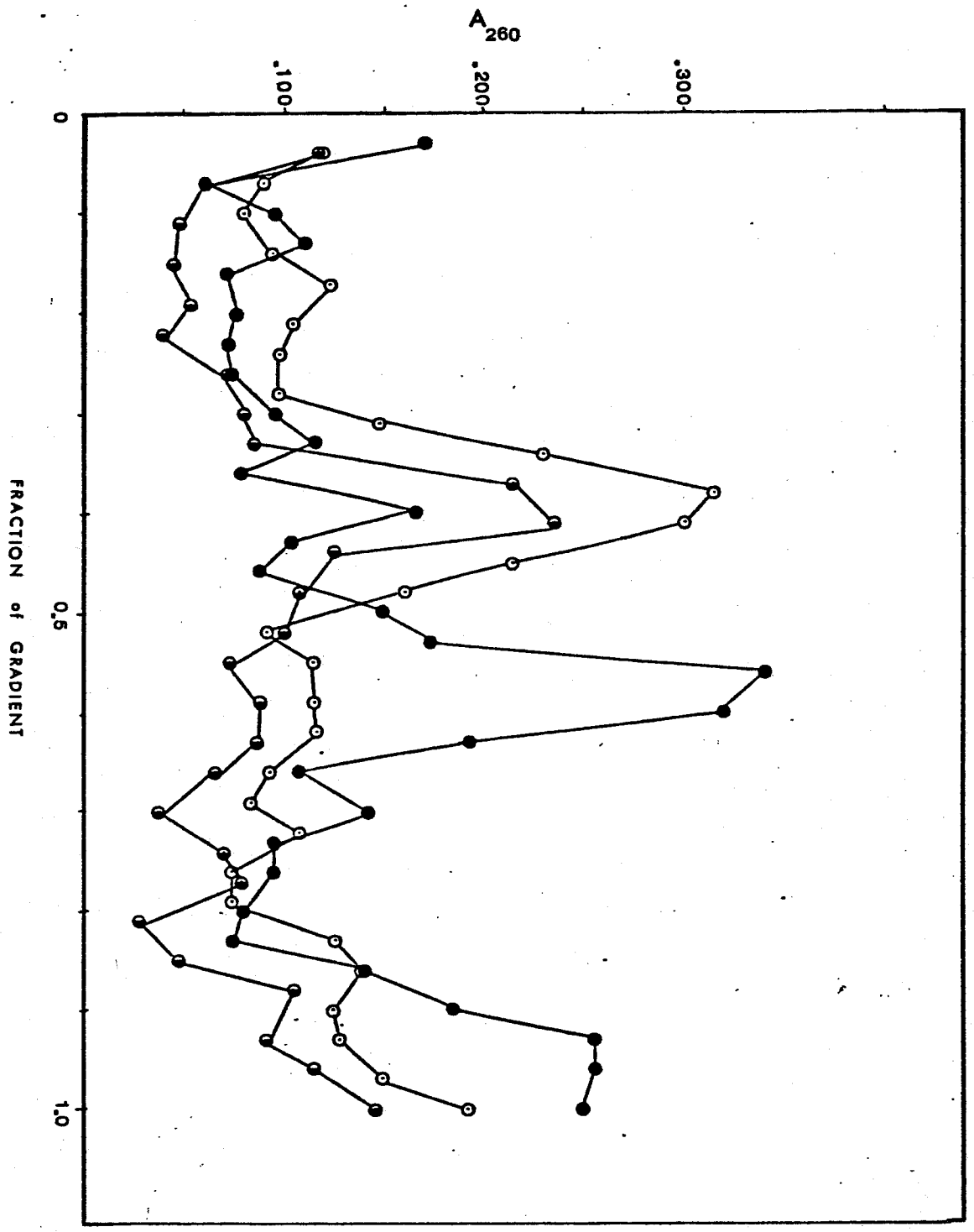

A_{260}
.100
.200
.300
0
0,5
1,0
FRACTION of GRADIENT

FIGURE 13

Effect of age on stability of strands. Isolated strands of different ages were run on an alkaline gradients to test stability.

(○) strands - 37 days old

(◑) strands - 50 days old

(●) strands - 252 days old

(△) strands - 37 days old (early dialysis)

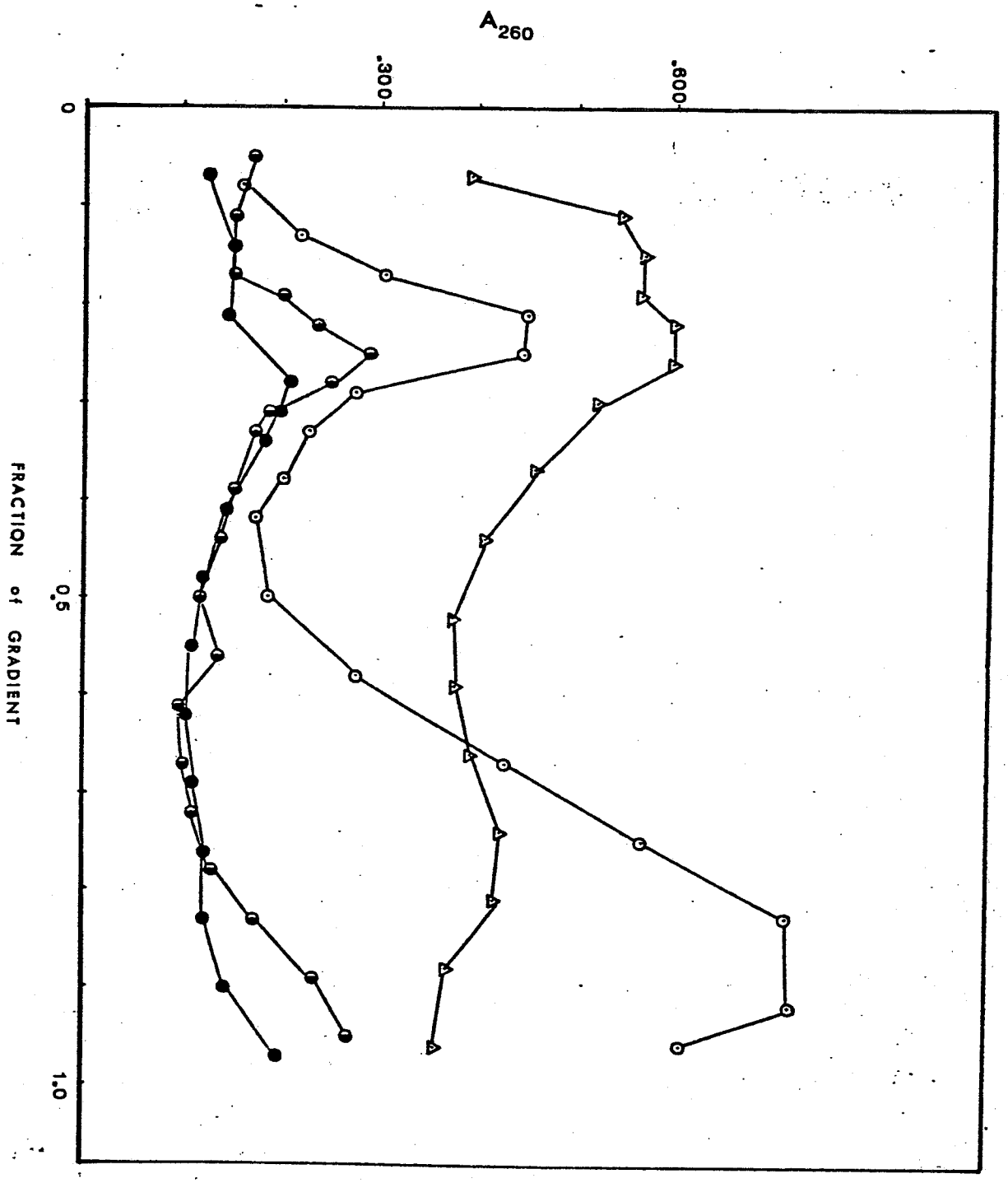

A_{260}
.300
.600
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 14

Effect of pH on formation of annealed DNA. Using λgal phage, homoduplexes were formed. The phage were denatured by the addition of NaOH (final concentration 10 mM) and incubated at 37°C for 30 minutes. Using tris-HCl pH 7.6, one sample was brought to a pH of 7.6. Another sample was made pH 9.5 by the addition of sodium glycinate pH 9.2. The annealed mixtures were then incubated at 65°C.

