

THE USE OF AGAROSE GEL ELECTROPHORESIS  
TO MEASURE THE SIZE OF DNA MOLECULES  
IN CRUDE CELL LYSATES

by

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M. Sc. Thesis

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ABSTRACT

In this thesis, horizontal slab gel electrophoresis is presented as an alternative to alkaline sucrose gradients for the detection and sizing of Okazaki fragments. When CV-1 cells were uniformly labeled with  $^{14}\text{C}$ -labeled thymidine, pulse-labeled for 30 seconds with  $[^3\text{H}]$ thymidine, lysed and run on 0.7% agarose gels, the large  $^{14}\text{C}$ -labeled bulk was found in a peak at the top of the gel, whereas the  $^3\text{H}$  was found in small molecular weight DNA at the gel bottom. This  $^3\text{H}$  peak representing Okazaki fragments comigrated with the bulk  $^{14}\text{C}$  label after pulse-chasing. Agarose gels thus display the typical pulse and pulse-chase characteristics of Okazaki fragments and can be used to separate Okazaki fragments from bulk following direct lysis of cells. Okazaki fragments released by direct lysis of cells and those obtained by various methods of purification were compared electrophoretically on 0.7% agarose gels and found to be similar in migration pattern. When the Okazaki fragments from the purified and crude lysate procedures were sized on agarose gels using Hind III digested SV40 as a marker, molecular weights of  $2.15 \times 10^5$ , and  $2.3 \times 10^5$  were determined for the fragments respectively.

L'UTILISATION DE L'ELECTROPHORESE SUR GEL D'AGAROSE POUR DETERMINER  
LA TAILLE DES MOLECULES D'ADN DANS LES LYSATS DE CELLULES

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RESUME

Dans cette thèse l'électrophorèse sur gel horizontal est présentée comme une alternative aux gradients alcalins de sucrose, dans le but de détecter et la déterminer la taille des fragments d'Okazaki. Lorsque les cellules CV-1 uniformément marquées sont alors pulsées à la thymidine  $^3\text{H}$  pour 30 secondes, puis lysées et déposées sur des gels de 0.7% agarose, nous avons constaté que la plus grande partie de  $^{14}\text{C}$  est restée à la base du gel, tandis que le  $^3\text{H}$  est retrouvé au front, dans l'ADN à faible poids moléculaire; celui-ci, représentant les fragments d'Okazaki. Après le pulse-chase tout le matériel tritié migre avec le  $^{14}\text{C}$  - les gels d'agarose montrent ainsi les caractéristiques particulières des fragments d'Okazaki lors du pulse et du pulse-chase. Les gels peuvent être utilisés pour les fragments d'Okazaki à partir d'extraits cellulaires bruts, directement après la lyse des cellules - les fragments d'Okazaki isolés à partir de lysats de cellules et ceux obtenus par d'autres méthodes de purification ont été comparés sur des gels de 0.7% d'agarose. Le mode de migration est similaire - les poids moléculaires, mesurés à partir de SV40 digéré par Hind III utilisé comme marqueur, sont de  $2.15 \times 10^5$  pour les fragments purifiés et de  $2.3 \times 10^5$  pour les fragments provenant de lysats.

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## INTRODUCTION:

The purpose of the experiments in this thesis is the development of a convenient, accurate, and reproducible technique to analyze DNA replication events in mammalian cells that can replace alkaline sucrose gradient analysis. In alkaline sucrose gradient analysis, one establishes a density gradient along the length of a centrifuge tube, layers a dilute solution of DNA on top of the gradient, and centrifuges the tubes at high speeds until the molecules of interest have sedimented part way along the length of the tube. In this way, molecules are separated according to their rate of sedimentation, which is expressed as the sedimentation coefficient  $s$ .

$s = \frac{dx}{dt}$ , where  $x$  is the distance from the centre of

$$w^2x$$

rotation,  $w$  the angular velocity in radians per second, and  $t$  the time in seconds. A sedimentation coefficient of  $1 \times 10^{-13}$  seconds is called a Svedberg unit, or simply a Svedberg, abbreviated S. The sedimentation coefficient is a function of both the weight and shape of the molecule in solution, and the distribution of S reflects the distribution of molecular sizes or states in a population. Alkaline sucrose gradient centrifugation allows convection-free sedimentation at very low DNA concentrations, and a wide variety of solvents can be employed (Studier, 1965). DNA labeled by extremely short pulses of  $[^3H]$ thymidine is partly in the form of molecules much shorter than bulk DNA, and migrates only a short distance into the gradient. This peak represents chains that initiated during the pulse (Okazaki fragments).

In this thesis, horizontal slab gel electrophoresis is presented as a useful alternative for studies of this type. It offers excellent resolution for DNA of molecular weights up to at least  $26.5 \times 10^6$ , with differences of

less than 10% molecular weight being readily resolved. Gel electrophoresis separates DNA according to length. The logarithms of known molecular weights when plotted against relative mobility in gels, give a linear plot by which other molecular weights can be accurately determined (McDonnell et al., 1977). When compared to the vertical slab gel system, the horizontal slab gel used in these experiments offers greater flexibility in gel handling for drying and staining procedures. Unlike tube gels, it permits the comparison of multiple samples on the same gel. Most important, gel electrophoresis overcomes some of the difficulties encountered in alkaline sucrose gradient work. In sucrose gradient centrifugation, there is difficulty in separating, and hence sizing, very small amounts of short chain DNA from the trailing edge of the great excess of larger DNA. However, with gel electrophoresis, small molecules move faster than large, and therefore it should be possible to separate short DNA chains free from contamination by larger molecular weight species even though the latter constitute the bulk of the DNA. Also, when cells are lysed on alkaline sucrose gradients, an artifact is seen that mimics the sedimentation properties of Okazaki fragments. The artifact is a small fraction of unincorporated radioactive precursor which coprecipitates with acid insoluble contaminating cellular proteins (Edenberg and Huberman, 1975).

The results of my experiments show that a gel electrophoresis system could be used as an alternative to alkaline sucrose gradient analysis for DNA replication events in mammalian cells. The slab gels display the conventional pulse and pulse-chase characteristics of Okazaki fragments. They can be used to separate Okazaki fragments from bulk DNA and also provide an accurate means of sizing DNA.

In this thesis, I will review the mechanism of discontinuous synthesis of DNA and then present my experimental results.

### Discontinuous Synthesis of DNA

The mechanism of DNA chain growth at the replication fork has long been a subject of interest. Early work by Meselson and Stahl (1958) indicated that replication of the E. coli bacterial chromosome proceeds sequentially. This led to the inference that both daughter strands of chromosomal DNA grow continuously, the direction of synthesis being  $5' \rightarrow 3'$  on one strand, and  $3' \rightarrow 5'$  on the other. This presents a fundamental problem: no known DNA polymerase can synthesize in the  $3' \rightarrow 5'$  direction i.e. add nucleotides to the 5' end of the chain (Kornberg, 1960). The work of Okazaki (to be discussed in detail below), using a prokaryotic system, provided a model whereby the above difficulty could be avoided. He suggested that DNA was synthesized in vivo by a discontinuous method. This model assumes that in either one or both template strands, short stretches of DNA (generally called Okazaki fragments) are synthesized in the  $5' \rightarrow 3'$  direction, and then linked together so that the overall direction of synthesis appears to be  $3' \rightarrow 5'$ .

From this model of discontinuous replication, 2 predictions were made which provide the basis for much of the future work done in this area. These are:

- 1) The most recently replicated portion of 1 or both daughter strands can be isolated after denaturation as short DNA chains distinct from large DNA molecules derived from the rest of the chromosomes. No such difference in molecular size would be expected from a mechanism of continuous synthesis.

- 2) Selective and temporal inhibition of the enzyme for the formation of phosphodiester linkages between DNA chains will result in a marked accumulation of the nascent short chains. (Okazaki et al., 1968a).

To clarify the mechanism of DNA chain growth (i.e. continuous or discontinuous), it is necessary to determine the structure of the most recently replicated portion of the chromosome; that is the portion selectively labeled by an extremely short radioactive pulse (Okazaki et al., 1968b). Sakabe and Okazaki (1966) uniformly labeled E. coli 15T cells with [ $^{14}\text{C}$ ]thymidine for several generations, and then, after a brief period of thymidine starvation, pulse-labeled these cells with [ $^3\text{H}$ ]thymidine for 10 seconds at 20°C. The extracted DNA was denatured in alkali and subjected to preparative zone sedimentation in an alkaline sucrose gradient. Analysis of the radioactivity showed that while  $^{14}\text{C}$ -labeled DNA was found almost exclusively at the bottom of the centrifuge tube, most of the  $^3\text{H}$  was recovered in a slowly sedimenting band. The average sedimentation coefficients of  $^{14}\text{C}$  and  $^3\text{H}$ -labeled DNA in their denatured conformations were 40S and 7S respectively. When the 10 second pulse-labeling was followed by a 2 minute chase with unlabeled thymidine, the  $^3\text{H}$  band moved towards the bottom of the tube, and little difference was found between the distributions of  $^{14}\text{C}$  and  $^3\text{H}$ -labeled DNAs after a 20 minute chase. Therefore,  $^3\text{H}$ -labeled DNA from the pulse-labeled cells is characteristic of the replicating region of the chromosome. Its sedimentation pattern bears out the first prediction made from the model of discontinuous replication, whereby short stretches of DNA are synthesized by a 5'  $\rightarrow$  3' reaction at the replicating point, and then subsequently connected to the growing strand.

Testing of the second prediction was made possible by the identification of two T4 phage mutants, temperature sensitive for the formation of the enzyme polynucleotide ligase. When E. coli B/5 cells, infected at the permissive temperature with mutant T4tsA80, were shifted to

the non permissive temperature, and pulse-labeled with ( $^3\text{H}$ )thymidine for 40 - 60 seconds, the majority of radioactivity was found in short DNA chains, with little  $^3\text{H}$  appearing in the fast sedimenting material. When the same experiment was performed using wild type phage, most of the radioactivity was in larger DNA chains with a sedimentation coefficient of greater than 30S. Thus, in the temperature sensitive mutants, the short DNA chains are produced but their joining is inhibited almost completely (Okazaki et al., 1968a). Therefore, Okazaki's results are in accordance with the idea that in prokaryotes, DNA replicates in vivo by a discontinuous mechanism.

Current evidence suggests that a similar process occurs in eukaryotes (Schandl and Taylor, 1970; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). However, discontinuous replication needs frequent initiation of synthesis of new chains, and no known DNA polymerase can carry out de novo chain initiation. Present evidence suggests the existence of an RNA primer that can be extended by a DNA polymerase. Schematically the succession of events leading to chain growth are: 1) initiation of new fragments by an RNA polymerase; 2) elongation of the initiating RNA strand by a DNA polymerase; 3) removal of the RNA, possibly by an RNase-H; 4) elongation of the "old" DNA strand by a gap-filling DNA polymerase; 5) ligation of the final phosphodiester bond by a DNA ligase (Pigiet et al., 1974).

#### Formation of Replication Intermediates

In spite of the general acceptance of the above scheme, it is necessary to note other possible mechanisms which could lead to the formation of replication intermediates. These include:

- 1) endonuclease cleavage of newly synthesized DNA during the isolation

procedure. The work of Blumenthal and Clark (1977a), using *Drosophila* embryo DNA, does not support this mechanism. Using a variety of stringent cell lysis conditions, they produced the same size-distribution of replication intermediates on sedimentation velocity gradients. They also assessed endonuclease activity (assayed by the nicking of SV40 form I) and found it to be negligible under their experimental conditions. Okazaki et al. (1968a) have also obtained similar results when they used different experimental procedures in a number of different systems (including an endonuclease I-deficient *E. coli* strain). It was also shown that degradation of large DNA molecules is not responsible for the small DNA molecules isolated. The behaviour of large DNA molecules was observed on alkaline sucrose gradients along with lysates containing DNA pulse-labeled with [<sup>3</sup>H]thymidine for 30 seconds. Approximately 11% of the uniformly labeled <sup>14</sup>C DNA sedimented in the 0-10S region along with 60% of the <sup>3</sup>H counts. Subjecting the lysate to high shear force didn't affect the percentage of <sup>14</sup>C DNA molecules in the 0-10S region (Blumenthal and Clark, 1977b). On the other hand in support of the endonuclease hypothesis, Lehmann and Ormerod (1969) have stated that there is no need to invoke any special intermediate in DNA replication in order to explain the small and intermediate sized-DNA observed after a pulse-label. They have shown that when DNA radioactively labeled at the end(s) (as expected for a pulse-label) is sheared randomly, the pulse-label appears to be of lower molecular weight than the bulk of the DNA. This artifact, created when pulse-labeled DNA is sheared, would also mimic the behaviour of the intermediate sized-DNA in that the pulse-label would appear to move to bulk size upon further incubation or upon chasing.

2) Replication intermediates could be formed by a DNA repair process. Tye et al. (1977) have proposed that incorporation of uracil bases into newly replicated DNA, in place of thymine bases, caused the accumulation of 4-5S pieces in bacterial cells. The repair of the incorporated uracil bases could result in endonuclease -- or alkali-catalyzed cleavage of phosphodiester bonds and in the transient production of fragments from newly synthesized DNA. Lynch et al. (1972) do not exclude the possibility that the elongation of DNA chains in their in vitro system is a repair, rather than a replication process. They suggested that the DNA which is newly formed, but not the bulk of the DNA, may be susceptible to nucleases that act during isolation or incubation of the nuclei to produce new points of initiation for deoxyribonucleotide incorporation.

#### Okazaki Fragment Size

Detection of Okazaki fragments and measurement of their size are subject to many problems. Some studies have failed to detect Okazaki fragments as intermediates in DNA synthesis (Lehmann and Ormerod, 1969; Lynch et al., 1972). Still others, have detected incorporation of pulse-label mostly into DNA strands much greater in size and longer lived than the short strands of Okazaki (Hyodo et al., 1970; Berger and Irvin, 1970). However, most recent studies have reported Okazaki fragments in mammalian cells. Some of these have been conducted in vivo (Schandl and Taylor, 1970; Nuzzo et al., 1970; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). In vitro work was done by Kidwell and Mueller (1969) and Friedman (1974), who demonstrated Okazaki fragments in the nuclei of S phase HeLa cells, whereas Hershey and Taylor (1974) isolated fragments from S phase CHO nuclei. Okazaki fragments have also been detected in Polyoma-infected cells both in vivo and in vitro (Magnussen et al., 1973;



Pigiet et al., 1973; Pigiet et al., 1974; Francke and Hunter, 1974; Hunter and Francke, 1974), and in SV40 infected cells (Fareed and Salzman, 1972; Salzman et al., 1973). In the replication of these viruses in infected cells, only one DNA function is believed to be virus-specified, the initiation of synthesis at the genome origin. All subsequent DNA synthetic events, including Okazaki piece synthesis, most probably are the same as in uninfected cells.