A (○) control λgal phase

(◑) annealed DNA treated with tris-HCl pH 7.6

B (○) control λgal phage

(◑) annealed DNA treated with sodium glycinate pH 9.5

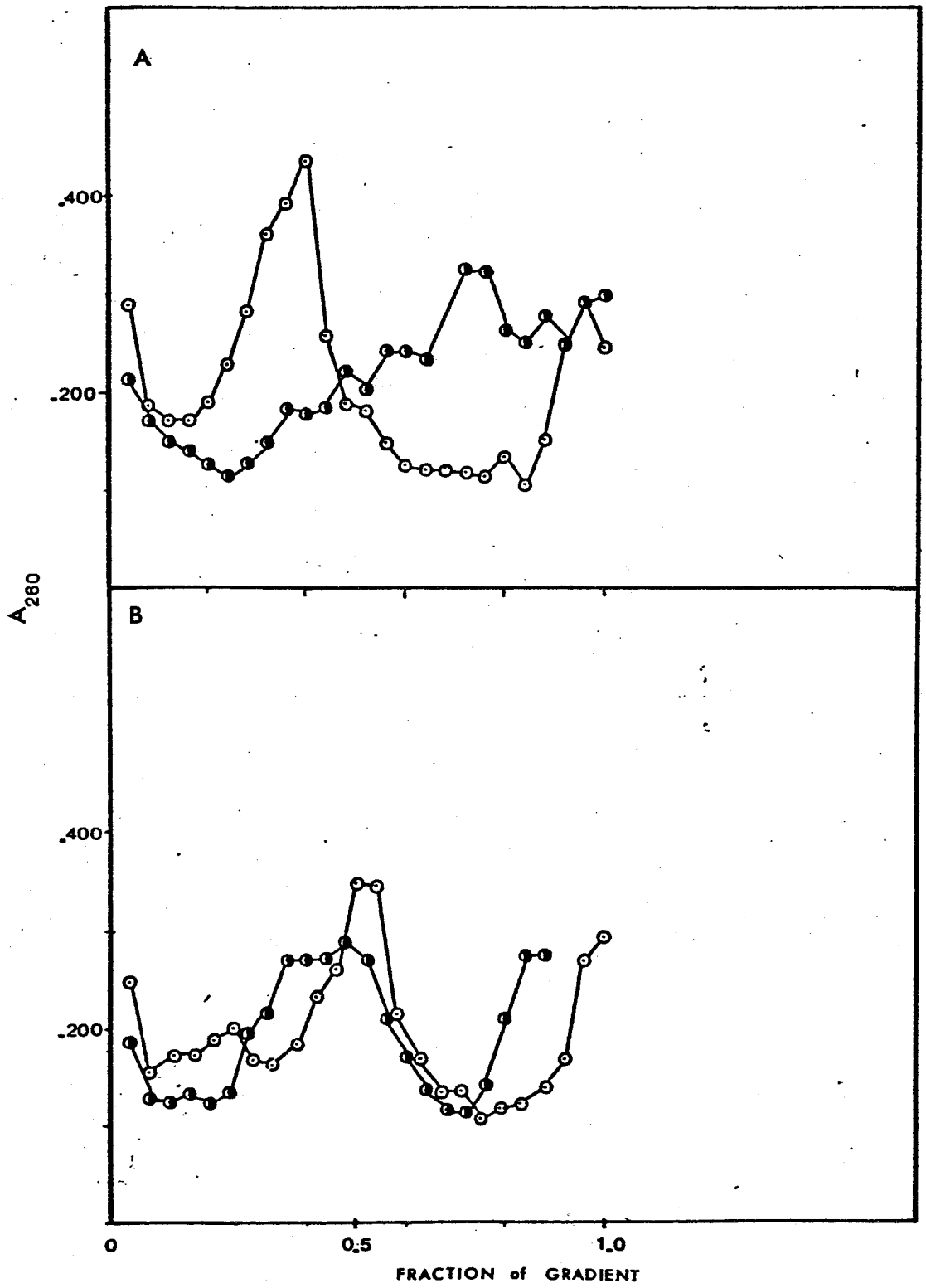

A
.400
.200
A260
B
.400
.200
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 15

Effect of pretreatment of polyallomer tubes on adsorption of DNA to the tubes. Using an annealed λc_1 homoduplex the effect of rinsing tubes with a DNA solution or of boiling tubes with a mixture of DNA-EDTA-PPi was tested. In each case, 100 μl samples containing 10 μg DNA were denatured and then run on alkaline sucrose gradients.

(○) annealed DNA

(◑) annealed DNA in rinsed tubes

(●) annealed DNA in boiled tubes.

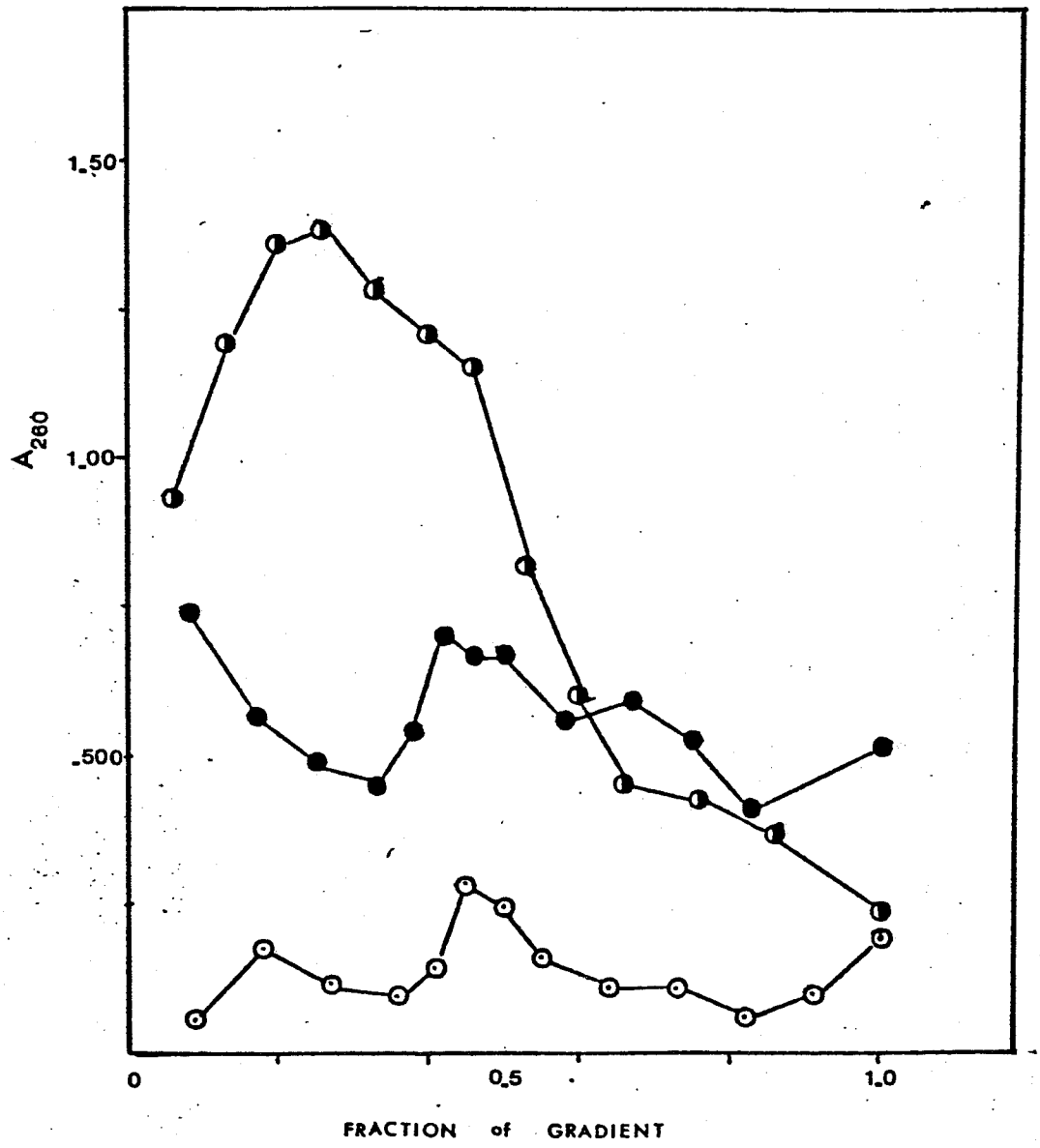

1.50
1.00
.500
A_{280}
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 16

Effect of silicone grease on adsorption of DNA to polyallomer tubes. 100 μl aliquots of λc_1 phage concentration 1780 μg/ml were tested with ungreased and greased polyallomer tubes. A tenfold diluted sample of phage was also run to see its effect.

(○) ungreased polyallomer tube

(◑) silicone greased polyallomer tube

(●) greased tube with dilute sample 1:10 dilution.

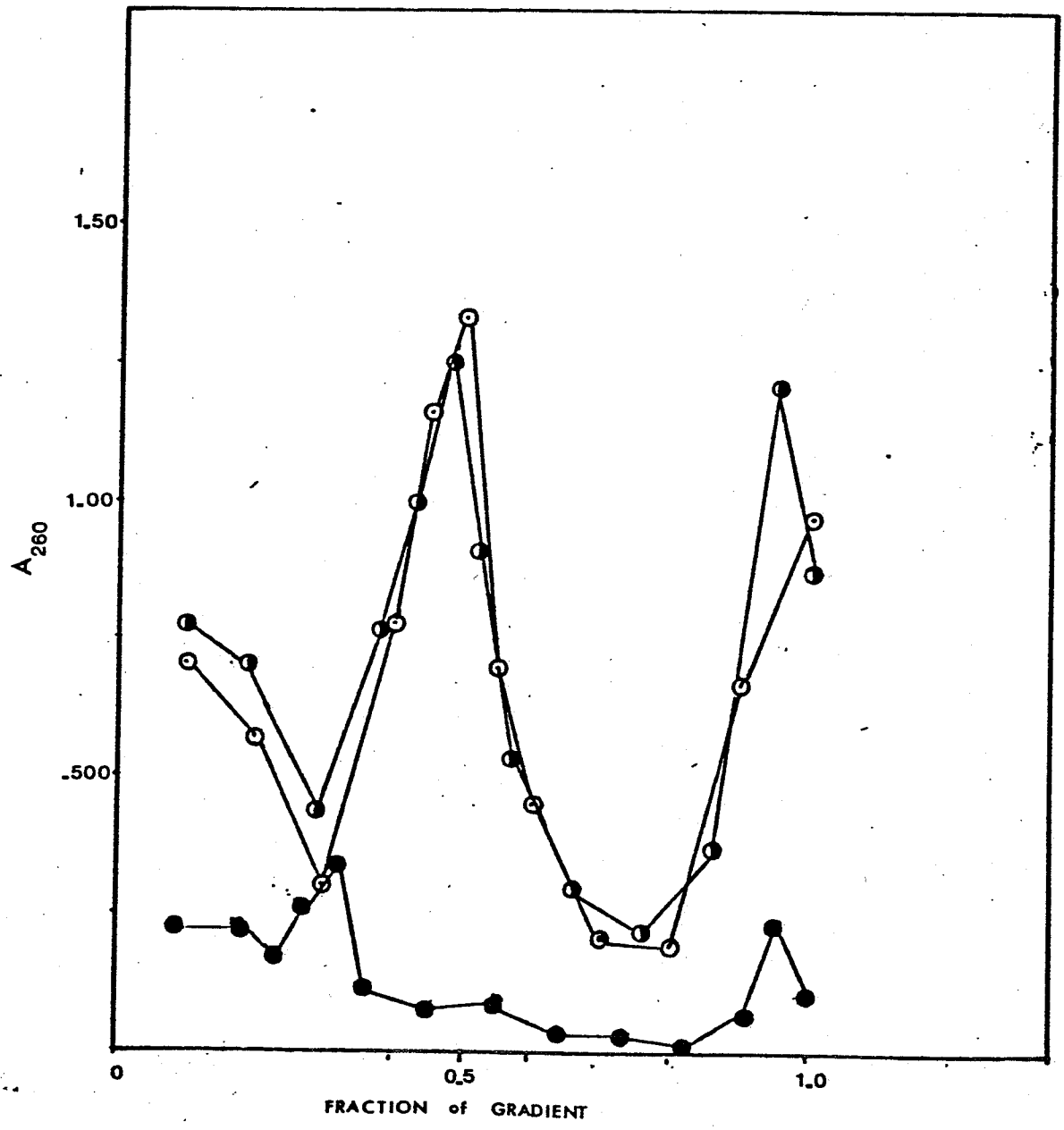

1.50
1.00
.500
A_{260}
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 17

Effect of DNA on formation of peaks. 100 μl aliquots of λc_1 phage, annealed λc_1 homoduplexes and λc_1 strands were tested on alkaline sucrose gradients.

(○) λc_1 phage (178 μg)

(◑) λc_1 strands (12.3 μg)

(●) λc_1 annealed homoduplex (10.2 μg)

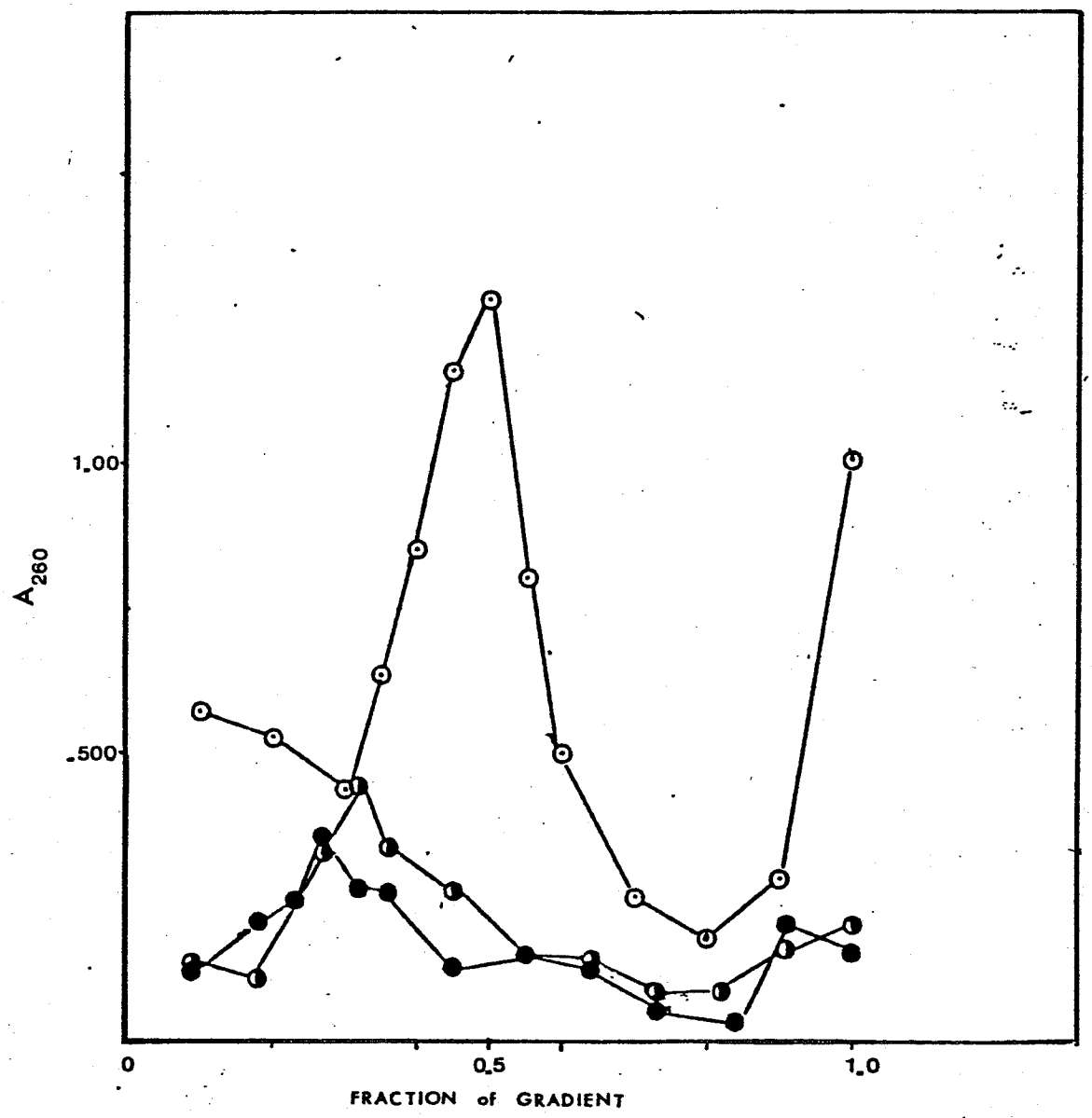

1.00
.500
A_{260}
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 18

Effect of buffer and Zn^{++}ion on formation of DNA peaks. Using 100 μl annealed λc_1 homoduplex (concentration 102 μg/ml) the effect of sodium acetate buffer pH 4.5 and Zn^{++}ion was tested.

(○) annealed DNA with sodium acetate buffer

(◑) annealed DNA with sodium acetate buffer and Zn^{++}ion

(●) annealed DNA with buffer, Zn^{++}ion and EDTA 10 mM

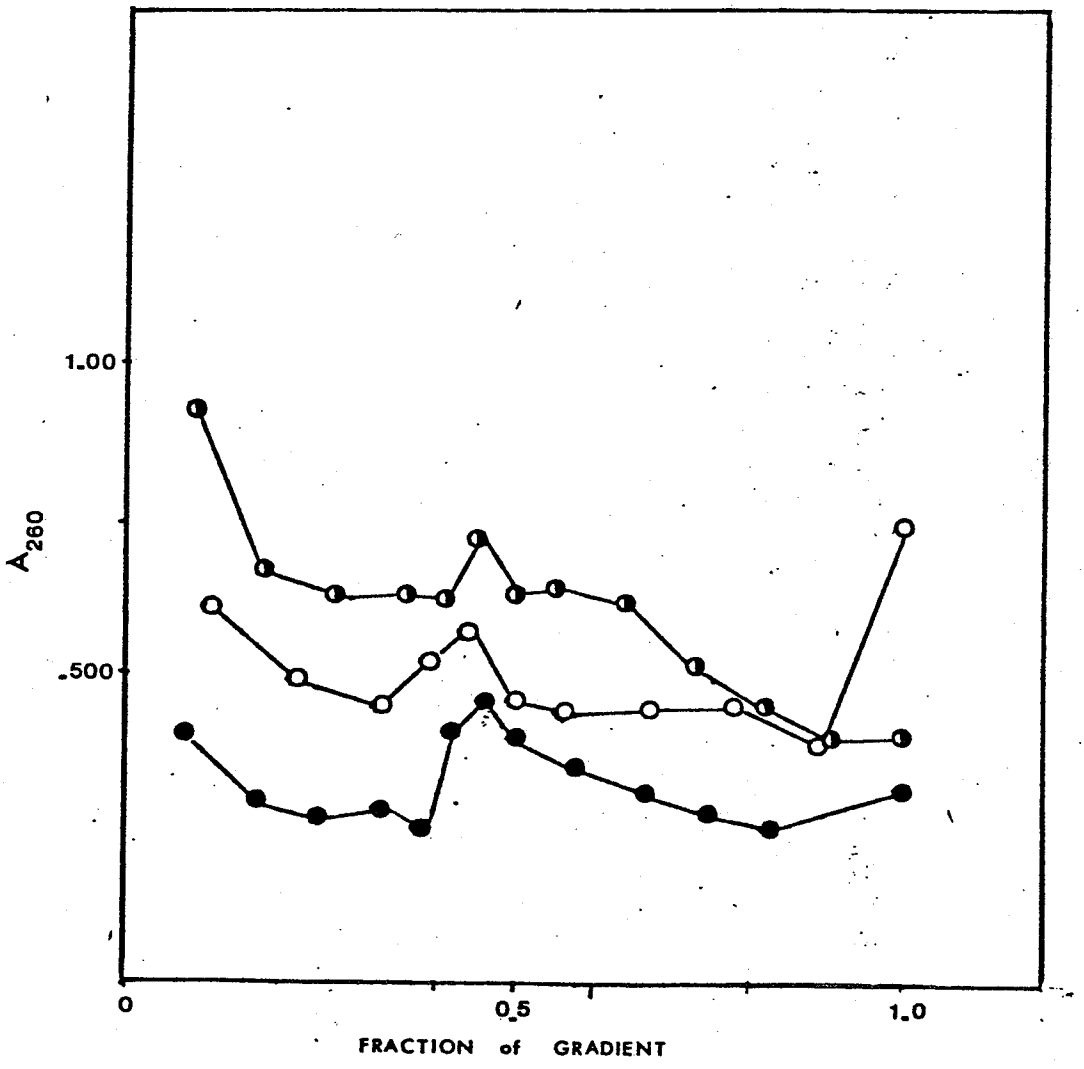

1.00
.500
A_{260}
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 19

Effect of denaturation conditions on DNA peaks. Using 200 μl aliquots isolated strands (6.4 μg DNA) made 50 mM in tris-HCl pH 7.6 and 100 mM in $MgCl_2$, the profile of the DNA on alkaline gradients was tested.