A great variation in sizes has been reported for Okazaki fragments. Some have described fragments of 4-5S (Nuzzo et al., 1970; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975). Others have estimated 5-10S (Schandl and Taylor, 1969; Schandl and Taylor, 1970) and 10S (Kidwell and Mueller, 1969; Goldstein and Rutman, 1973). There have also been reports of replication intermediates formed in discrete size classes which eventually give rise to high molecular weight DNA (Kidwell and Mueller, 1969; Hyodo et al., 1970). Blumenthal and Clark (1977b) isolated 3 discrete size classes of 61, 125 (3.8S) and 240 (4.8S) nucleotides in *Drosophila* DNA.

Much of the sizing work has been done with Polyoma and SV-40, whose replicating molecules can be isolated and purified. The replication intermediates consist of 1) covalently closed parental strands with superhelical turns in the non-replicated regions and 2) linear daughter strands hydrogen bonded to the parental strands. DNA replication in both viruses occurs by a discontinuous mechanism. Chain elongation involves the synthesis of short fragments (approximately 4S) as intermediates (Magnussen, 1973a and 1973b; Pigiet et al., 1973; Salzman and Thoren, 1973). Therefore the size of the viral Okazaki fragments closely agrees with the measurements of Gautschi and Clarkson (1975); Tseng and Gouliau (1975a) and Fox et al., (1975) for mammalian cells. Gautschi and Clarkson (1975) found replication

intermediates of 4S whether they pulsed for 15 seconds at 37°C or 60 seconds at 25°C, showing that Okazaki fragment size was independent of temperature. Thus the shortest strands formed during DNA replication in mammalian cells  $5 \times 10^4$  MW are 10-20 times shorter than Okazaki fragments of prokaryotes, which are about  $5 \times 10^5$  MW (Okazaki et al., 1968a).

#### Okazaki Fragments: Kinetics of Synthesis and Ligation

The kinetics of synthesis and ligation of Okazaki fragments are difficult to study. In vivo, Okazaki fragments are synthesized rapidly compared to the time to saturate the internal pools of deoxynucleoside triphosphates during very short pulses (Francke and Hunter, 1974). Also, in vivo studies on mammalian cells have shown extremely rapid labeling of Okazaki fragments. Gautschi and Clarkson (1975) state that the longest incubation time that can be used to label fragments only, is about 30 seconds at 25°C. After this, the peak of radioactive profiles is shifted to higher sedimentation values.

When DNA synthesis in SV40 was studied using brief pulses, a bimodal distribution of the newly synthesized DNA was observed on alkaline sucrose gradients. There was one well defined 4S peak and the remaining radioactivity sedimented in the 6-16S region. After a 2 minute chase, almost no 4S was detectable (Fareed and Salzman, 1972). Blumenthal and Clark (1977a) showed that a 10 second pulse-label in *Drosophila* cells resulted in  $\frac{1}{4}$  or greater of the  $^3\text{H}$  counts in molecules greater than 16S. Tseng and Goulian (1975a) estimate that the in vivo lifetime of Okazaki fragments in human lymphocytes is about 2-4 seconds, since only 10% of the label after 20-40 seconds of labeling is in the Okazaki fragment range.

Many in vivo and in vitro studies have indicated that the fragments are ligated to form high molecular weight DNA (Painter and Schaeffer, 1969;

Hunter and Francke, 1974; Tseng and Goulian, 1975a). Kidwell and Mueller (1969) isolated 10 and 24S DNA segments, in addition to 40S non replicating bulk in their in vitro system, after a short pulse. Pulse-chase experiments indicated that the 10S was a precursor of the 24S product. In vitro studies have proven useful in this area, since there is rapid equilibration of added label, and the rate of [ $^3\text{H}$ ]thymidine incorporation is about 12% the in vivo rate (Tseng and Goulian, 1975a). Huberman and Horwitz (1973) showed that the shortest strands synthesized in a 30 second pulse at 37°C formed a somewhat heterogeneous but distinct peak of 100 nucleotides. The position of this peak did not vary significantly for times ranging from 10-90 seconds. This suggests the existence of a build up of completed fragments which are subsequently joined rapidly onto strands of much greater length. Okazaki et al. (1968a) infected E. coli cells with phage T4 mutant, temperature sensitive for ligase formation. At the non permissive temperature, little radioactivity was found in fast sedimenting material even after pulse-labeling of up to 60 seconds duration. Magnusson (1973a and 1973b) treated Polyoma infected cells with hydroxyurea (HU) and found an accumulation of newly synthesized 4S fragments. Similar fragments were found upon incubation of nuclei isolated from Polyoma infected cells and were shown to be intermediates in replication. This accumulation of short fragments in the presence of HU might be caused by a preferential inhibition of one of the reactions involved in the joining of the fragments, a gap filling DNA polymerase being a possible target. Similar findings were reported by Laipis and Levine (1973), and Salzman and Thoren (1973), for SV40 cells treated with HU and 5-fluorodeoxyuridine (FdUrd) respectively.

Kidwell and Mueller (1969) and Friedman (1974) examined the role of ATP in DNA synthesis. Both found an accumulation of short fragments when ATP

was omitted from their in vitro system. Its absence may have inhibited the ligation step.

Okazaki Fragments: Semi or Totally Discontinuous Synthesis?

Studies to determine whether or not there is discontinuous synthesis on one or both strands of DNA have yielded conflicting results. Two approaches are generally used for investigation. 1) One approach is a kinetic one. If synthesis were totally discontinuous (both strands synthesized discontinuously), a kinetic study would show more label in Okazaki fragments than in longer strands at short pulse-labeling times; if semi-discontinuous (only one strand synthesized discontinuously), there should be the same amount of label in both classes at short times. On this basis, many in vivo and in vitro studies have been interpreted as showing totally discontinuous synthesis. Early work by Okazaki et al. (1968a) showed that for phage infected E. coli, virtually all radioactive label was found in short chains after an extremely short pulse. Huberman and Horwitz (1973) demonstrated the same in CHO cells. Gautschi and Clarkson (1975) have presented evidence using both alkaline sucrose gradients and agarose gel electrophoresis which showed that in mouse P-815 cells, all incorporation was into Okazaki fragments at the shortest pulse time. Magnussen (1973a and 1973b) and Salzman and Thoren (1973) treated pulse-labeled Polyoma and SV40 infected cells with HU and FdUrd respectively. In both systems, there was an accumulation of only 4S fragments. In contrast, Francke and Hunter (1974) demonstrated semi-discontinuous synthesis in Polyoma. Hershey and Taylor (1974) proposed semi-discontinuous replication in their S phase CHO nuclei. 2) A second potentially more reliable approach to this question is to determine whether the Okazaki fragments can anneal either to each other, or to specific template regions from both sides of each of the two growing forks.

If synthesis were totally discontinuous, one should observe either extensive self annealing of purified Okazaki fragments, or equal hybridization of Okazaki fragments to regions from both sides of the replication forks depending on the method used.

Both methods have yielded conflicting results. Perlman and Huberman (1977) hybridized Okazaki pieces and intermediate-sized DNA from SV40 infected monkey cells to the separated strands of two SV40 DNA restriction fragments, one lying to either side of the origin of bidirectional DNA replication. They found that 5 fold more pieces hybridized to the 3'→5' DNA strand than to the 5'→3' strand. A reverse asymmetry was detected with respect to the hybridization of intermediate-sized DNA. The simplest interpretation of the results is that SV40 is synthesized semi-discontinuously. By comparison, Fareed, Khoury and Salzman (1973), using the method of self annealment, reported that nascent 4S strands isolated from replicating SV40 self anneal extensively (70-92%), indicating discontinuous synthesis at both strands. Similarly, for Polyoma, Pigiet et al. (1973) reported discontinuous synthesis of both strands after witnessing that the Okazaki fragments reannealed with an efficiency of 80-90%. Hunter et al. (1977) reported semi-discontinuous synthesis for Polyoma, after observing 4 fold more Okazaki pieces hybridized to the 3'→5' strand, than to the 5'→3' strand of the Polyoma DNA template.

The ambiguities in the above results may be in part attributed to the many difficulties encountered in a study such as this. The kinetic approach may give misleading results if excess Okazaki fragments are synthesized and then destroyed or if the deoxyribonucleoside triphosphate pools on each side of the fork equilibrated with exogenous nucleosides at different rates (Edenberg and Huberman, 1975). A source of error in the determination of

self complementarity might be a contamination by degraded Polyoma-specific mRNA. Such a contamination would probably result in an overestimation of the amount of self-complementarity (Magnusson, 1973a). Also, if the same DNA sequence were synthesized by forks moving in different directions in different molecules, the resulting Okazaki fragments would self-anneal even if synthesis were semi-discontinuous (Edenberg and Huberman, 1975). Lastly, Francke and Vogt (1975) found high self annealing values for Polyoma if virus stocks generating defective DNA were used for infection. They also pointed out that there was more self annealing in the larger Okazaki fragments than in the smaller ones and they argued that this may have been due to contamination of the Okazaki fragments by short continuously synthesized strands.

#### Okazaki Fragments: Destabilized Nature of the Replication Fork

Nascent DNA differs in several ways from the bulk of the DNA. Friedman and Mueller (1968) stated that replicating DNA differed from non replicating DNA by its partition to the interphase during extraction with phenol or chloroform. They also found that the replicating DNA floated in CsCl gradients. This is a property of single-stranded DNA. On neutral sucrose gradients, however, it separated into 2 fractions. One behaved as rapidly sedimenting material, and the other as slowly sedimenting material. Okazaki et al. (1968b) suggested a unique state during replication to explain these sedimentation characteristics, whereby the secondary structure of the replicating region containing these newly formed units is abnormally unstable. It may also indicate functioning of the newly synthesized units or the complementary portions of the parental strands as templates for RNA synthesis.

A large but variable proportion of nascent DNA can sometimes be extracted in single-stranded form (Schandl and Taylor, 1969; Fox et al., 1973) or in partially single-stranded form (Berger and Irvin, 1970). Tsukada et al. (1968) found newly replicated DNA extracted from rat liver nuclei at 0°C entirely double-stranded. Habener et al. (1970) extracted DNA by a variety of methods from intact HeLa cells pulse-labeled and examined for the presence of single and double-strandedness. Like Okazaki, they suggested that a portion of the newly replicated DNA is present in the nucleus not as free single strands but in a "destablized" state and that depending on the conditions of extraction, it can be converted to either the single-stranded or double-stranded form. Nuzzo et al. (1970) confirmed this finding. They found that with mild extraction procedures, there was extensive degradation of the DNA, as judged by the sedimentation coefficient of the bulk DNA. When more drastic extraction procedures were used there was less evidence of degradation.

The significance of these findings is not clear. Some single-stranded DNA is expected at the replication fork where Okazaki fragments have not yet been synthesized. Blumenthal and Clark (1977b) showed this with electron microscopic studies of replication forks in *Drosophila* DNA.

#### Okazaki Fragments: RNA Priming

Discontinuous replication needs frequent initiation of synthesis of new chains. As mentioned, the known mammalian DNA polymerases cannot carry out de novo chain initiation. However, they can extend certain synthetic primers, and the  $\alpha$ -polymerase can extend natural primers (Spadari and Weissbach, 1975). Therefore, one solution to the problem of initiating Okazaki fragments is for an RNA polymerase to synthesize a short stretch of RNA that serves as a primer for a DNA polymerase (Sugino et al., 1972).

In this area of study there have also been conflicting results. Evidence consistent with the idea that RNA primes Okazaki fragments has been reported in Polyoma. Short fragments of DNA isolated from Polyoma replication-intermediates pulse-labeled in vitro were shown to have RNA covalently attached by 3 criteria: 1) Such fragments were slightly denser than bulk viral DNA. Isolated Polyoma replication-intermediates banded at densities slightly higher than DNA in neutral  $\text{Cs}_2\text{SO}_4$  gradients. Such a shift didn't occur after treatment with alkali (Magnusson et al., 1973; Hunter and Francke, 1974). The 4-5S pieces as opposed to the longer pieces were mainly responsible for the observed asymmetry on the gradient. 2) They could be labeled with  $\alpha$ - $^{32}\text{P}$  labeled ribotriphosphates, thereby demonstrating the presence of RNA directly. This fraction of  $^{32}\text{P}$  radioactivity is reproducibly found at DNA density on  $\text{Cs}_2\text{SO}_4$  gradients (Hunter and Francke, 1974; Tseng and Goulian, 1977). 3) A modified nearest neighbor analysis experiment showed that when Okazaki fragments were labeled with  $\alpha$ - $^{32}\text{P}$  deoxyribonucleoside triphosphates, purified and treated with alkali to convert the RNA to 2'(3') ribonucleoside monophosphates, a small proportion of the incorporated  $^{32}\text{P}$  was transferred to the ribonucleotides, indicating the presence of covalent phosphodiester linkages between ribonucleotides and deoxyribonucleotides. The  $^{32}\text{P}$  transfer occurred primarily from Okazaki fragments with a frequency consistent with one RNA-DNA link at the 5' end of the DNA. When the link was examined for base specificity, all 4 common ribo- and deoxyribonucleotides were present with close to equal frequency (Hunter and Francke, 1974; Magnusson et al., 1973; Tseng and Goulian, 1975b and 1977).



Kaufmann et al. (1977) examined nascent DNA chains of less than 200 nucleotides in the cell free SV40 DNA replication system. They identified RNA-DNA covalent linkages using  $^{32}\text{P}$  transfer experiments, as defined above. Potassium iodide gradient centrifugation showed that only a portion of the Okazaki pieces appeared to contain RNA, whereas the remaining pieces contained only DNA. They estimated the size of the RNA to be at least 7 residues. In further experiments with this system, analysis of Okazaki pieces recovered from hybrids with various Hind II and Hind III restriction fragments of SV40 DNA showed that rN-P-dN sequences were uniformly distributed around SV40 (RI) DNA. Therefore, most if not all of the RNA primers served to initiate Okazaki pieces rather than DNA replication at the origin of the genome.

There is other evidence implicating an RNA primer. Alkaline sucrose gradient centrifugation revealed that ribonucleoside triphosphate specifically increased the formation of short chains after five minutes of incubation. This suggests that ribonucleotides are involved in the formation of short chains (Magnusson et al., 1973). Tseng and Goulian (1975a) have also witnessed this stimulatory effect. Similarly, the RNA-DNA junctions were lost during chases at a rate similar to the chasing of Okazaki fragments into DNA (Tseng and Goulian, 1977). Magnusson et al. (1973) have also witnessed this transient association of RNA with nascent DNA.