(○) strands with 180 mM NaOH

(◑) strands with 180 mM NaOH, 50 mM EDTA

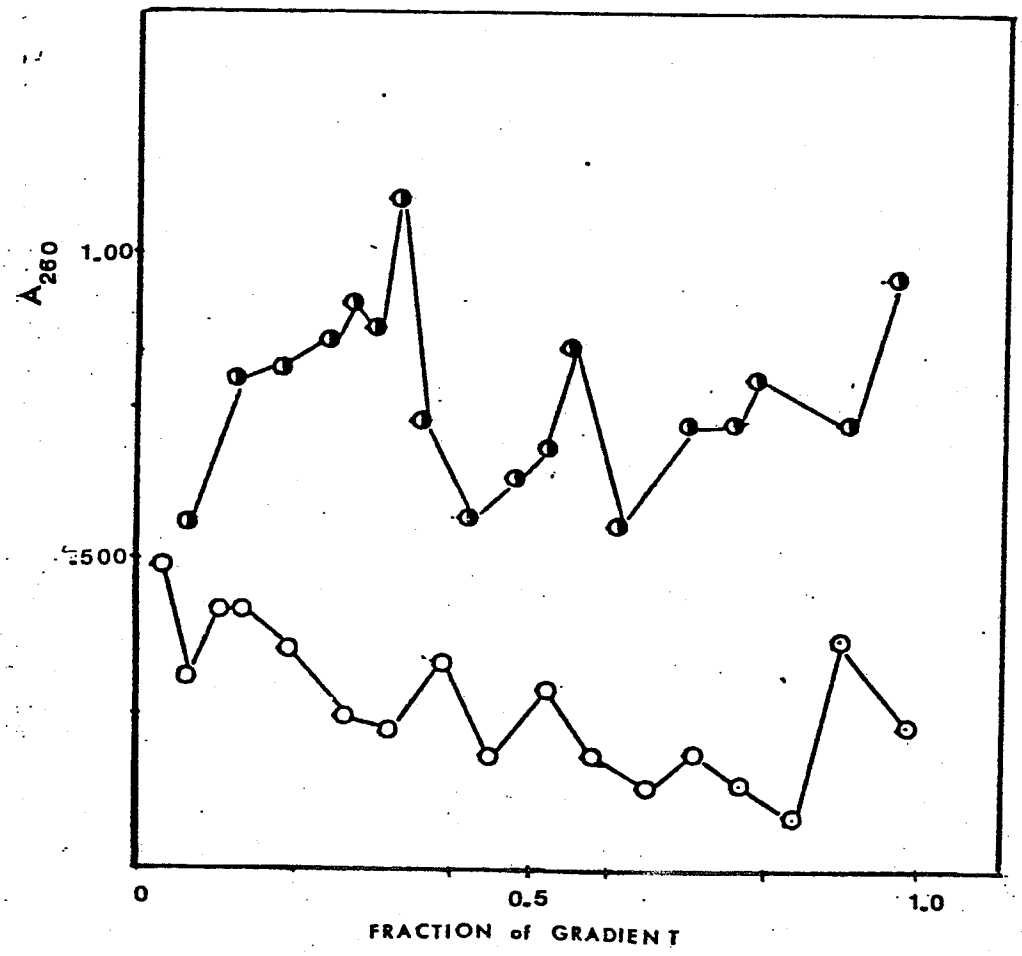

A_{260}
1.00
.500
0
0.5
1.0
FRACTION of GRADIENT

FIGURE 20

Effect of denaturation conditions on DNA profile. Using 110 μl aliquots of λc_1 strands (5.8 μg DNA) made 50 mM in tris-HCl pH 7.6, and 10 mM in $MgCl_2$, different procedures of denaturation were tried.

A (○) 50 mM EDTA, 180 mM NaOH, 5 minute incubation boiled reagents.

B (○) 25 mM EDTA, 180 mM NaOH, 5 minute incubation boiled reagents.

C (○) 25 mM EDTA, 180 mM NaOH, 10 minute incubation boiled reagents.