Neubort and Bases (1974) demonstrated covalently linked RNA-DNA complexes in HeLa cell cultures. In mouse P-815 cells, no evidence for ribonucleotides at the 5' end of Okazaki fragments was obtained either in isopycnic  $\text{CsCl}$  or  $\text{Cs}_2\text{SO}_4$  gradients or after incubation with polynucleotide kinase and [ $\gamma\text{-}^{32}\text{P}$ ] ATP (Gautschi and Clarkson, 1975).

Reichard et al. (1974) and Tseng and Goulian (1977) further characterized the 5' ends of growing progeny strands of replicating Polyoma. Reichard found that the RNA started with either ATP or GTP. Also, digestion of progeny strands with pancreatic DNase released labeled RNA that on gel electrophoresis gave a distinct peak in the position expected for a decanucleotide. Tseng and Goulian (1977) isolated an oligonucleotide 8-11 nucleotides in length with a triphosphate group at the 5' end and 2 or 3 deoxynucleotides at the 3' end that were not removed by DNase.

Reichard called the decanucleotide "initiator RNA". It was quite homogeneous in size, but had no unique base sequence. He proposed that the switch from RNA to DNA synthesis during chain elongation may thus depend on the size of initiator RNA, rather than a specific base sequence.

#### Okazaki Fragments: Gap Filling

There is some evidence that two DNA polymerases and a ligase are required to permit DNA chain growth (Salzman and Thoren, 1973; Magnusson, 1973a and 1973b). One of these is responsible for synthesizing the Okazaki fragment, and the other for the gap left by the degradation of the RNA primer. Okazaki et al., (1970 and 1971) described a DNA polymerase I deficient mutant of E. coli in which newly replicated short DNA is joined at about 10% of the rate of wild-type strains. Since DNA synthesis occurs, but there is very little joining of the fragments, he suggested a gap filling function for this enzyme.

Accumulations of short fragments (4S) were also seen in SV40 or Polyoma treated with either HU or FdUrd (Magnusson, 1973a and 1973b; Laipis and Levine, 1973; Salzman and Thoren, 1973). Addition of HU to the medium gives a very rapid depletion of the intracellular dGTP pool followed by a slower decrease of the dATP pool, whereas FdUrd reduces the pool of dTTP in the

cell. Because the  $\alpha$  and  $\beta$  DNA polymerases have a lesser affinity for deoxyribonucleoside triphosphates than does the  $\delta$ -polymerase (Spadari and Weissbach, 1974), and the drugs mentioned affected these pools, it would appear that the  $\alpha$  and  $\beta$  polymerase are more affected by them. This would imply that  $\alpha$  and  $\beta$  are the gap filling enzymes, and  $\delta$ , the enzyme that synthesizes the Okazaki fragment. Others have suggested that it is the  $\alpha$  polymerase which synthesizes the Okazaki fragments since it shows quantitative alterations with stages of the cell cycle, and with the cell growth rate (Laipis and Levine, 1973). In support of this, is that  $\alpha$  is the only polymerase found capable of extending a natural RNA primer (Spadari and Weissbach, 1975). Hunter and Francke (1975) tested the effects of 1- $\beta$ -D arabinofuranosyl CTP (ara-CTP) on in vitro Polyoma DNA synthesis. Two effects were found: 1) predominant labeling of short chains with reduced amounts of radioactivity in the longer growing viral DNA strands; 2) a 1/3 to 1/2 reduction in size for short DNA chains. Since the polymerase is more sensitive to ara-CTP, they postulated that  $\alpha$  is the gap filling enzyme, and that it may also be responsible for continuous extension of the Okazaki fragment, since there is a reduction in the size of the fragment. Further elucidation of these points is needed.

#### Okazaki Fragments: Nucleosomes as a Signal for their Initiation

The chromosomal material or chromatin, contains five proteins called histones, that are complexed with DNA in a repeating pattern. The repeat unit of the pattern is termed a nucleosome. The nucleosome contains a core of 140 base pairs (bp) surrounding a histone octamer and a spacer or linker of about 60 bp of DNA less intimately associated with the octamer. The octamer contains two copies each of the slightly lysine-rich histones H2A and H2B, and the arginine-rich histones H3 and H4. H1 is not part of the

nucleosome, but is associated with it. The amount of DNA per histone octamer can vary between 140 and 240 bp, depending upon the organism and tissue from which the nucleosomes are isolated. The evidence for this variability comes from studies in which nuclei are digested briefly with staphylococcal nuclease. The variability of the length of DNA in the nucleosome repeat derives from variation in the length of the spacer region. Physical studies conducted on the nucleosome have concluded that it is a cylindrical structure of about 100 Å in diameter, and 50 Å in height, with the DNA wrapped around it to form a pair of rings at the top and bottom (Kornberg, 1977; Felsenfeld, 1978).

The DNA of the nucleosome is susceptible to internal cleavage by nucleases. Although the spacer DNA is the preferred target, the nucleosome core is also attacked but at a slower rate (Axel, 1974). Hewish (1976) and Rosenberg (1976) suggested that nuclease accessibility is a measure of accessibility to cellular factors involved in DNA functions (e.g. polymerases, regulatory proteins). There is thus a regular alternation of DNA regions which are either readily accessible or inaccessible.

Hewish (1976) and Rosenberg (1976) have suggested that the periodic structure of chromatin has two basic biological functions. These are:

- 1) It may play an important role in determining the initiation of a function which is not coded by a specific base sequence: the synthesis of Okazaki pieces during DNA replication;

- 2) Substantial reduction of the amount of DNA which must be scanned by agents which interact at coded sites on the DNA, thereby decreasing errors and enabling the cell to produce lower concentrations of required transcriptional and replicational factors. A corollary of this, is that because of the lack of specificity in nucleosome location, coded control

sequences in DNA must be redundant to ensure their accessibility.

Several experimental results are consistent with the idea that the nucleosome provides the signal for initiation of Okazaki fragments. Firstly, the RNA primer of Okazaki fragments is not specific. The DNA intermediates in eukaryotes are initiated by the synthesis of short RNA primers (Edenberg and Huberman, 1975), the base sequence of which is not specific (Reichard et al., 1974). Also, there is no base specificity at the RNA-DNA junction (Reichard et al., 1974; Tseng and Goulian, 1975). This non specificity supports the above model which postulates that accessibility of the RNA polymerase to the template is determined solely by the association of the DNA with chromatin proteins. Secondly, it has been shown that newly synthesized histones form completely new nucleosomes, and there is preferential association of new synthesized histones with nascent DNA (Freedlender et al., 1977, Felsenfeld, 1978). This is consistent with Hewish's (1976) prediction that only one of the two template strands remains associated with parental chromatin and it is this association that specifies the linker DNA as the initiation site of Okazaki piece synthesis. Thirdly, there is evidence that the eukaryotic DNA is probably synthesized semi-discontinuously (Perlman and Huberman, 1977; Hunter et al., 1977). This is consistent with Hewish's (1976) statement that nucleosomes segregate asymmetrically.

Kaufmann et al. (1977) support the above model for initiation of Okazaki fragments. However, there is some evidence against this hypothesis. These have been reviewed by Hand (1978). Nucleosome DNA has been estimated to range from 154-241 bp (Kornberg, 1977). If Okazaki fragment size is determined by the length of nucleosome DNA, then some of the most recent estimates of less than 150 bp (Perlman and Huberman, 1977) are too small to

be accounted for by the hypothesis. Blumenthal and Clark (1977a) have provided evidence for the existence of three discrete size classes of replication intermediates in *Drosophila* cells. A *Drosophila* nucleosome contains about 200 nucleotides of DNA, 60 in the spacer and 140 in the core particle. Their 61 nucleotide intermediate corresponds to the spacer region, but none of the other replication intermediate lengths can be correlated with the parameters of the nucleosome structure.

In conclusion, there remains much to be explained concerning events at the replication fork, since even the existence of Okazaki fragments as a replication intermediate is still in question. Because of the  $3' \rightarrow 5'$  polarity of one of the daughter strands and the  $5' \rightarrow 3'$  direction of all known polymerases, there is a need to invoke some mechanism which accounts for how DNA is synthesized. Experimentally it has been shown that in many prokaryotic and eukaryotic systems, nucleotides are incorporated onto very short (approx. 100 nucleotides) short lived strands, and that these short strands are subsequently joined onto longer intermediate strands. The fact that these short fragments have been isolated, and that they are consistent with our most plausible explanation of DNA synthesis, lends support to these fragments being Okazaki pieces or intermediates in replication. Other hypotheses such as endonuclease cleavage of newly synthesized DNA or an excision repair mechanism of incorporated uracil bases have not, in my opinion, satisfactorily explained this consistent finding. Even if Okazaki fragments do exist, there still are many other questions which still remain: How many fragments are there per fork? Is there semi or totally discontinuous synthesis? What are the kinetics of synthesis and ligation? Is there an RNA primer? To help answer these questions, good consistent techniques of Okazaki fragment detection and sizing are needed, and it is in

this area that my system can make its contribution.

## Materials and Methods

The line derived from African green monkey kidney cells, CV-1, was used for all experiments. The cells were grown in Eagle's minimal essential medium (MEM) with 10% fetal calf serum (FCS), supplemented with 0.25 ug/ml amphotericin B, 50 ug/ml gentamicin, and 60 ug/ml tylocine. Cells were grown in 75 cm<sup>2</sup> tissue culture flasks at 37°C until confluent, at which point  $2 \times 10^5$  cells were transferred to fresh flasks.

### (<sup>3</sup>H)Thymidine Labeling and Cell Lysis

For experiments, unsynchronized cells in logarithmic growth were used. Forty-eight hours before the experiment, Petri dishes were seeded at a density of  $6 \times 10^5$  cells/dish. The following day, they were labeled with (<sup>14</sup>C)thymidine (0.1 uCi/ml; 40 - 60 mCi/mmol), for approximately 24 hours. On the day of the experiment, the radioactive medium was removed, the cells were washed with prewarmed phosphate buffered saline (PBS) and were subsequently incubated in fresh MEM for 2 hours. FdUrd  $2 \times 10^{-6}$  M was then added for 1 hour, after which the cells were either pulse-labeled, or pulse-labeled and chased with MEM. Pulse-labeling was done using (<sup>3</sup>H)thymidine (100 uc/ml; 40 - 60 Ci/mmol) for 30 seconds at 37°C. The pulse was stopped by placing the Petri dishes on ice, and then washing twice with ice-cold PBS. Cells were trypsinized immediately, centrifuged and the pellet resuspended in 0.3 mls of cold PBS. Cells were lysed in 0.5 M NaCl, 0.015 M sodium citrate, 1% sarcosyl for 1 hour at room temperature, and the lysates then lyophilized and dissolved in a small volume of electrophoresis buffer. This preparation served as the "crude lysate" of DNA used for subsequent agarose gel electrophoresis experiments. Crude lysates were stored for not longer than 24 hours at -20°C so as to avoid DNA degradation. Cells which were pulsed and chased, were pulsed with (<sup>3</sup>H)thymidine



as described above and chased for 1 hour at 37°C with MEM, 10% FCS which contained  $2 \times 10^{-6}$  M FdUrd and  $2 \times 10^{-6}$  M thymidine. They were then trypsinized as described. Cell density at the time of pulse-labeling was approximately  $1.5 \times 10^6$  cells/dish as determined by hemocytometer counting.

#### Preparation of Purified DNA

Samples of purified DNA were prepared to compare with the crude lysate DNA preparations described above. Two procedures, one based on that described by Gautschi and Clarkson (1975), and the other, a modified Marmur DNA extraction (Britten et al., 1974) were used for subsequent agarose gel electrophoresis experiments. A third procedure, based on that of Miyamoto and Denhardt (1977) was also performed. The DNA isolated by this stringent method of purification was compared with that obtained in the above two procedures.

In the method of Gautschi and Clarkson, CV-1 cells were pulsed with [ $^3$ H]thymidine and lysed as outlined above. Pipetting during lysis reduced viscosity. To the cell lysate, a solution of 5 M NaCl was added to a final concentration of 1 M NaCl, and left overnight at 4°C. The precipitate was removed by centrifugation for 15 minutes at 20,000 revolutions per minute (rpm) at 10°C in an SW 56 rotor of the Beckman L65B centrifuge. With this procedure, approximately 90%  $^3$ H cpm were recovered in the supernatant. After NaCl extraction, samples were incubated for 30 minutes at 37°C with pronase (250 ug/ml, self digested beforehand for 30 minutes at 37°C) and the remaining proteins were removed by 2 rounds of extraction with chloroform-isoamyl alcohol (24:1 v/v) that had been equilibrated with standard saline citrate, 10m M Tris, 10m EDTA, pH 7.5. The deproteinated sample was then precipitated with 2 volumes of ethanol at -20°C overnight, and centrifuged at 15,000 rpm for 1 hour at 4°C, in the SS34 rotor, of the Sorvall RC3

centrifuge. The resulting pellet was well dried and suspended in the appropriate buffer.

In the modified Marmur procedure, CV-1 cells were pulsed with [ $^3\text{H}$ ]thymidine and lysed as outlined above. To the resulting pellet, 2 mls of Tris 0.05M, NaCl 0.15 M, EDTA 0.05M, pH8 (TNE), and 2 mls of protease B (Sigma Chemical Co. 10 mg/ml in TNE, self digested for 3 hours at 37°C) was added for 3 hours at 37°C. To this, 1/10 volume 5M sodium perchlorate ( $\text{NaClO}_4$ ) was added and the solution mixed. Proteins were then removed with 2 rounds of extraction with phenol that had been equilibrated with 0.05M Tris pH8 and chloroform-isoamyl alcohol (24:1 v/v). To the deproteinated sample, 1/10 volume of RNase was added, incubation for 3 minutes at 37°C carried out and a final chloroform-isoamyl alcohol (24:1 v/v) extraction done. The DNA was precipitated with 2 volumes of ethanol at -20°C overnight, and centrifuged at 15,000 rpm for 1 hour at 4°C in the SS 34 rotor of the Sorvall RC3 centrifuge. The pellet was dried and suspended in the appropriate buffer. Recovery of  $^3\text{H}$  and  $^{14}\text{C}$  cpm approached 85% and 50% respectively.

In the procedure of Miyamoto and Denhardt, CV-1 cells were grown in Petri dishes and pulse-labeled with [ $^3\text{H}$ ]thymidine as outlined above. The pulse was stopped by pouring out the radioactive media and adding 10 ml of a boiling solution containing 2% sodium dodecyl sulfate (SDS), 3% distilled phenol and 10m M EDTA, pH 7.0. Each plate was then scraped with a rubber policeman containing 3.3 ml of the same boiling solution. Each solution was heated until it just returned to boil and then chilled on ice for about 30 minutes. This stopping procedure is considered very important since it should inactivate all the cellular enzymes, and denature the DNA. About 2 minutes elapsed from the point of pulse-labeling until chilling.

Centrifugation at 15,000 rpm, in the Sorvall RC3 rotor for 30 minutes at 5°C was then done to pellet the high molecular weight DNA. The resulting supernatant contained greater than 90% of the  $^3\text{H}$  cpm and approximately 30% of the  $^{14}\text{C}$  cpm. The supernatant was extracted with one half volume of phenol-chloroform (1:1) which has been previously equilibrated with 50m M borate pH 9.2. The mixture was shaken vigorously on the vortex for about 3 minutes at room temperature, and then centrifuged to attain separation of the phases. The aqueous phase was collected, leaving the interphase which was washed with 50m M Tris-HCl pH 8.1, and the resulting aqueous phase pooled with the first. The pooled phases were mixed with 1/10 volume of 3 M sodium acetate pH 5.5, 2 volumes of isopropanol and left overnight at -20°C. The nucleic acids were then precipitated in an RC3 centrifuge for 1 hour at 5°C at 5000 rpm. The pellet was dried and prepared for reprecipitation with cetyltrimethylammonium bromide (CTAB), a cationic detergent (a product of Sigma Chemical Co.). The pellet was resuspended in equal volumes of 0.025 M Tris - HCl - 0.025 M NaCl pH 7.6, 2.5 M  $\text{KPO}_4$  pH 8 and 2-methoxyethanol (ethylene glycol monoethyl ether). It was then vortexed intermittently for 3 minutes at 0°C and centrifuged at 10,000 rpm for 10 minutes in the Sorvall HB-4 rotor. The upper phase was aspirated, and an equal volume of 0.2 M sodium acetate pH 5.5 added. One half volume of 1% CTAB (W/V) was added slowly, and after 60 minutes at 0°C, the sample was centrifuged at 10,000 rpm for 30 minutes in the Sorvall HB-4 rotor. The pellet was dried, washed with 10 ml 70% ethanol containing 0.1 N sodium acetate pH 5.5 and centrifuged at 10,000 rpm for 20 minutes in the Sorvall HB-4 rotor. This process was repeated using 95% ethanol. The  $^3\text{H}$  recovery was approximately 60% after this procedure. The pellet was dissolved in 10 mM Tris - HCl pH 8.1, 0.1 M EDTA, heated to 100°C for 1 minute, combined

with 1/5 volume of 3 M KCl and then applied to a nitrocellulose column of approximately 3 ml bed volume. A "high salt" solution (0.5 M KCl, 10m M Tris - HCl, 0.1m M EDTA, pH 7.4) followed by a "low salt" solution (10m M Tris - HCl, 0.1m M EDTA, pH 7.4) was passed through the column. Purification by the column operates on the principle that single stranded DNA will adhere to the nitrocellulose in high salt, whereas RNA and most proteins will pass through. The DNA elutes in low salt. The columns were loaded and eluted using gravity-induced flow. Fractions were collected and assayed for radioactivity. The recovery of  $^3\text{H}$ -labeled DNA in the low salt fraction was usually about 80% of the DNA that was applied. The DNA in the low salt fraction was recovered by isopropanol precipitation and centrifuged to equilibrium in CsCl gradient. The nucleic acid was dissolved in 50m M Tris-HCl pH 7.6, and 5m M EDTA to yield a final solution weighing 5.7 grams; it was then heated (100°C, 30 seconds) and added to a polypropylene ultracentrifuge tube containing 7.3 grams CsCl. The refractive index was verified to be 1.3996 for CsCl (density 1.7 grams/ml). Centrifugation was performed at 40,000 rpm for about 60 hours at 10°C in the A-321 rotor of the IEC B60 ultracentrifuge. Fractions of 0.3 ml were collected from the top with a Buchler Auto Densi Flow apparatus. Fractions from the CsCl gradient containing the DNA were diluted five-fold with 50m M Tris - HCl pH 8.1 and precipitated with isopropanol and sodium acetate. The pellet was suspended in 0.05 M Tris - HCl pH 8.1, heated for 30 seconds at 100°C and layered on top of a 5 ml 5 - 20% sucrose gradient containing 1 M NaCl, 1m M EDTA and 50m M Tris - HCl pH 8. The DNA was centrifuged in the Beckman SW 50.1 rotor at 50,000 rpm for 3 hours at 20°C. Fractions of 300 ul were collected from the bottom of the tube and assayed directly for radioactivity in Triton - toluene scintillation fluid (Triton x-100 (Sigma) and toluene in a 1:2 v/v

ratio with 4 grams of 2,5-diphenoxazole per litre). Single-stranded RF II  $\phi$ X-174 was used as a sedimentation marker.

#### Agarose Gel Electrophoresis

A horizontal slab gel system for electrophoresis on agarose gels, as described by McDonnell et al. (1977), was used in these experiments. The buffer used for neutral gel electrophoresis was Buffer E, (0.04 M Tris -<sup>s</sup> pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA). To prepare gels, 1.4% agarose in water (SeaKem, Marine Colloids Inc.) was melted, mixed thoroughly and cooled at 50°C. An equal volume of twice concentrated Buffer E warmed to 50°C was then added, giving a final agarose gel concentration of 0.7%. Samples containing  $1 \times 10^5$  cells were lysed as described above, and the final lyophilized sample suspended in 25  $\mu$ l of Buffer E. They were then heated for 5 minutes in boiling water and applied to the gel. 0.025% bromophenol blue (BPB) was run in a separate well as a marker dye. Electrophoresis was carried out at room temperature, until the BPB had migrated to a distance of 5 cm, generally 4 - 5 hours at 40 millivolts. After electrophoresis, the gels were soaked in 10% trichloroacetic acid (TCA) - 0.5% sodium pyrophosphate ( $\text{NaPP}_i$ ) for 1 hour, to precipitate the DNA and to complex free unincorporated  $^3\text{H}$ . The gel was cut into 1 mm slices using stacked razor blades. Slices were placed into scintillation vials containing 0.5 ml of distilled water, autoclaved for 3 minutes to dissolve the agarose and analyzed for radioactivity by scintillation counting.

For alkaline gel electrophoresis, the gel buffer consisted of 30m M NaOH, 2m M EDTA 12.2 (McDonnell et al., 1977). Samples were dissolved in neutral E buffer. All other conditions were the same as outlined above.

#### Velocity Sedimentation in Alkaline Sucrose Gradients

Gradients of 5 ml of 5-20% sucrose were formed over a cushion of 70%

sucrose. Gradients were 0.9 M in NaCl, 0.3 M in NaOH, and 0.001 M in EDTA (Sheinin, 1976). Samples of  $1 \times 10^5$  cells (25  $\mu$ l) in 0.3 ml of 0.2 M NaOH - 0.01 M EDTA were layered carefully over the gradients. When purified DNA samples were run,  $4 \times 10^4$   $^3\text{H}$  cpm (approximately 100  $\mu$ l) were used. Cell lysis and DNA denaturation were allowed to proceed at  $4^\circ\text{C}$  for 8 to 12 hours. Centrifugation was carried out in an SW 50.1 rotor of the Beckman L 65B centrifuge at 26,000 rpm for 16 hours at  $4^\circ\text{C}$ . Fractions of 0.2 ml were collected by gravity from the bottom of the tube using a constant volume fractionator (MRA Corp. Boston, Mass.), and were analyzed for radioactivity using 95% aquasol. ( $^3\text{H}$ )-labeled SV40 DNA was used as a sedimentation marker. It sediments at 16 S under these conditions.

#### Preparation of SV40 DNA

Confluent MA-134 monolayers in 100 mm plastic Petri dishes were infected with SV40 at a multiplicity of 5 plaque forming units (PFU) per cell. The inoculum was allowed to adsorb to the cells for 90 minutes. At 22 hours after infection, ( $^3\text{H}$ )thymidine was added (10  $\mu\text{C}/\text{ml}$ ; 40 - 60 Ci/mmol). Cell lysis and extraction of DNA was performed at approximately 70 hours after infection according to the method of Hirt (1967). The Hirt supernatant was extracted twice with chloroform-isoamyl alcohol (24:1). To the resulting aqueous phase 1/10 volume of 0.5 mg/ml. RNase was added and the tube put at  $37^\circ\text{C}$  for 30 minutes. A final chloroform-isoamyl alcohol (24:1) extraction was then performed, 2.5 volumes of 95% ethanol added, and the preparation kept at  $-20^\circ\text{C}$  overnight. The sample was then centrifuged for 1 1/2 hours at  $-20^\circ\text{C}$  at 17,000 rpm in the SS-34 rotor of the Sorvall RCB centrifuge. The pellet was dried, suspended in TNE pH 7.4, and then centrifuged to equilibrium in CsCl-ethidium bromide. Centrifugation was at 38,000 rpm, at  $4^\circ\text{C}$  in a 40.2 rotor of the Beckman L265B ultracentrifuge.

The fractions containing virus were pooled, an equal volume of CsCl saturated isopropanol added, and the upper phase containing the ethidium bromide was discarded. This was repeated once, the final sample was dissolved in TNE and 2 volumes of 95% ethanol, and left overnight at  $-20^{\circ}\text{C}$ . It was centrifuged at 15,000 rpm for 1 hour at  $-20^{\circ}\text{C}$  in an SS-34 rotor of the Sorvall RC3 centrifuge. The final pellet was dried, dissolved in twice concentrated 0.01 M Tris pH 7.5, 50m M NaCl, 0.5m M EDTA and 1 volume of glycerol. The specific activity of [ $^3\text{H}$ ]thymidine-labeled closed, circular SV40 DNA was approximately  $5 \times 10^4$  cpm/ug.

#### Cleavage of SV40 DNA with H. Influenza Restriction Endonuclease

Hind III enzyme preparation was obtained from Chemical Credentials Inc. One unit of enzyme was defined as that amount of enzyme required to completely digest 1 ug of Lambda phage DNA in 1 hour at  $37^{\circ}\text{C}$ . For preparation of complete digests, SV40 covalently closed circular DNA was incubated with Hind III restriction endonuclease at  $37^{\circ}\text{C}$  in a reaction mixture containing 50m M NaCl, 6m M Tris-HCl pH 7.5, 6m M  $\text{MgCl}_2$ , and 5 ug bovine serum albumen (BSA). Generally, incubation of 1 ug of SV40 DNA with 1 unit of Hind III in a volume of 0.05 ml for 3 hours at  $37^{\circ}\text{C}$  gave complete digestion, giving 6 DNA fragments as determined by electrophoresis on agarose gels of the products. The fragments ranged in size from  $1.3 \times 10^5$  to  $1.1 \times 10^6$  MW (Danna et al., 1973). All reactions were terminated by addition of EDTA to 0.025 M. For agarose gel electrophoresis, when SV40 DNA was labeled with ( $^3\text{H}$ )thymidine, the gel was assayed for radioactivity by gel slicing and scintillation counting as described above. When it was unlabeled, the gel was soaked in electrode buffer, made 0.4 ug/ml in ethidium bromide and visualized under UV light.

## Results

The first experiment was conducted to see whether or not an agarose gel electrophoresis system could be used as an alternative to alkaline sucrose gradients for the detection of Okazaki fragments. CV-1 cells were prelabeled with [ $^{14}\text{C}$ ]thymidine for 24 hours and then pulsed for 30 seconds, pulse-chased for 1 hour and lysed. Samples were applied to 0.7% agarose gels, which were prepared in an alkaline buffer in order to maintain the denatured conformation of the DNA. The resulting radioactivity profile showing incorporated  $^3\text{H}$  and  $^{14}\text{C}$  cpm versus migration distance (mm) is shown in Fig. 1. In Fig. 1a, the  $^{14}\text{C}$  prelabel, (65% of total  $^{14}\text{C}$  cpm) migrates as a smooth peak at the top of the gel, indicating its presence in large molecular weight or bulk DNA. After a 30 second pulse at  $37^\circ\text{C}$ , 37% of the  $^3\text{H}$  label is seen to migrate at the bottom of the gel, corresponding to DNA of low molecular weight and 12% of counts comigrate with the  $^{14}\text{C}$  peak in bulk DNA. The remaining counts are incorporated into intermediate sized DNA. Some of this has most likely resulted from breakdown of bulk, as seen from the  $^{14}\text{C}$ -DNA profile. Denatured SV40-DNA, used as a marker, migrates behind the  $^3\text{H}$  peak. A 30 second pulse of [ $^3\text{H}$ ]thymidine at  $37^\circ\text{C}$  followed by a 1 hour chase is shown in Fig. 1b. As before, approximately 74%  $^{14}\text{C}$  cpm migrates as a distinct peak at the top of the gel, with the remaining 35% in progressively smaller sized DNA. The  $^3\text{H}$  peak in smaller molecular weight DNA is no longer seen at an equivalent area at the bottom of the gel. The  $^3\text{H}$  cpm previously in the peak at the gel bottom and in intermediate size DNA appear as a peak of  $^3\text{H}$  comigrating with the  $^{14}\text{C}$  peak, and representing 72% of total  $^3\text{H}$  cpm. The remaining  $^3\text{H}$  counts are seen in intermediate sizes as before. The  $^3\text{H}$  peak at the gel bottom present after a 30 second pulse, represents the "Okazaki fragments" or smallest