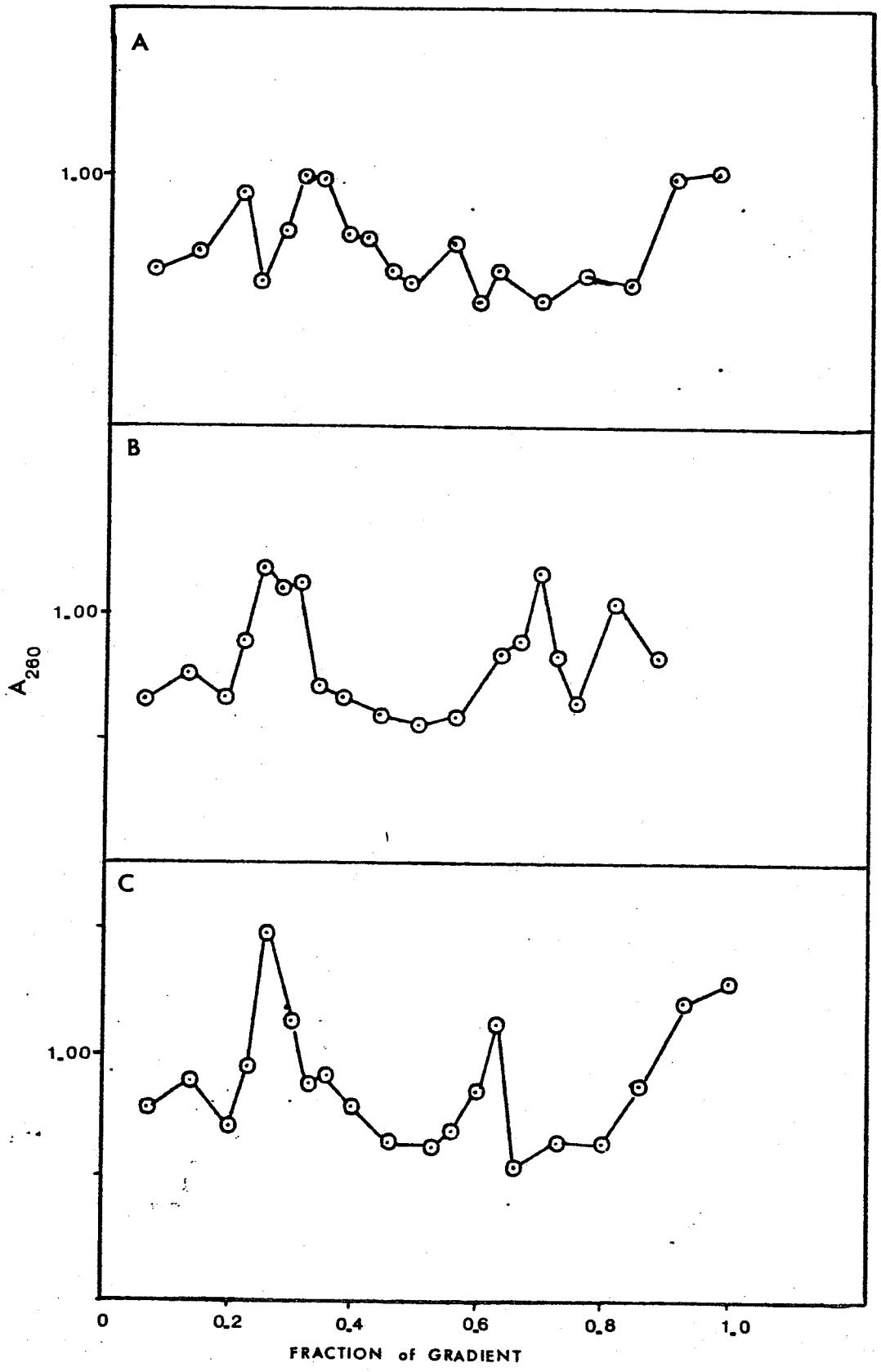

A
B
C
1.00
1.00
1.00
A_{280}
0
0.2
0.4
0.6
0.8
1.0
FRACTION of GRADIENT

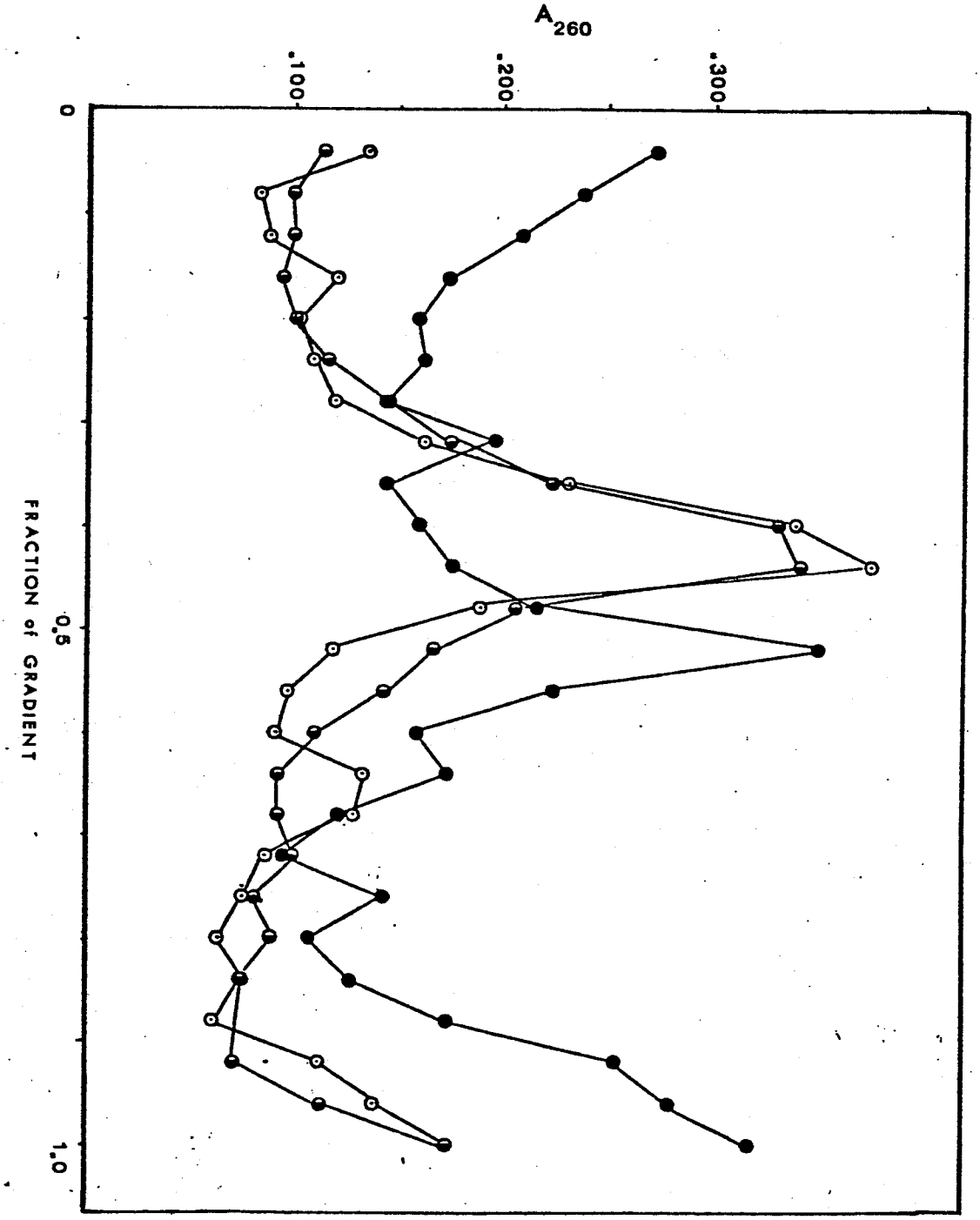
FIGURE 21

Standard profile of strands, annealed DNA and S1 treated annealed DNA. A heteroduplex was prepared using 100 mM sodium glycinate pH9.2 as the buffer. The pH was adjusted to a final value of 9.5 by the addition of 1N. HCl. After annealing the DNA an aliquot was removed and treated with S1 nuclease (100 mM sodium acetate pH 5.0, 10 mM $ZnSO_4$). The standard procedure is outlined in Methods. The profile of these samples on alkaline sucrose gradients was determined.

(○) control - untreated strands

(◑) annealed heteroduplex

(●) annealed DNA treated with S1.


A_{260}
.100
.200
.300
0
0.5
1.0
FRACTION of GRADIENT

S1 FRAGMENT CLEAVAGE SITE

Before work could proceed with the repair of the S1 nuclease treated fragment, it was necessary to determine if S1 produced butt-ended duplexes or removed all non hydrogen bonded nucleotides.

One experiment was devised where lambda DNA was treated with S1 prior to strand separation. This would remove the cohesive ends from the molecule. Following separation, these treated λc_1 strands were annealed to either a λgal strand (DNA-A), a complementary λc_1 strand (DNA-B) or to the fragment mixture isolated after S1 digestion of a heteroduplex (DNA-C). The fragment mixture from S1 digestion of a heteroduplex was also examined (DNA-D). These four samples were mixed with DNA dependant DNA polymerase I (pol I) and a repair cocktail. It was found that the samples of DNA-A and DNA-C catalysed repair reactions while DNA-B and DNA-D would not undergo repair. (Figure 22). This result indicated that there had been a "clean" cleavage by S1 nuclease leaving no nucleotides to act as a template for pol I.

To confirm this conclusion that the S1 nuclease removed all non hydrogen bonded nucleotides, the 5'-termini of DNA before and after S1 cleavage were analysed. Since the sequence for the cohesive ends of lambda was already known[126], the extent of S1 cleavage before and after enzyme treatment could be determined. By simple 5'-nucleotide analysis using λc_1 DNA and λc_1 S1-treated DNA, the 5'-ends were labelled with ^{32}P using $[\gamma^{32}P]$-ATP and polynucleotide kinase. Following removal of excess ATP, the 5'-nucleotide was determined by combined pancreatic DNase and snake venom phosphodiesterase digestion

and paper chromatography. Unfortunately, the results were not conclusive, as both the control and S1 treated strands yielded varying amounts of different nucleotides (Table 6). This was ultimately shown to be the result of an endonuclease in the polynucleotide kinase[127]. While this work was in progress, a report appeared which confirmed that S1 nuclease did remove all non-hydrogen bonded nucleotides from DNA[128].

CONDITIONS FOR REPAIR

The sequence of reactions required that the S1 cleaved heteroduplex fragments be annealed to a whole DNA strand and subsequently repaired or extended with DNA polymerase I. Therefore, initially the report conditions were analysed using an enzyme preparation supplied by Dr. H. G. Khorana. All of the reagents including the calf thymus DNA substrate and the cocktail components were checked with no improvement in the repair reaction. The enzyme itself needed to be analysed.

Using three different preparations of pol 1, provided by Dr. H. G. Khorana (Sample A), Dr. H. van de Sande (Sample B), and a crude extract prepared in our lab according to Englund[107] (Sample C), a repair reaction was set up. The sample A enzyme did not incorporate any nucleotides while Sample B enzyme exhibited excessively high levels. This indicated a contaminating nuclease was present in the B enzyme preparation. The Sample C preparation alone showed a good level of ^{32}P incorporation, which indicated that the repair

conditions were suitable but that the enzyme was the problem. Consequently, the B preparation of pol I was run on a G-100 column, 1 x 45 cm. The eluted fractions were assayed for polymerase. After isolating the active peak, the nuclease effect was no longer present during repair experiments. This indicated the presence of exonuclease III in the untreated preparation.

Since the repair conditions were functioning, the repair of the annealed complex of the S1 cleaved fragment and a whole $\lambda c_1 1$ strand was attempted. This complex would allow repair only into the att site as the polymerase repairs in a 5' → 3' direction. The labelled product produced was isolated and analysed to determine the 3'-termini (i.e., the attachment site P sequence). Unfortunately, the results were not significant, although the "A" value was slightly higher than other values.

FIGURE 22

Repair of several DNA duplexes to determine if S1 nuclease produced a butt ended cleavage. DNA-A was a duplex of λc_1 strand and a λgal strand. DNA-B was the λc_1 strand annealed to a complementary λc_1 strand. DNA-C was the λc_1 strand annealed to an isolated fragment of DNA obtained after S1 cleavage. DNA-D was the S1 cleaved fragment alone.