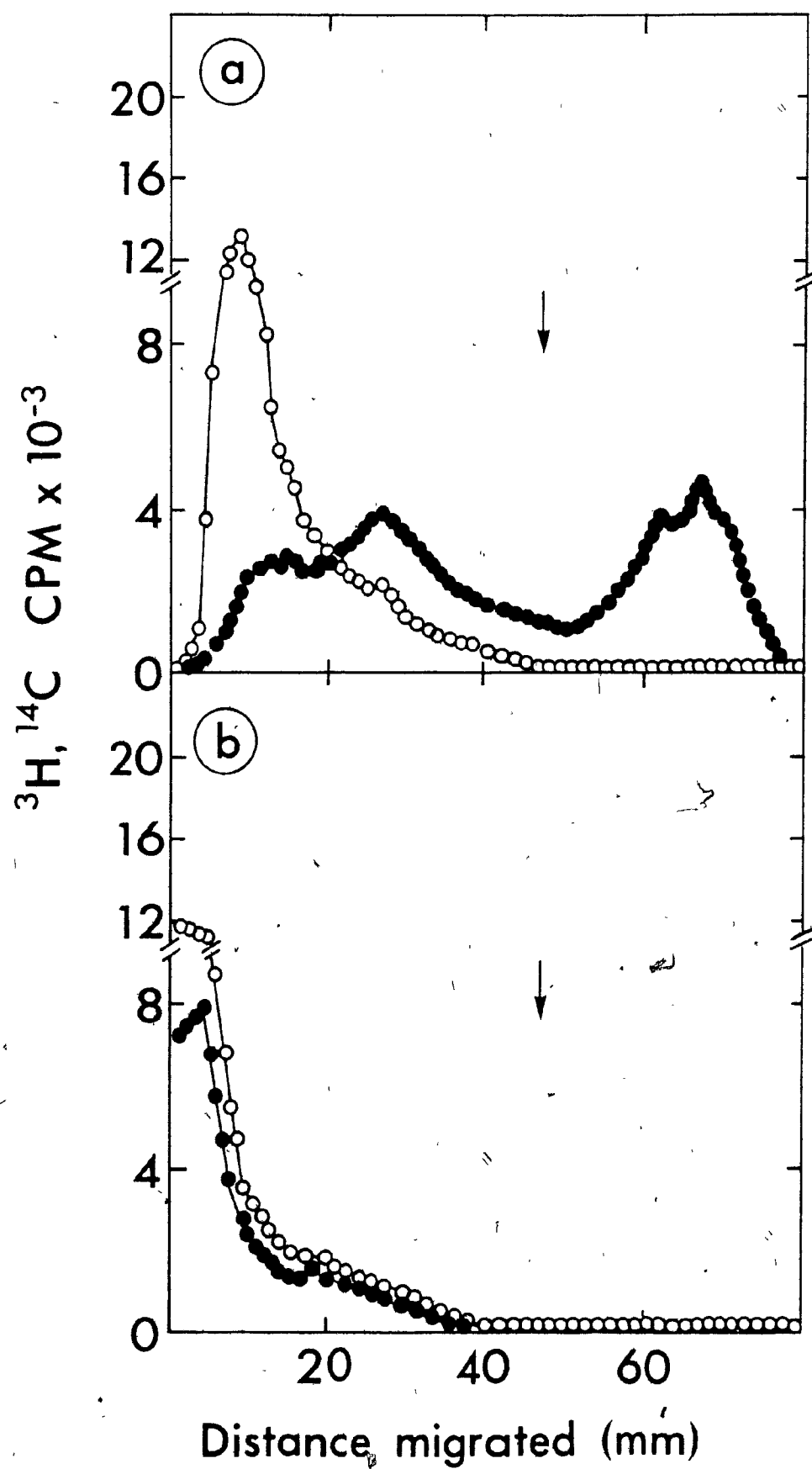


Figure Legend

Fig. 1: Electrophoresis of crude lysates of DNA on alkaline 0.7% agarose gels.

Exponentially growing CV-1 cells were uniformly labeled with [ $^{14}\text{C}$ ] thymidine for 24 hours, washed with prewarmed PBS, incubated with FdUrd for 1 hour, and then pulse-labeled with [ $^3\text{H}$ ]thymidine (Fig. 1a) or pulse-labeled and chased (Fig. 1b). Cells were lysed, lyophilized and dissolved in buffer as in Materials and Methods. The position of the SV40 DNA marker is shown.

Migration was from left to right    ● ,  $^3\text{H}$ ;    ○ ,  $^{14}\text{C}$ .  
gels.



replication, which are later ligated into larger DNA pieces (Okazaki et al., 1968a) and not seen after a 1 hour chase. Therefore, the slab gels accurately display the typical pulse and pulse-chase characteristics of Okazaki fragments. They can be used to separate Okazaki fragments from bulk DNA following direct lysis of the cells.

Fig. 2 shows neutral 0.7% agarose gels which were run using identical pulse and pulse-chase samples to those in alkaline gel experiments. The method of sample denaturation consisted of boiling for 5 minutes at  $100^{\circ}\text{C}$ , followed by rapid cooling on ice, and immediate electrophoresis on neutral gels.

In the pulse experiment, Fig. 2a, 67% of the total  $^{14}\text{C}$  cpm migrate as a distinct peak at the top of the gel. The  $^3\text{H}$  cpm have a wider range of distribution, 45% are present in a peak at the bottom of the gel in small molecular weight DNA and the remaining counts are present in intermediate sized DNA.

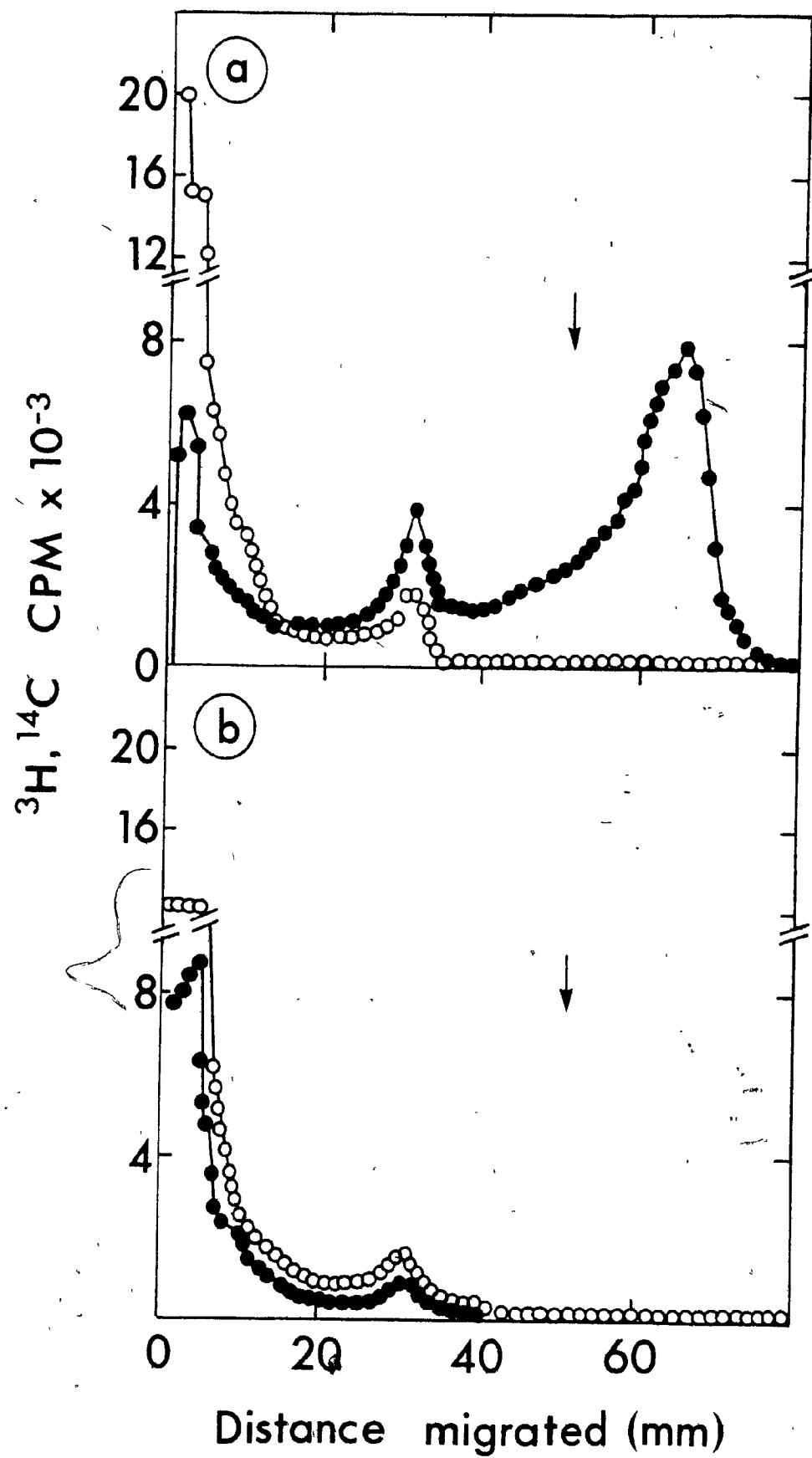
In the pulse and chase experiment profile, Fig. 2b, DNA prelabeled with  $^{14}\text{C}$  migrates as a smooth peak at the top of the gel, containing 65% of the total  $^{14}\text{C}$  cpm. The  $^3\text{H}$  peak present at the gel bottom after pulsing is no longer seen. The  $^3\text{H}$  is found in a peak at the gel top comigrating with the  $^{14}\text{C}$  and represents small MW DNA which has been chased into large size. The chase, however, was not complete, since the small MW peak contained 90,000 cpm and only half of these appear to be represented in the large MW  $^3\text{H}$  peak (60,000 cpm). That is, chasing has taken place, but some loss of  $^3\text{H}$  cpm has occurred and is not represented in the chase peak. Please note different scales on axis.

Therefore, between 65 - 75% of total  $^{14}\text{C}$  cpm are present in 1 peak of large molecular weight DNA, in pulse and pulse-chase experiments, in both

Figure Legend

Fig. 2: Electrophoresis of crude lysates of DNA on neutral 0.7% agarose gels.

Logarithmically growing CV-1 cells were uniformly labeled with [ $^{14}\text{C}$ ] thymidine for 24 hours, washed with prewarmed PBS, incubated with FdUrd for 1 hour, and then pulse-labeled with [ $^3\text{H}$ ]thymidine (Fig. 2a) or pulse-labeled and chased (Fig. 2b). Cells were lysed, lyophilized and dissolved in buffer, as in Materials and Methods. Samples were denatured by boiling for 5 minutes at  $100^\circ\text{C}$ , followed by rapid cooling on ice. The position of the SV40 DNA marker is shown. Migration was from left to right     ● ,  $^3\text{H}$ ;  
○ ,  $^{14}\text{C}$ .



alkaline and neutral gel systems. Similarly, for  $^3\text{H}$  in pulse experiments, between 37 - 45% of  $^3\text{H}$  cpm constitute the Okazaki peak in both gel systems and after chasing most of  $^3\text{H}$  cpm comigrate with bulk. Both the radioactivity profiles and proportion of counts present in each peak are similar, showing that simple boiling of DNA followed by immediate electrophoresis is sufficient to separate the small fragments from bulk DNA.

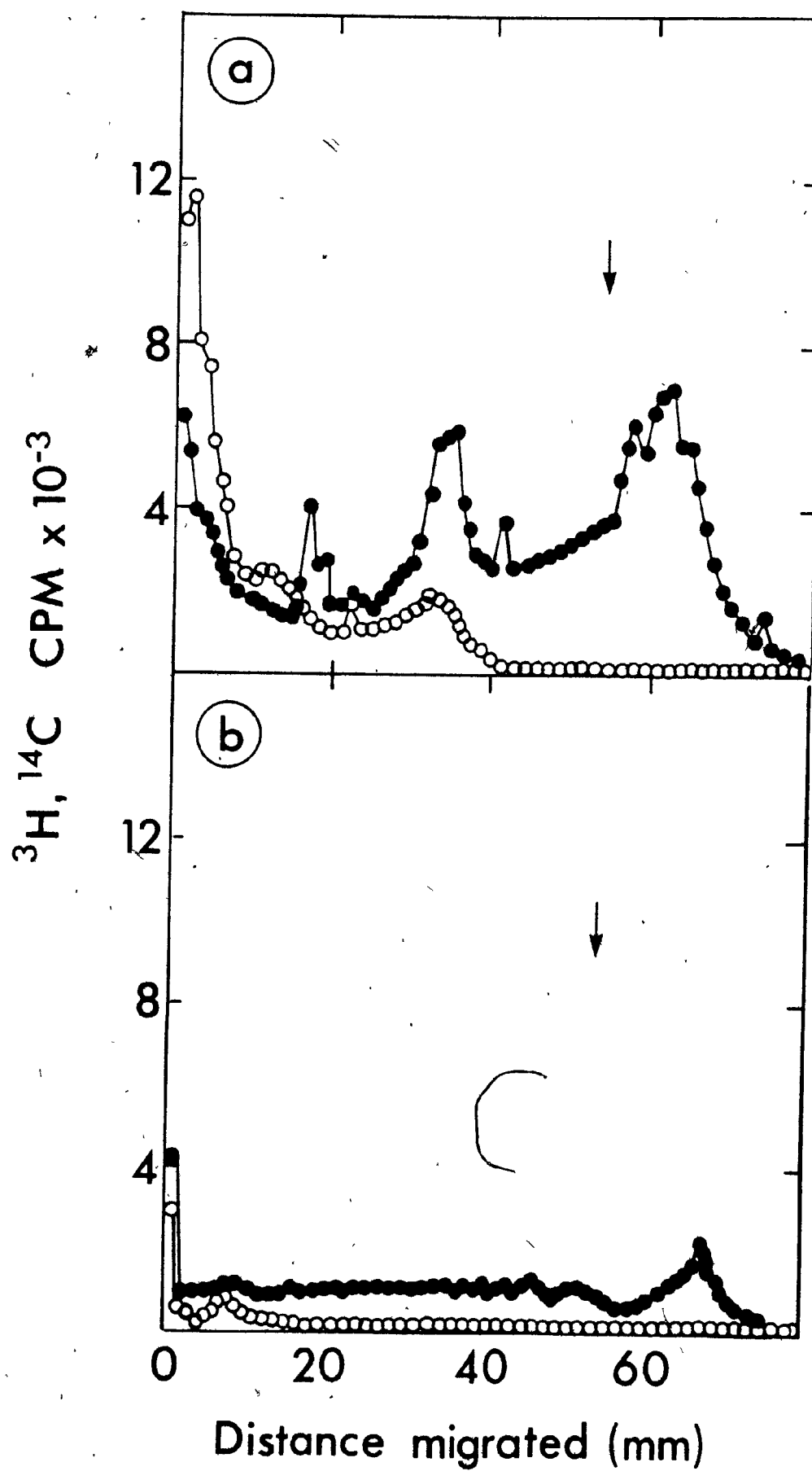
Okazaki fragments were isolated using 2 methods of DNA purification and their electrophoretic behaviours were examined on neutral 0.7% agarose gels (Fig. 3, 4 and 5). Crude lysates were compared with these to see if purified Okazaki fragments had similar mobility in the gels to the fragments released by direct lysis.

The radioactivity profile of a crude lysate of DNA (Fig. 3a) is compared to that of a sample purified by a modified Marmur technique (Fig. 3b) after a 30 second pulse with [ $^3\text{H}$ ]thymidine. Please note differing scales on axis. These profiles are similar. The  $^{14}\text{C}$  cpm are predominantly found in bulk sized DNA in a peak at the top of the gel. The peak migrates similarly in both the crude and purified preparations, approximately 1 - 2 mm migration distance into the gel. The  $^3\text{H}$  profiles are also similar. 10% of  $^3\text{H}$  cpm comigrate with the  $^{14}\text{C}$  bulk peak at 1 mm distance into the gel in both samples. A  $^3\text{H}$  peak at the bottom of the gel representing Okazaki fragments is found in both preparations, at similar migration distances. The proportion of counts found in these peaks are 35% and 20% in the crude lysate and purified sample, respectively. In the purified preparation, this peak has a span from 59 - 72 mm migration distance with an absolute peak at 67 mm. In the crude lysate, the peak spans fractions 55 - 72, with an absolute peak at 62 mm. In the purified sample, 70% of the  $^3\text{H}$  cpm is found in intermediate sized DNA. A small proportion of these counts may be

### Figure Legend

Fig. 3: Relative electrophoretic mobilities of a crude lysate of DNA and DNA purified by a modified Marmur procedure in a pulse experiment.