(◑) DNA-A

(○) DNA-B

(●) DNA-C

(□) DNA-D

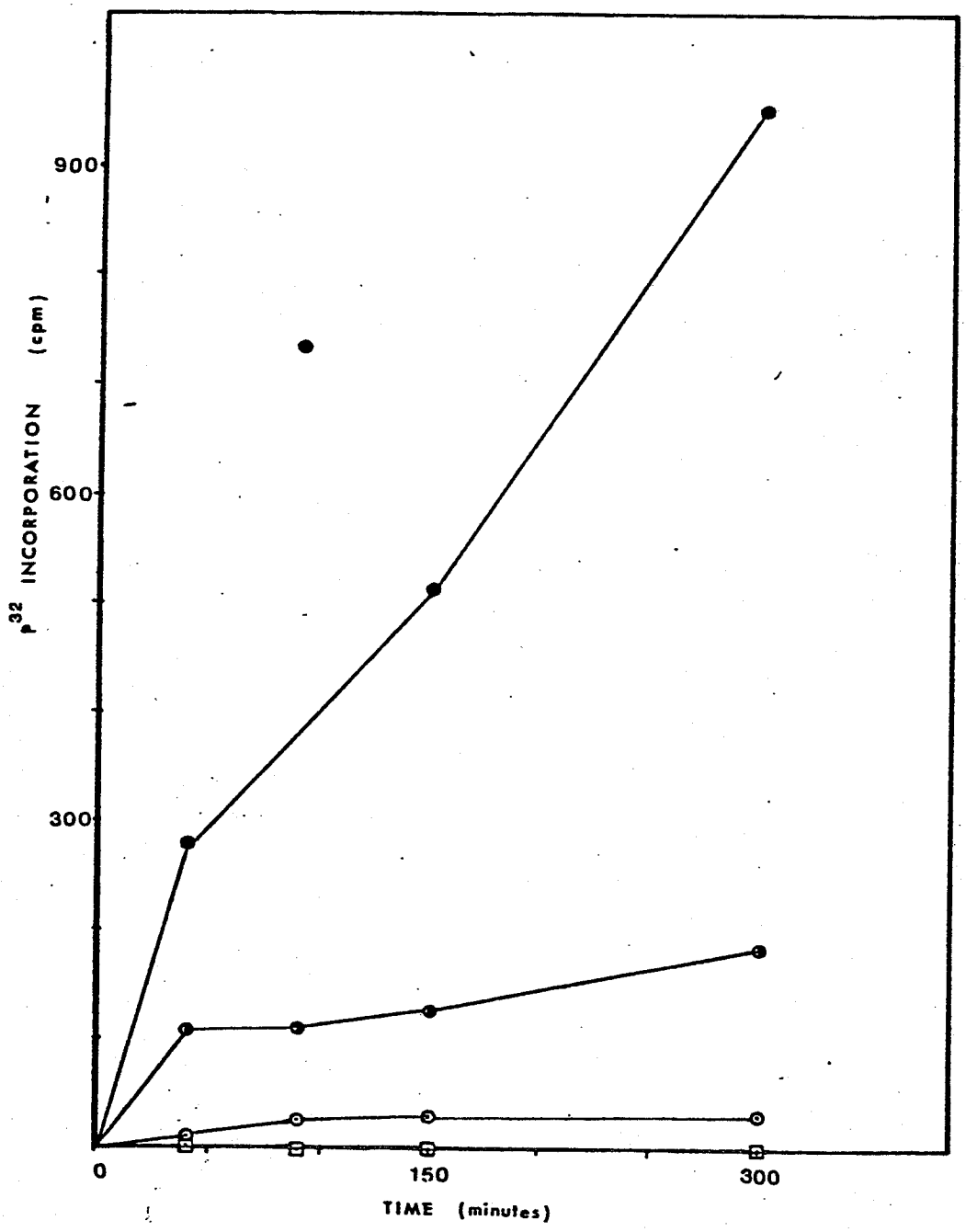

900
600
300
P^{32} INCORPORATION (cpm)
0
150
300
TIME (minutes)

TABLE 6

Results of 5'-termini analysis after polynucleotide kinase labelling. The counts for each respective nucleotide are listed. The experiment was repeated four times generating the data shown. Details of the experiment are outlined in Methods.

a values are in counts per minute.

TABLE 6

		A	G	T	C
λc_1	1	22[a]	28	16	14
	2	25	40	46	41
	3	8864	7108	6626	4333
	4	876	630	833	326
$\lambda c_1 S1$	1	880	780	609	352
	2	111	70	147	77
	3	4169	3398	2329	1729
	4	405	410	340	305

METHOD II

CIRCLES PREPARATION AND ISOLATION

Protocol I (Figure 23a) which involved polymerase repair of an S1 cleaved fragment and a $\lambda c_1 1$ DNA strand, was difficult to exploit. Although the system was functional, there was an inherent difficulty due to the number of manipulations involved. The net result was a limited amount of material to work with.

Consequently, a different approach was attempted (Figure 23b). The principle was to prepare circles of lambda heteroduplexes, and subsequently treat the circles with S1 nuclease. The result would be a linear molecule which had the attachment at each end. By using polynucleotide kinase and $[\gamma^{32}\text{-P}]$ ATP the 5'-ends could be labelled, and these labelled fragments could then be isolated after partial nuclease digestion.

Since the annealing procedure had been optimized, the first step to be analysed in detail was the formation of circles. Several methods have been published on the formation of circles[129-131]. Paoletti's[129] procedure or modifications of it were used to prepare circles. Initial work was done with the phage as the source of DNA, until certain problems were resolved. The procedure as outlined in Methods has some critical steps. First, the cohesive ends of the duplex must form a hydrogen bonded complex, which requires low concentrations of DNA to prevent the formation of aggregates. Then the sample must be concentrated without damaging the circles. Finally the ends must be sealed using polynucleotide ligase.

The ultimate consideration was the yield of circles, which was generally low. To overcome this, the conditions were varied. The method of concentration was changed from dry sucrose to air evaporation. The incubation was also modified. It was determined that milder conditions were more favorable. Thus, when sealed with ligase, 20°C was used for ligase activity, followed by 55°C for 90 minutes to inactivate the enzyme. The ligase was previously inactivated by incubation at 75°C for 10 minutes. The former treatment tended to favor the formation of linear molecules rather than circular ones. After preparing circles, it was difficult to determine whether the preparation was effective or to isolate the circles.

Therefore, initially the circle preparations were analysed by the use of alkaline sucrose gradients. A gradient profile could be obtained running a SW50.1 rotor at 38K for 75 minutes. Under these conditions a greater separation between linear and circular DNA could be obtained (Figure 24). There were two problems with the alkaline gradients. First, the peaks for the circular DNA were very low, making it difficult to isolate any DNA and making it apparent that only a portion of the sample was converted to circular form. Covalently closed circular DNA was difficult to prepare. Secondly, the use of alkaline gradients required the subsequent reannealing of any isolated circles, an additional manipulation.

One alternative method for assaying circle formation was by intercalation of ethidium bromide into DNA molecules[132]. It is known that closed circles cannot accept as many ethidium bromide molecules

due to topological restrictions. However, when the fluorimetric assay was tried, no alteration in the fluorescence could be found. This could be caused by the failure of the assay or by the amount of circular DNA being too low. It was therefore necessary to find a different procedure for the analysis and isolation of circles and agarose gels were tested to determine if they could separate linear and circular DNA.

Initially, the agarose gel was prepared using 100 mM potassium phosphate pH 6.9, with an agarose concentration of 0.8%. The DNA was visualized by staining with ethidium bromide, followed by scanning with UV lights. Annealed DNA remained at the origin after three hours of electrophoresis with 125 mA current. Although the time of electrophoresis was increased to 16 hours (overnight) only the isolated strands would enter the gel. Therefore, the conditions of electrophoresis and the concentration of the agarose were altered until the DNA moved freely into the gel. The final conditions were a 0.6% agarose solution in 100 mM potassium phosphate pH 6.9, and five to sixteen hours of electrophoresis.

All of the preliminary work was done with slab gels 0.3 cm x 20 cm x 20 cm. These slab gels were difficult to use because the wicks dried out during long electrophoresis times, the gel troughs could not be loaded easily, and the DNA tended to stream randomly throughout the gel. To overcome these difficulties, disc gels were tested and found to greatly improve the results since the gels ran evenly, producing clear bands.

Some modifications were made to facilitate the transition from slab to disc gels. First, due to the slippery nature of the agarose, nylon mesh holders were added to the bottom of the gel tubes. Secondly, the sample was loaded (1-2 μg, 10-50 μl) then overlayed with an equal volume of 20% sucrose, 0.001% bromphenol blue. The sucrose overlay helped to keep the sample in position, and to run into the gel evenly. With these improvements, all further work was done with the disc gels.

Another problem was located when it was found that the ethidium bromide solutions age, becoming less effective for intercalation. When a fresh solution was used, the best concentration for the stain is 0.5 to 1.0 μg/ml.

Another fact found was the correct length of electrophoresis to obtain good separation of samples without loss or degradation. Although a time course experiment was done ranging from 2.75 hours to 12.5 hours at 50 ma, no particular time could be chosen as the samples progressed through the gel with a gradual increase in separation, and with some variation in mobility from experiment to experiment. Hence the internal standard of bromphenol blue was found to be useful and when the tracking dye reached the bottom of the gel regardless of the time period, the electrophoresis was stopped.

During the course of modifying the system for gels, the patterns of DNA were found to be unique. There were different results for linear strands, annealed DNA and for circles. The strands were very

mobile, while the annealed DNA was restricted to slight movement. The circles were intermediate between the strands and annealed DNA. The DNA samples would respond quite differently if denatured with alkali. The DNA solutions were made 0.2 M in NaOH, then incubated at 37°C for 10 minutes to denature them. When denatured, the annealed DNA had the same banding pattern as the strands, which corresponded to the previous work with gradients. The standard profile can be seen in Illustration 1, where the native sample of circular DNA is very significant. The circle preparation contains an extra band of DNA which has greater mobility than the others. The presence of circles as an extra band can be seen in Illustration 2. This readily identifiable band was useful in analysis of circle preparations, which definitely contained both linear and circular DNA.

When the DNA preparations were treated with S1 nuclease before electrophoresis, some new patterns emerged. The S1 cleavage of the heteroduplex circles would be expected to produce a linear molecule slightly smaller than the annealed complex as a whole. After S1 treatment, it was found that the DNA was degraded to small fragments of varying lengths, possibly because the concentration of the sample was sufficiently low for double stranded cleavage to occur. In addition, the presence of linear DNA in the population masked the products formed making it necessary to isolate the circles before any S1 treatment.