Crude lysate was prepared by uniformly labeling exponentially growing CV-1 cells with [ $^{14}\text{C}$ ]thymidine for 24 hours, washing with prewarmed PBS, incubating with FdUrd for 1 hour, and then pulse-labeling with [ $^3\text{H}$ ]thymidine. Cells were lysed, lyophilized and dissolved in buffer as described in Materials and Methods (Fig. 3a). For the purified samples, cells were pulse-labeled and lysed as in crude lysate. The sample was treated with protease, extracted with phenol, treated with RNase, then extracted with chloroform-isoamyl alcohol as described in Materials and Methods (Fig. 3b). The position of SV40 marker is indicated. Migration is from left to right     ● ,  $^3\text{H}$ ;     ○ ,  $^{14}\text{C}$ .



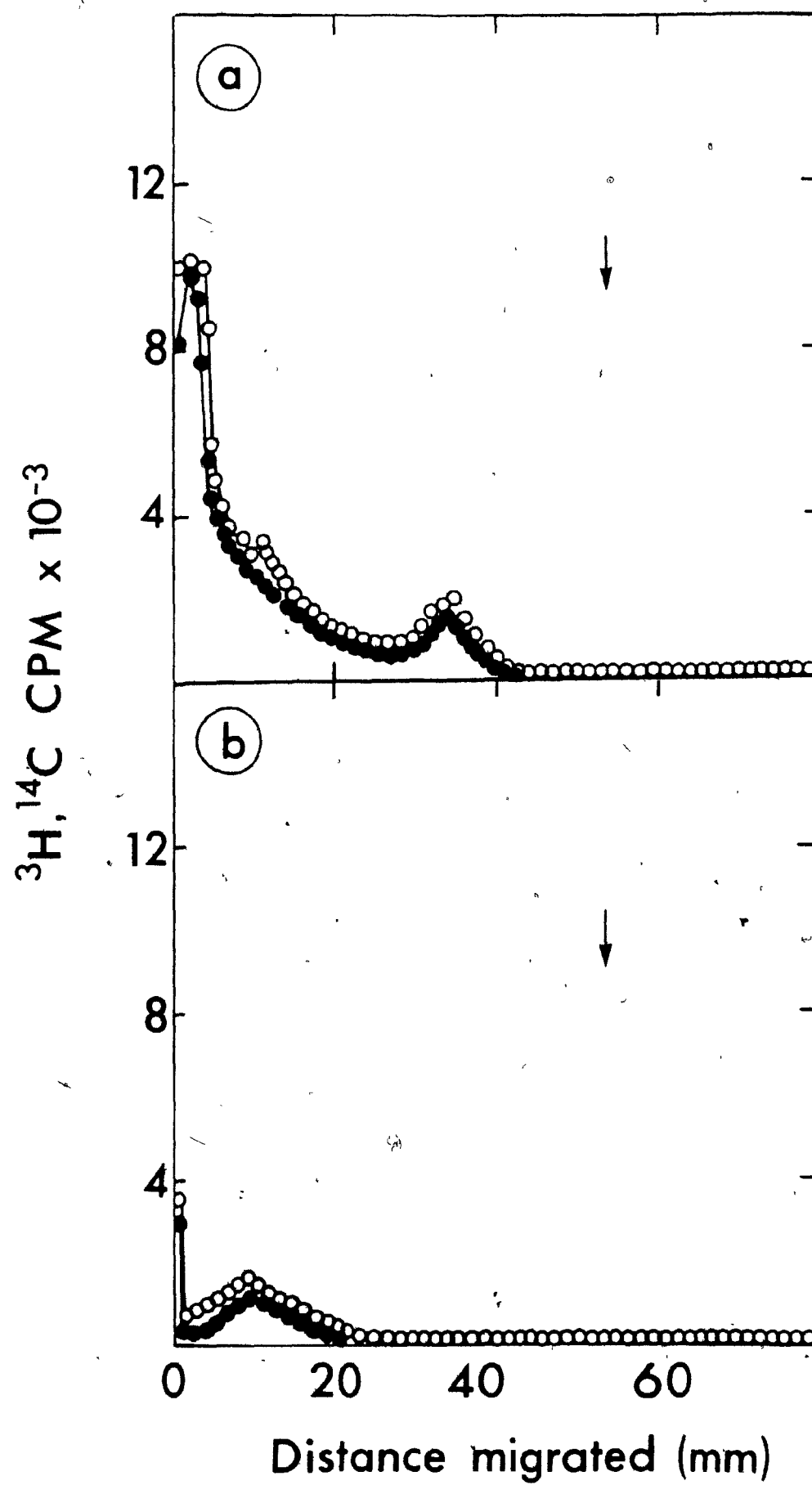


accounted for by breakdown of bulk DNA during purification as shown by the presence of  $^{14}\text{C}$  label. For the pulse experiments therefore, the  $^{14}\text{C}$  and  $^3\text{H}$  in bulk DNA show identical migration patterns. The  $^3\text{H}$  cpm found in small molecular weight DNA show similar migration in terms of the total span of the peak, although the absolute peak differs by 5 mm between the two. Also, there is some loss of the Okazaki fragments in the purification procedure as evidenced by the lesser proportion of  $^3\text{H}$  cpm in the small molecular weight DNA peak at the gel bottom (20% versus 35% in the crude lysate). Fig. 4a and 4b show the same comparison of crude lysate versus DNA purified by a modified Marmur procedure in a pulse and chase experiment. As in Fig. 3, the profiles are similar.  $^{14}\text{C}$  cpm are found to label bulk DNA and the peak of both samples falls at 1 mm migration distance into the gel. Similarly,  $^3\text{H}$  cpm migrates with the  $^{14}\text{C}$  peak, at migration distances of approximately 1 - 2 mm into the gel in pure and crude lysate preparations. In this experiment, the peak of  $^3\text{H}$  representing the Okazaki fragments appears to have been chased into the first 10 fractions of the gel. Please note differing scales on axis. Fig. 5a shows the radioactivity profiles of a crude lysate of DNA compared with that of DNA purified by the method of Gautschi and Clarkson (1975) in a pulse experiment. In this case, profiles of crude lysate and purified sample are almost identical. The  $^3\text{H}$  peaks at the bottom of the gel show a wide spread in both cases, 36 - 70 mm migration distance into the gel for the crude lysate sample versus 39 - 70 mm for the purified preparation. The absolute peaks are at 51 mm and 52 and 54 mm, respectively. The proportion of total  $^3\text{H}$  cpm found in this peak is about 60% in both cases. In both cases, there is also a peak of  $^3\text{H}$  migrating at the top of the gel in gel in large molecular weight DNA. The proportion of  $^3\text{H}$  cpm found here is 10% and 5% in the crude and pure samples, respectively.

### Figure Legend

Fig. 4: Relative electrophoretic mobilities of a crude lysate of DNA and DNA purified by a modified Marmur procedure in a pulse and chase experiment.

Crude lysate was prepared by uniformly labeling exponentially growing CV-1 cells with [ $^{14}\text{C}$ ]thymidine for 24 hours, washing with prewarmed PBS, incubating with FdUrd for 1 hour, then pulse-labeling with [ $^3\text{H}$ ]thymidine and chasing. Cells were lysed, lyophilized and dissolved in buffer as described in Materials and Methods (Fig. 4a). For the purified samples, cells were pulse-labeled and chased, and lysed as in the crude lysate. The sample was treated with protease, extracted with phenol, treated with RNase, then extracted with chloroform-isoamyl alcohol as described in Materials and Methods (Fig. 4b). The position of SV40 marker is indicated. Migration is from left to right    ● ,  $^3\text{H}$ ;    ○ ,  $^{14}\text{C}$ .

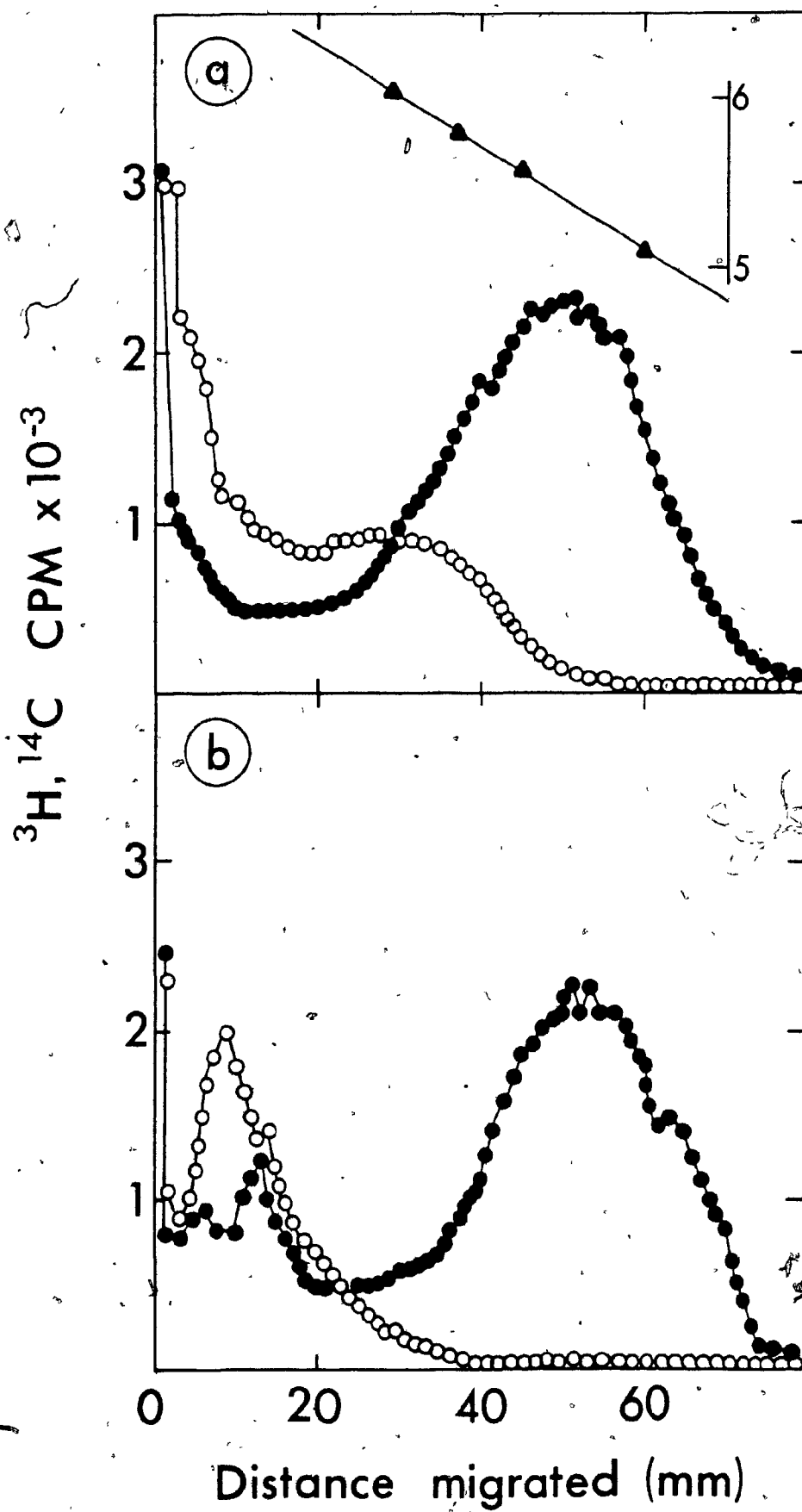


### Figure Legend

Fig. 5: Relative electrophoretic mobilities of a crude lysate of DNA and DNA purified by the method of Gautschi and Clarkson in a pulse experiment.

Crude lysate was prepared by uniformly labeling exponentially growing CV-1 cells with [ $^{14}\text{C}$ ]thymidine for 24 hours, washing with prewarmed PBS, incubating with FdUrd for 1 hour, and then pulse-labeling with [ $^3\text{H}$ ]thymidine. Cells were lysed, lyophilized and dissolved in buffer as described in Materials and Methods (Fig. 5a). For the purified sample, cells were pulse-labeled and lysed as in crude lysate. To the lysate, 5M NaCl was added, and centrifugation performed. The supernatant was treated with pronase and extracted with chloroform-isoamyl alcohol (Fig. 5b). Logarithm molecular weight of SV40 digested with Hind III restriction endonuclease is shown plotted against migration distance. Migration is from left to right

● ,  $^3\text{H}$ ;    ○ ,  $^{14}\text{C}$ .



SV40 digested with Hind III restriction endonuclease was run on the same gel as a marker for sizing the small molecular weight or Okazaki fragment. peak in both the purified and crude lysate samples. The log molecular weight of the resulting SV40 fragments are plotted versus migration distance on agarose gels (see Fig. 5). For the purified sample, using 52 mm as migration distance peak, corresponds to a molecular weight of  $2.15 \times 10^5$  for the Okazaki fragments, whereas for the crude lysate, using 51 mm as peak, a molecular weight of  $2.3 \times 10^5$  is obtained. Please note differing scales on axis.