The next step was to develop a method of using the agarose gels preparatively. Initially, larger gels were tried. These gels were

4 cm. x 15 cm. as opposed to regular disc gels of 0.5 cm. x 7 cm. Although the conditions were the same, there was a problem in the DNA entering the gel. The area was probably too large or possibly the resistance was too high but the DNA was fragmented in the gel.

Further studies were done on the small disc gels to see if they could be used preparatively. After testing the capacity of the gels, it was determined that volumes up to 100 μl per tube and samples up to 4 μg would give clear bands. This value was reasonable and would allow a circle preparation to be isolated after one or two experiments.

In order to use the gels preparatively, it was necessary to elute the isolated band of circular DNA from the gel. Sections of the gels were tested with and without the presence of ethidium bromide stain. The sections of gel were placed in SM-Mg buffer with or without gel homogenization. The content of DNA in the eluted sample was very low, about 20% recovery. Further work would have to be done to overcome the loss of DNA at this stage.

FIGURE 23

Schematic diagram of protocols involved to obtain attachment sequence.

 single stranded DNA

 double stranded DNA

 labelled DNA

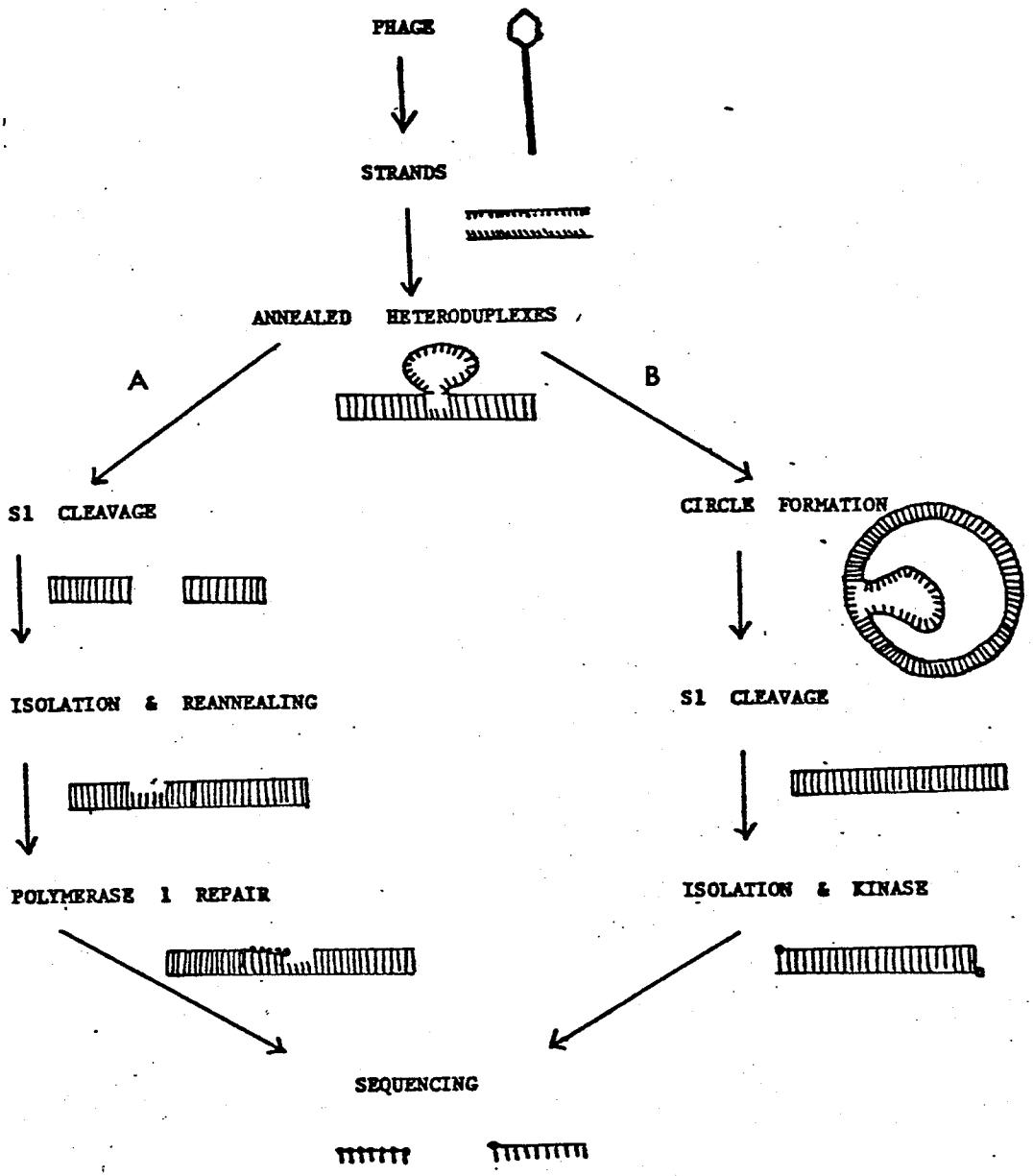

PHAGE
STRANDS
ANNEALED HETERODUPLEXES
A
B
S1 CLEAVAGE
ISOLATION & REANNEALING
POLYMERASE 1 REPAIR
CIRCLE FORMATION
S1 CLEAVAGE
ISOLATION & KINASE
SEQUENCING

FIGURE 24

Profile of annealed DNA and circular DNA on alkaline sucrose gradients. DNA was run on alkaline sucrose gradients for 75 minutes at 38K (SW50.1).

(○) annealed DNA λc_1RgalL

(◐) circular DNA λc_1LgalR

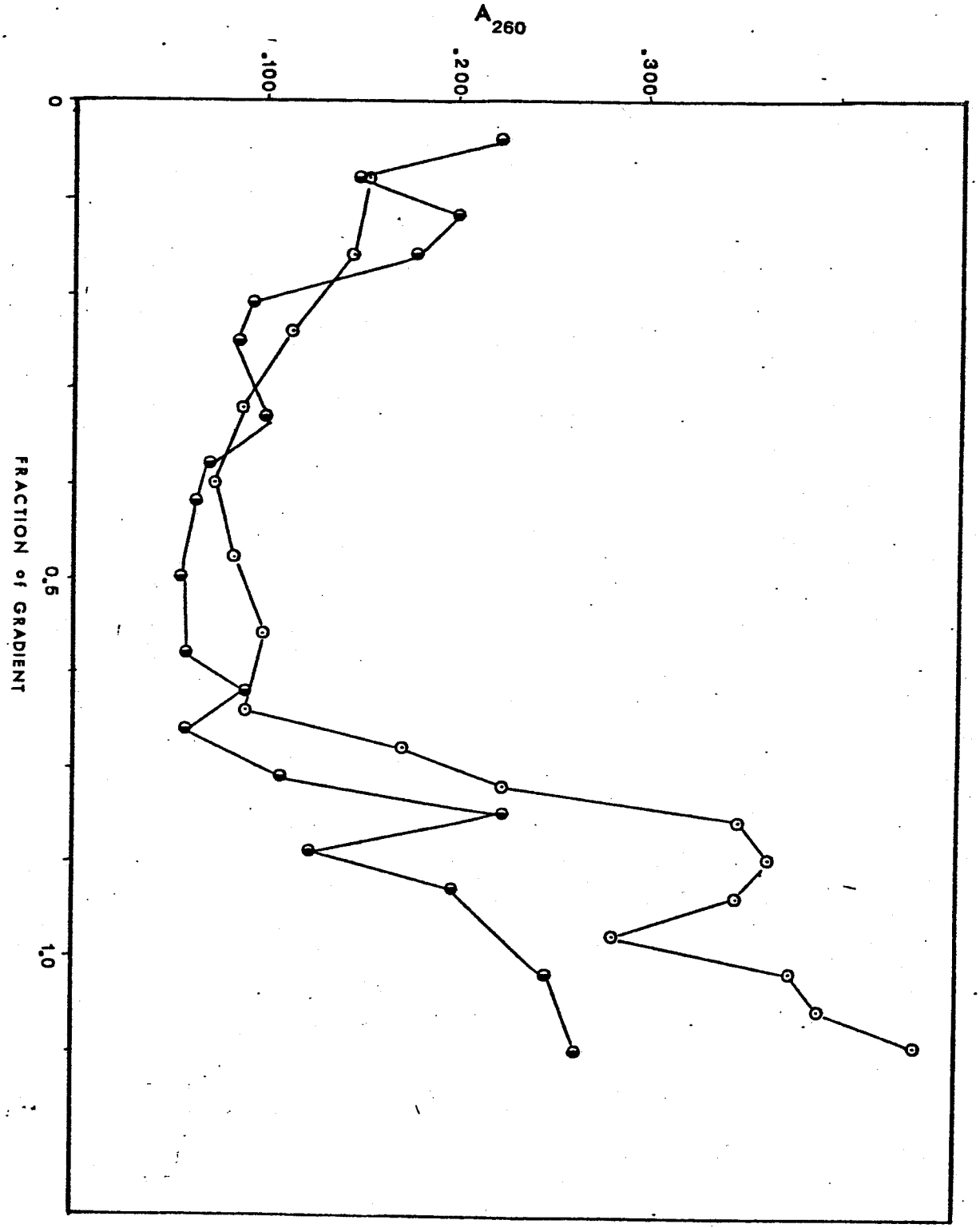

A_{260}
.100
.200
.300
0
0.5
1.0
FRACTION of GRADIENT

ILLUSTRATION 1

Diagram of DNA banding in agarose gels. Samples of DNA were treated and loaded on the agarose gels as described in Methods. The diagram shows flourescent bands on the gels.

1. $\lambda\, c_1L$ strands.
2. Annealed DNA heteroduplex, $\lambda\, c_1L$galR
3. Annealed DNA homoduplex, $\lambda\, c_1Lc_1R$
4. Circles λc_1LgalR
5. $\lambda\, c_1L$ strands
6. Annealed DNA heteroduplex, $\lambda\, c_1L$galR
7. Annealed DNA homoduplex, $\lambda\, c_1Lc_1R$
8. Circles λc_1LgalR

Samples 1 - 4 were denatured before loaded.

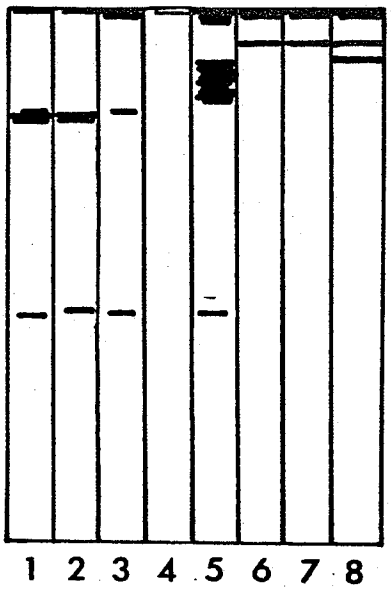
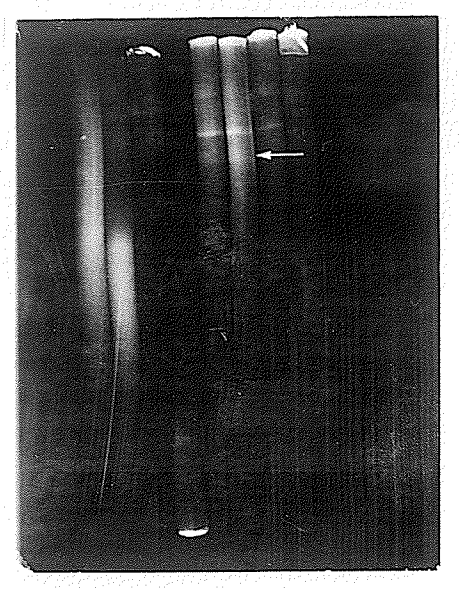
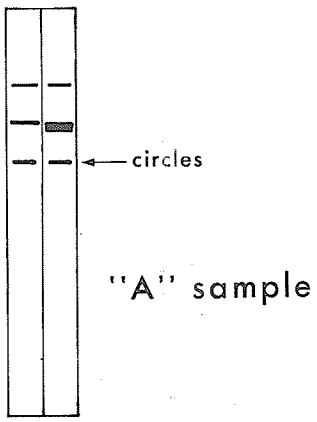

1 2 3 4 5 6 7 8

ILLUSTRATION 2

Photograph of agarose gel bands. The gels were photographed in the presence of UV light after staining with ethidium bromide. Sample "A" has an extra band of DNA, which corresponds to circular DNA.





"A" sample

DISCUSSION

DISCUSSION

The approaches proposed for sequencing the λ attachment site were theoretically feasible and employed techniques which were currently available for nucleic acid manipulation when the work commenced. The basic shortcoming of both approaches lay in the large number of manipulations necessary before the stage of sequence analysis was reached.

As outlined in Figure 23, the primary approach involved cleavage of the single strand regions of heteroduplexes containing non base pairing regions adjacent to the attachment site followed by repair elongation of the DNA fragments. The concept while simple, involves a large number of steps starting with the growth of three different phage stocks, continuing through strand separations, annealings, enzyme digestions, further annealings, repair and finally sequence analysis of the repaired fragments. Losses were inevitable at each stage and even small losses are quickly magnified in a series of consecutive manipulations.

The second approach, which was effectively a modification of the first, also involved nuclease cleavage of the nonbase paired regions of heteroduplexes, but necessitated the formation of DNA circles which have never been produced in high yield. It was thought that a lower yield would be acceptable since the subsequent labelling with polynucleotide kinase and sequence analysis would be simpler than the repair sequence method. However, there proved to be such a large loss at this stage that the procedure proved to be impractical.

This thesis describes the optimization of the various steps involved in the sequencing protocol. In many cases, techniques were employed directly with little modification from the literature. In other cases, the published procedures did not work as described, resulting in low yields or else the protocol was inappropriate for use with the large amounts of material necessary for sequence analysis. In these cases, extensive modification of the procedures were required.

Even the very first step of phage stock preparation was not trouble free, and necessitated fairly extensive searches for better growth procedures and mutant selection. The published procedures for phage preparation are not particularly detailed and since good stocks of phage were required, considerable work was done optimizing the phage yield. Such things as Difco yeast extract, host specificity, multiplicity of infection and preadsorption of the phage to bacteria were all found to affect the yield. Ultimately, yields of 10^{13} -10^{15} phage per ml were obtained for $\lambda c_1 857S7$.

The λgal presented different problems from those found in the λc_1 preparations. In spite of higher Mg^{++}ion concentration and the use of DNase and chloroform to break up cell debris, yields of only 10^{11} phage per ml were achieved. The problem was finally traced to λ gal S7 in fact not containing the amber mutation (S7) which consequently allowed leakage of the phage. This was not discovered initially because most of the available host strains were either suppressors or leaky and only the arrival of a suppressor free strain solved the problem. Ultimately, using λgal the yields were also about 10^{11} phage per ml, which made it necessary to continually restock λgal.

One of the peripheral studies generated by the gal growth problem was several genetic experiments to obtain a lysogen. Several procedures were tried which were unsuccessful. One complication to the work was the need for nonaggressive procedures, as secondary mutations were undesirable. Consequently, selection and enrichment procedures were the only approaches possible. The difficulty with these methods was the need for screening large populations in order to compensate for the low natural mutation rate[133]. Using selective or differential media helped to locate potential lysogens.

The next step in the protocol was the strand separation for which the procedures had been published. No problems were found except that the isolated strands had a definite shelf life of several weeks after which fragmentation had occurred. This necessitated regular strand separation of both phage types involving 48 hours CsCl centrifugations.

Subsequent reannealing of strands to form the heteroduplexes also involved optimization. The high renaturation temperature of 65°C lowered the pH of tric-HCl pH7.6 buffer sufficiently that fragmentation of the DNA occurred. The use of sodium glycinate buffer pH9.2 eliminated this problem. This effect could be due to the presence of a small amount of contaminating nuclease which only becomes evident at extremely low concentration. Alternatively a critical ratio of enzyme and substrate could be essential to enzyme action. Another critical factor was the reaction pH since the low pH of 4.5 to 5.0 required for nuclease action would possibly cause depurination of the DNA. Fortunately, short incubation times were sufficient for nuclease action without unwanted side reactions.

Following nuclease digestion, the DNA fragments had to be purified by alkaline sucrose gradients and significant losses occurred. Pretreatment of the polyallomer tubes with a mixture of DNA-EDTA-PPi as well as using larger amounts of DNA per gradient seemed to overcome this problem.

Had the nuclease created butt-ended DNA fragments? Preliminary experiments using DNA repair suggested that in fact this was the case and while this work was in progress, a report appeared in the literature[128] confirming this conclusion. As a result, no further work was carried out on this problem.

Following another annealing step, the final step in the protocol was the DNA polymerase I catalyzed repair reaction. Only preliminary experiments were carried out at this stage. The low yields and problems in earlier steps made it very difficult to arrive at the final step with sufficient material to carry out comprehensive experiments.

This was the rationale for attempting the second approach using polynucleotide kinase. The kinase has the advantage of involving just one reaction and very high specific activity [γ-^{32}p] ATP was available which would make large amounts of substrate less necessary.

The major problem encountered here was the very poor yield of circles from linear DNA. Yields of 10-30% compare favorably with the maximum literature yield of 50%[129]. Isolation of the circles from linear DNA proved to be an even greater problem. Alkaline sucrose gradients were unsatisfactory while agarose gel provided good resolution of the two fractions. S1 nuclease treatment either produced fragments which masked the circular DNA or fragmented in circles.

Therefore, isolation of the circles prior to S_1 treatment was necessary. Attempted recovery of the circular DNA from the gels proved to be the major block, since yields of only 10-20% were obtained. This meant that for every OD unit of annealed heteroduplexes, only 0.20 D unit formed circles and 80% of this was lost on the gel leaving only 0.04 OD unit for the kinase treatment. This was a much lower yield than had been anticipated and forced the abandonment of the project.

In summary then, the major problem with the repair protocol was simply time. Yields were reasonable but there were so many manipulations that the final yield of DNA was invariably low. As a result, a continuous cycle of phage growth, strand separations and DNA annealings had to be maintained. Any interruption even for a few days disrupted the whole protocol. This protocol can be viewed as pyramid with the continuous necessity for phage preparation at the base.

The second protocol had similar disadvantages but the major block was the low yields at the circularization stage.

Since this work has been completed, the sequence of the attachment site has been determined. In an elegant series of reports[134,35,136] deletion mutants and subsequently isogenic strains were used to locate unique fragments of DNA. The various strains of DNA were subjected to digestion by restriction enzymes. The unique fragments isolated were then analysed by the recently developed sequencing method of Maxam and Gilbert[90]. This method involved partial cleavage of DNA at specific nucleotide residues followed by complete cleavage of the DNA backbone. The fragments generated are isolated and analysed using acrylamide slab gels.

The sequence determined showed a common core region in the att site of 15 bases. This area could undergo a staggered cut during site specific recombination as suggested previously[59,60]. Alternatively, the OP' arm has some special sequence patterns which could be significant.

Further work in this area will no doubt clarify the nature of site specific recombination.

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