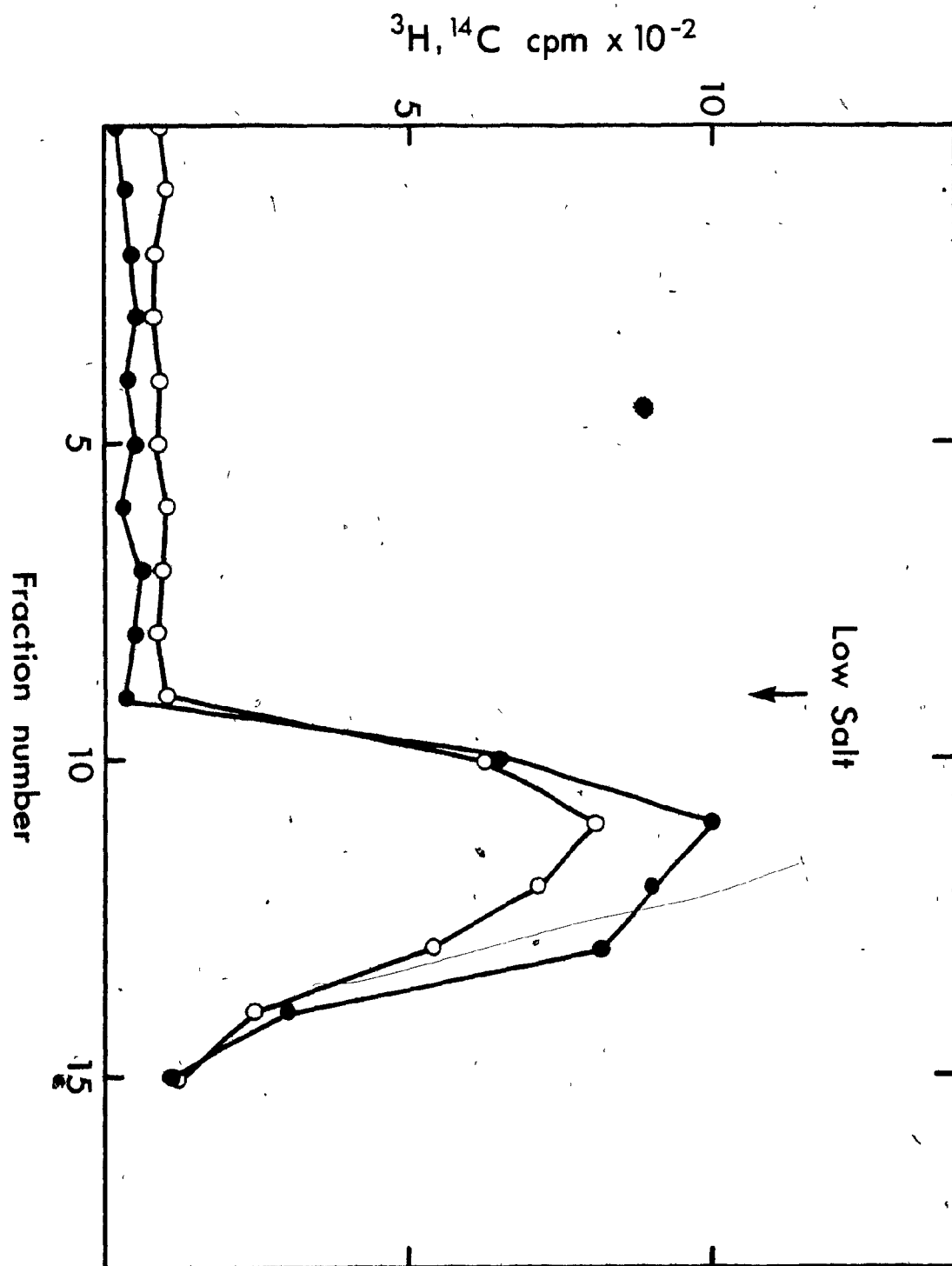
A stringent method of DNA purification based on that of Miyamoto and Denhardt (1978) yielded a purified DNA sample whose size was determined on a sucrose gradient. Included in this procedure, was purification of DNA by nitrocellulose column chromatography, the elution profiles of which are shown in Fig. 6. With this method, single stranded DNA should adhere to the nitrocellulose in high salt, whereas RNA and most proteins should pass through. The DNA elutes in low salt. Accordingly, approximately 80% of the applied  $^3\text{H}$  cpm were isolated in the low salt fraction and almost all the radioactivity isolated in the high salt fraction was alkali labile. Next, the low salt fractions from the nitrocellulose column, containing the labeled DNA, were pooled, the DNA precipitated with isopropanol and prepared for equilibrium density centrifugation in CsCl. Banding in CsCl is shown in Fig. 7; the peak of  $(^3\text{H})$  DNA was at a density of 1.7 g/cc.

To fractionate the DNA according to its size, it was heated (100°C, 30 seconds) and centrifuged on a 5 - 20% neutral sucrose gradient, containing 1M NaCl, 1mM EDTA and 50 mM Tris - HCl pH 8. Using single stranded RF II  $\times$  174 DNA as a sedimentation marker, the  $(^3\text{H})$  DNA was estimated to be  $2.8 \times 10^5$  molecular weight as shown in Fig. 8.

### Figure Legend

Fig. 6: Elution profile of DNA on nitrocellulose column.

CV-1 cells were pulse-labeled and the DNA extracted and treated as described in the method of Miyamoto and Denhardt (1978) in Materials and Methods. The pellets from the isopropanol precipitation of the phenol-chloroform extract was dried and prepared for reprecipitation with CTAB, a cationic detergent, as described in Materials and Methods. The pellet was reprecipitated with 70% ethanol, then 95% ethanol, dissolved in 10 mM Tris-HCl pH 8.1 0.1 M EDTA, heated to 100°C for 1 minute, combined with 1/5 volume of 3M KCl and applied to a nitrocellulose column of 3 ml bed volume. A "high salt" (0.5 M KCl, 10 mM Tris-HCl 0.1 mM EDTA pH 7.4) followed by a "low salt" solution (10 mM Tris-HCl, 0.1 mM EDTA pH 7.4) was passed through the column. The DNA was eluted in the low salt fraction, and was about 80% of the DNA that was applied. The DNA in the low salt fraction was recovered by isopropanol precipitation. ● , <sup>3</sup>H, ○ , <sup>14</sup>C.

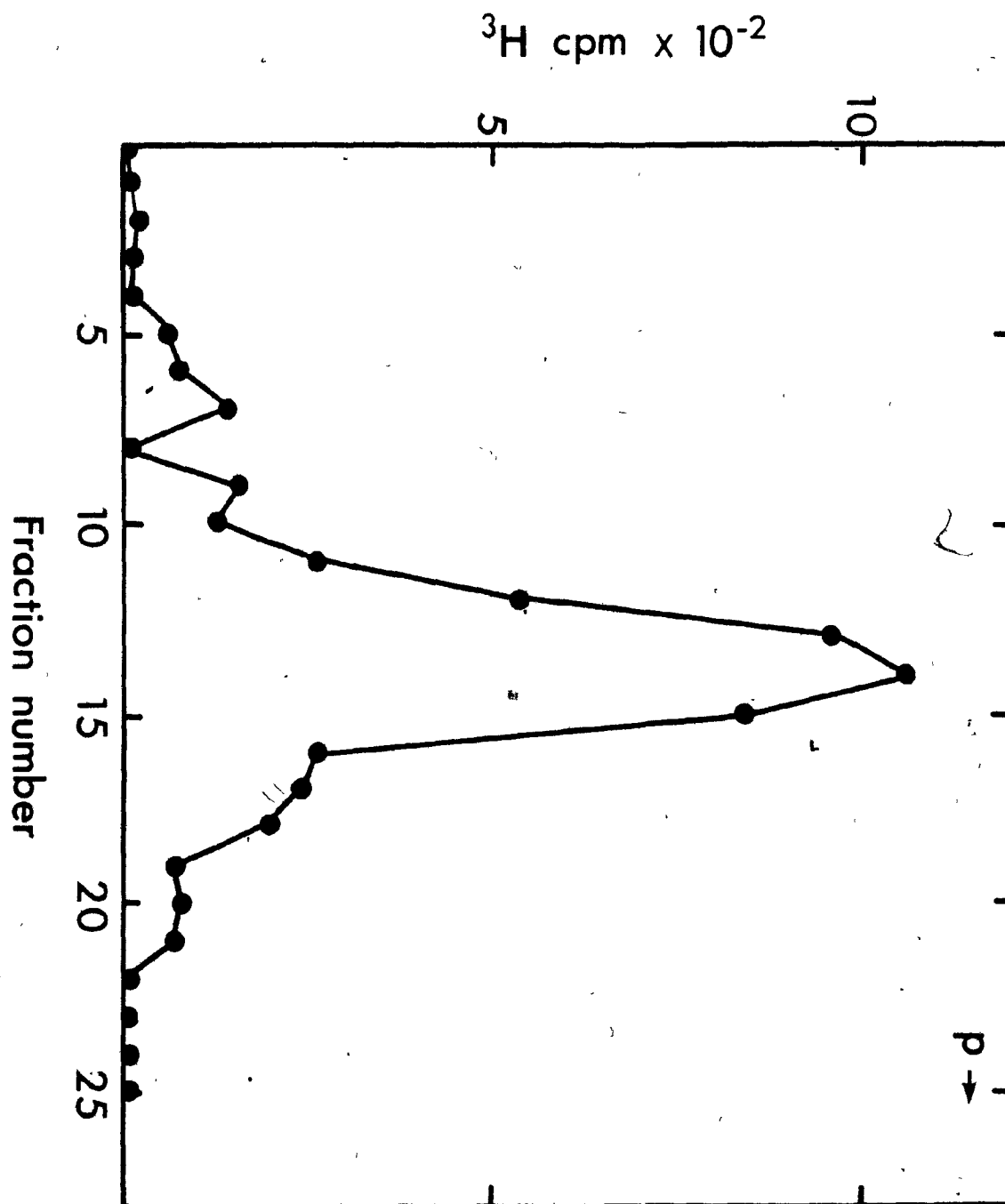




### Figure Legend

#### Fig. 7: Equilibrium buoyant density gradient centrifugation of DNA.

The low salt fractions from the nitrocellulose column containing the  $^3\text{H}$ -labeled DNA were pooled and the DNA precipitated with isopropanol. The precipitate was dissolved in 50 mM Tris-HCl pH 7.6 5 mM EDTA, heated at  $100^\circ\text{C}$  for 30 seconds and made up to a density of a 1.7 g/ml with CsCl. Centrifugation was performed at 40,000 rpm for approximately 60 hours at  $10^\circ\text{C}$  in the A321 rotor of the LEC B60 ultracentrifuge. Fraction 0.3 ml were collected from the top with a Buchler Auto Densi Flow apparatus. Aliquots of the fractions were assayed for  $^3\text{H}$  as described in Materials and Methods. ● ,  $^3\text{H}$ .

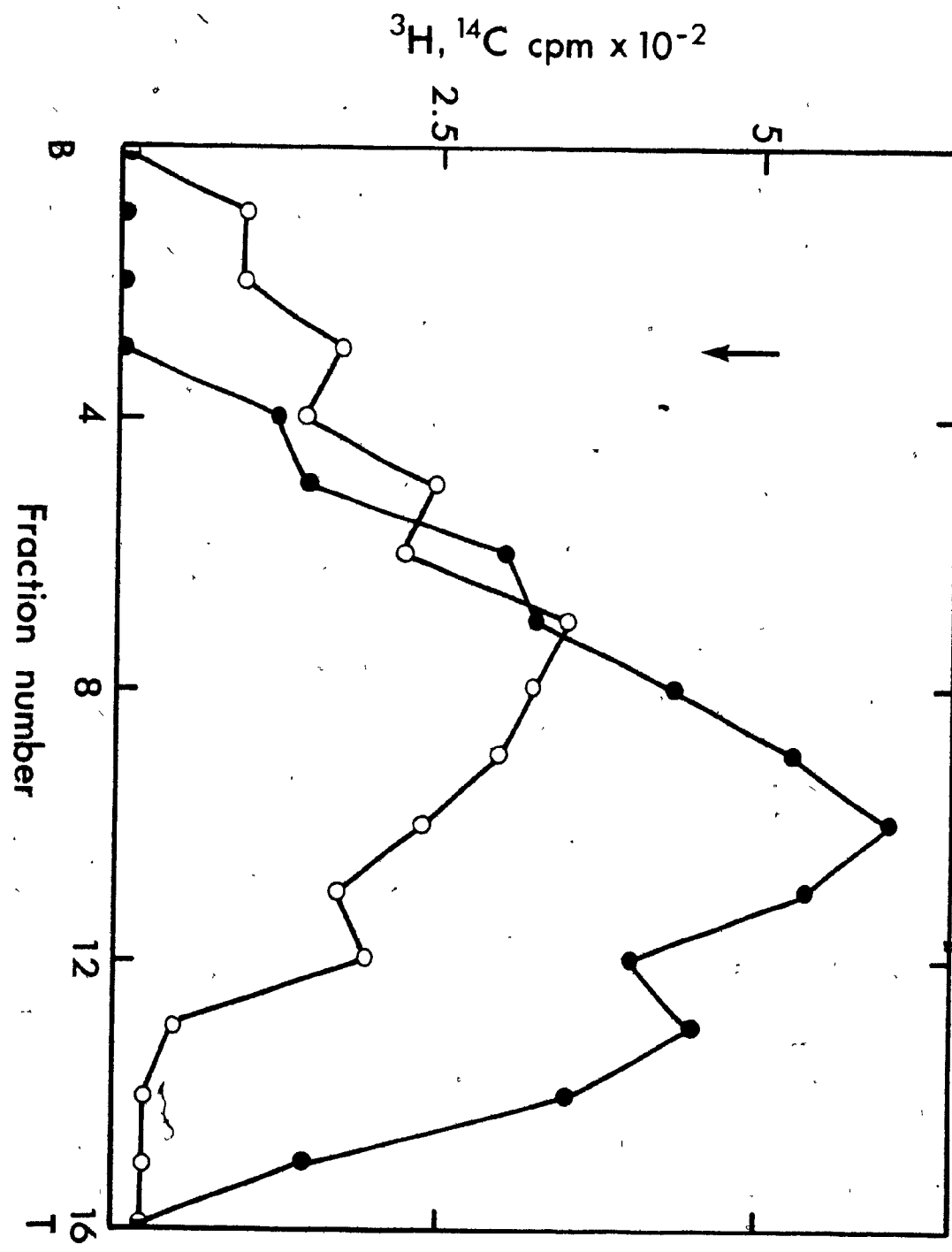


Alkaline sucrose gradients (5-20%) were run to compare the results of Okazaki fragments purified by the Gautschi and Clarkson method (Fig. 9a) with those obtained by direct lysis of pulse-labeled cells on the gradients (Fig. 9b). The significant result here is that in both cases, there is a peak of  $^3\text{H}$  cpm at the top of the gradient, present at the second fraction from the top. The [ $^{14}\text{C}$ ]-labeled bulk in contrast has migrated to the bottom of the gradient. An SV40 marker, which is 16 S under these conditions, migrates 5 fractions into the gradient. A size estimate of approximately 6.5 S - 7 S ( $1.6 - 2.3 \times 10^5$ ) is thus obtained for the small molecular weight DNA.

### Figure Legend

Fig. 8: Sedimentation velocity centrifugation of DNA molecules in a neutral sucrose gradient.

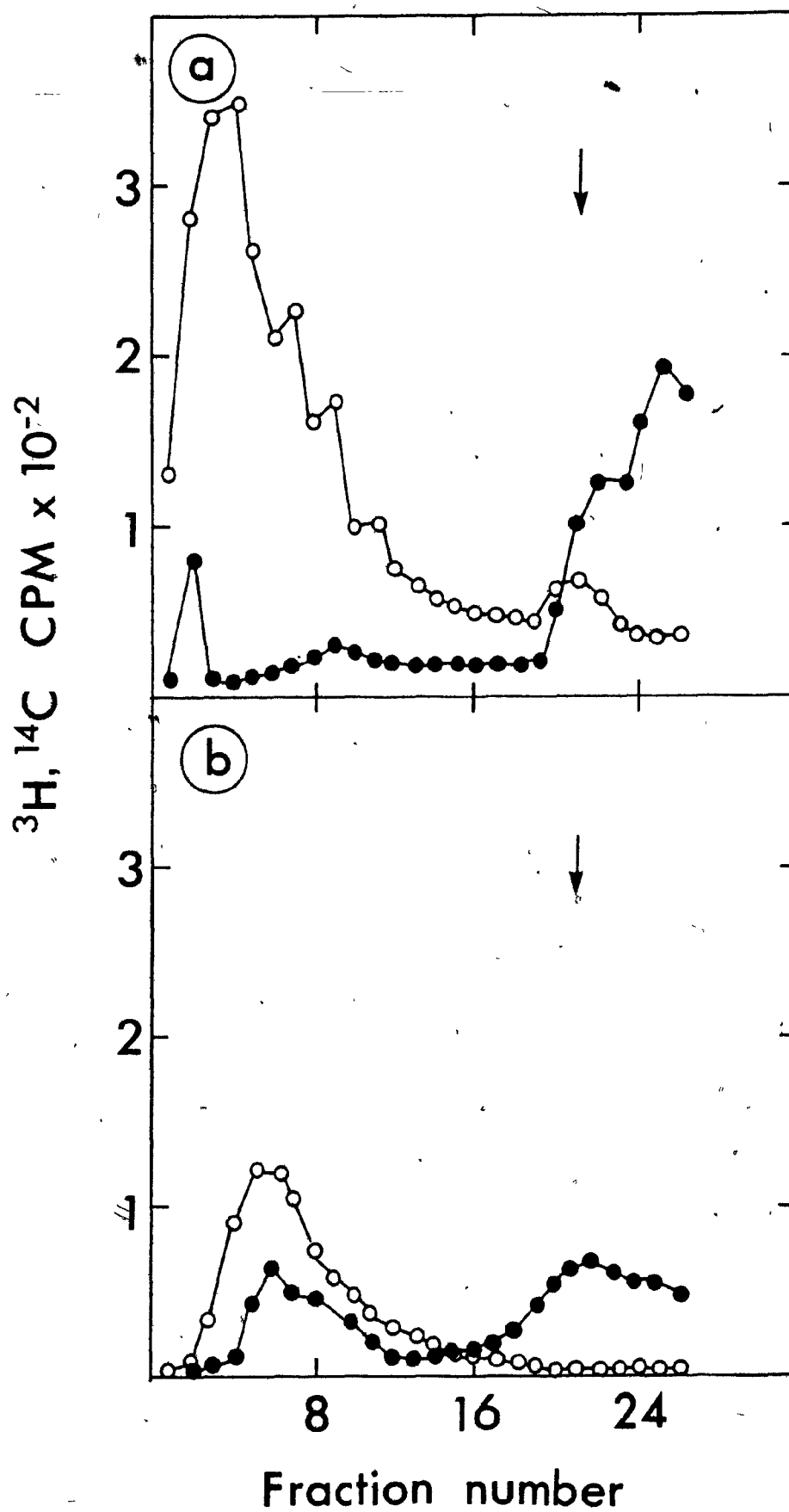
Fractions from the CsCl gradient containing the DNA were diluted 5-fold with 50 mM Tris-HCl pH 8.1 and precipitated with isopropanol. The pellet was suspended in 0.05 M Tris-HCl pH 8.1, heated for 30 seconds at 100°C, and layered on top of a 5 ml 5-20% sucrose gradient containing 1M NaCl, 1 mM EDTA and 50 mM Tris-HCl pH 8. The DNA was centrifuged in the Beckman SW 50.1 rotor at 50,000 rpm for 3 hours at 20°C. Fractions of 0.3 ml were collected from the bottom of the tube and assayed as described in Materials and Methods. Single-stranded RF 11 OX-174 used as a sedimentation marker is shown. ●, <sup>3</sup>H; ○, <sup>14</sup>C.



### Figure Legend

Fig. 9: Velocity centrifugation in alkaline sucrose gradients of crude DNA lysate (Fig. 9a) and DNA purified by the Gautschi and Clarkson method (Fig. 9b).

CV-cells were pulsed as in the crude lysate preparation in Materials and Methods. Samples of  $1 \times 10^5$  cells (25  $\mu$ l) in 0.3 ml of 0.2 M NaOH - 0.01 M EDTA were layered over 5 ml of 5-20% alkaline sucrose gradients. Gradients were 0.9 M in NaCl, 0.3 M in NaOH, and 0.001 M in EDTA. Cell lysis and DNA denaturation were allowed to proceed at 4°C for 8-12 hours. The purified samples were prepared according to the method based on that of Gautschi and Clarkson described in Materials and Methods. Approximately  $4 \times 10^4$   $^3\text{H}$  cpm (approximately 100  $\mu$ l) were used. Centrifugation was carried out in an SW 50.1 rotor of the Beckman L65B centrifuge at 26,000 rpm for 16 hours at 4°C. Fractions were collected and analyzed as described in Materials and Methods. [ $^3\text{H}$ ]-labeled SV40 DNA used as sedimentation marker is shown. ●,  $^3\text{H}$ ; ○,  $^{14}\text{C}$ .



## Discussion

This series of experiments showed that a gel electrophoresis system could be used as an alternative to alkaline sucrose gradient analysis for DNA replication events in mammalian cells. The results showed the following:

1) When CV-1 cells were prelabeled with [ $^{14}\text{C}$ ]thymidine, pulse-labeled with [ $^3\text{H}$ ]thymidine for 30 seconds at  $37^\circ\text{C}$ , then lysed and run on 0.7% agarose gels, the large [ $^{14}\text{C}$ ]-labeled bulk was found in a peak at the top of the gel, whereas the  $^3\text{H}$  was found in small molecular weight DNA at the gel bottom. One hour chases resulted in both the  $^3\text{H}$  and  $^{14}\text{C}$  cpm comigrating at the gel top. The  $^3\text{H}$  peak at the bottom of the gels present after a 30 second pulse represented the Okazaki fragments which were ligated or chased into large DNA pieces. Therefore, the agarose slab gels displayed the typical pulse and pulse-chase characteristics of Okazaki fragments. They could be used to separate Okazaki fragments from bulk DNA following direct lysis of the cells;

2) When pulse and pulse-chase samples run on alkaline gels were compared with samples run on neutral agarose gels that had been denatured by boiling for 5 minutes, the electrophoretic mobility pattern was the same. Thus, simple boiling immediately followed by electrophoresis was sufficient to separate the small fragments from bulk DNA;

3) Okazaki fragments purified by a modified Marmur procedure (Britten et al., 1974), and by a method based on that of Gautschi and Clarkson (1975) had similar mobility in gels to the fragments released by direct lysis. This same relationship was shown on alkaline sucrose gradients;

4) When the Okazaki fragments from the purified and crude lysate procedure were sized on agarose gels, using Hind III digested SV40 as a



marker, molecular weights of  $2.15 \times 10^5$  and  $2.3 \times 10^5$  were determined for the fragments respectively. A very stringent method of purification based on that of Miyamoto and Denhardt (1977) produced a similar molecular weight of  $2.8 \times 10^5$  for the fragments.

Therefore, gel electrophoresis of crude lysates of DNA represents a suitable alternative to alkaline sucrose gradients for the detection and sizing of Okazaki fragments. The results also infer that for the purposes of sizing, one need not perform extensive purification of DNA before electrophoresis as has been done in the past, since similar sizes were found in both crude and purified samples. This is useful since any purification will cause a loss of pulse-labeled DNA, an important point considering the size of the DNA dealt with and the short pulse-labeling times needed to generate them. Friedman and Mueller (1968) stated that replicating DNA could be lost because of its partition to the interphase fraction during extraction with phenol and chloroform, a prominent step in any purification procedure.

Agarose gel electrophoresis is also useful in that it overcomes some of the technical difficulties encountered in alkaline sucrose gradients. On gradients, low molecular weight DNA will migrate only a short distance into the gradient. It remains at the top along with cell proteins and other debris, which may interfere with good resolution of the fragments. Also, sizing the fragments presents a problem in that there is a lack of precisely characterized marker of small molecular weights. In gels, there is better detection of small molecular weight DNA as it migrates to the gel bottom away from non migratory bulk. Since migration is an exponential function, there is potential for large separation of low molecular weight DNA. Another difficulty was encountered by Horwitz (Edenberg and Huberman, 1975),

who found that when whole cells were lysed on alkaline sucrose gradients, an artifact was seen whose behaviour mimicked the sedimentation properties of true Okazaki fragments. This artifact consisted of a small fraction of unincorporated radioactive precursor which coprecipitated with contaminating cellular proteins that were acid precipitable. Partial purification of DNA prior to running alkaline sucrose gradients was necessary to eliminate this artifact. Apart from the fact that agarose gel electrophoresis eliminates a number of difficulties encountered in sucrose gradient work, it does have a number of positive attributes which make it an excellent technique for a study of this type. The electrophoretic mobility of DNA through agarose gels is a smooth function of the length of the DNA. It is common practice to plot the logarithm of known molecular weights against relative mobility, and obtain a linear plot by which to estimate other molecular weights. Excellent resolution can be obtained for DNAs of molecular weights up to at least  $26.5 \times 10^6$ , a difference of less than 10% being readily resolved even for molecules of this size. Also, gel electrophoresis can provide information about conformation as well as size of DNA. Testing electrophoretic mobility relative to standard DNAs over a wide range of ionic strength, pH, gel concentration, and voltage gradient should readily distinguish among native, single-stranded, linear and circular DNAs. Multiple samples can be compared on the same slab at a fraction of the time, effort, and expense of alkaline sucrose gradients. Also, large sample wells and thick gels can be used for preparative separations. An advantage of the horizontal system of slab gels used in these experiments, is that gels of very low concentration are adequately supported and do not distort during electrophoresis. This makes it possible to measure accurately relative mobilities of large DNAs. This horizontal system makes easy removal of the

gel possible for drying and autoradiography or for staining. Lastly, if ethidium bromide is present during electrophoresis, DNAs can be visualized and photographed without removing the supporting medium from the apparatus or disturbing it in any way. Alkaline sucrose gradients are not this flexible in their handling (McDonnell et al., 1977).

Aside from the use of gel electrophoresis, there are a number of other technical refinements possible in these experiments which could provide a basis for future work. Firstly, the DNA preparations were of necessity double-labeled, the bulk DNA and small molecular weight DNA being identified by [ $^{14}\text{C}$ ]thymidine and [ $^3\text{H}$ ]thymidine respectively. This necessitated the use of gel slicing and scintillation counting to determine radioactivity profiles rather than fluorography, which is a less tedious, higher resolution technique for the detection of radioactivity. It is also advantageous in that it does not destroy the sample (Bonner and Laskey, 1974). Future work should adapt this technique to fluorography, perhaps by using a well established system, where bulk DNA need not be labeled, and only pulse-labeling is necessary. The present experiments demanded double label to establish the presence of a working agarose gel system for Okazaki fragment detection and sizing. Secondly, because of the short pulse times necessary to isolate Okazaki fragments, the absolute number of cpm present in the sample was low. Lyophilization of the sample was performed to concentrate the radioactivity for gel electrophoresis. This is undesirable, in that it represents a form of DNA manipulation and shear, which is best avoided in studies like this (Lehmann and Ormerod, 1969). It is unlikely, however, that bulk DNA would be sheared to Okazaki fragment size and alter the proportion of  $^3\text{H}$  cpm found in that peak (Blumenthal and Clark, 1977a). Conditions which increase the specific activity of the sample, perhaps by

favouring greater incorporation of isotope, or by use of a higher emission energy isotope, should be investigated.

It is valuable to compare the data obtained here for Okazaki fragment size, with the estimates derived from other eukaryotic cell systems. It has been mentioned that there exist many difficulties in studies of this type, both in the detection of Okazaki fragments using alkaline sucrose gradients and in the experimental conditions needed to generate them. First, Okazaki fragments are short-lived. Although readily detected after a 30 second pulse (37°C) of [<sup>3</sup>H]thymidine, they are almost impossible to see after a 2 minute pulse (Huberman and Horwitz, 1973). A second difficulty involves the inability to control the initiation of DNA synthesis. Ideally, in order to detect such intermediates in pulse-label experiments, the start of the pulse and the initiation event should be simultaneous. When this condition is not fulfilled, end labeling of partially replicated molecules obscures the presence of any true DNA intermediates. Kurek and Taylor (1977) attempted to overcome this problem by establishing a block in the cell cycle at the G1/S boundary using FdUrd. They showed that at 10 uM FdUrd, there was no synthesis of TdR via the de novo pathway and that the cells ability to synthesize DNA was primarily limited by the availability of free TdR from the medium. Additional difficulties which concern the use of alkaline sucrose gradients, have been discussed above. Therefore, it is not surprising that so much variation exists in the reported sizes for Okazaki fragments. Table I illustrates some of the sizes estimated in different mammalian and viral systems.

Edenberg and Huberman (1975) state that the most recent and reliable estimates of Okazaki fragment size are about  $5 \times 10^4$  -  $1 \times 10^5$  (4-5S), (Magnusson et al., 1973; Francke and Hunter, 1974; Gautschi and Clarkson,

1975). In the CV-1 system, the smallest replication intermediates have been sized at  $2.3 \times 10^5$  (6.5-7 S) and  $2.15 \times 10^5$  (6.5-7 S) for the crude and purified samples respectively. These sizes compare favourably to but are somewhat larger than the currently accepted value of  $5 \times 10^4 - 1 \times 10^5$  (4-5S). This may be due to the fact that pulse-labeling was done for 30 seconds at 37°C. Shorter pulses may have resulted in the isolation of a smaller replication intermediate.

Table I

<u>Organism</u>	Replication	Sedimentation	<u>Reference</u>
	Intermediate Size (Molecular Weight)	Coefficient in Alkaline Sucrose Gradients	
Human (heteroploid cell line EUE)	$1 \times 10^5$	5 S	Nuzzo et al., 1970
Human (HeLa)	$5 \times 10^5$	10 S	Kidwell and Mueller, 1969
Human (lymphocyte cell line 8866)	$5 \times 10^4$	4 S	Tseng and Goulian, 1975
Chinese Hamster (B14 FAF 28-G3)	$3.3 \times 10^5$	9 S	Schandl and Taylor, 1969
Chinese Hamster (CHO)	$5 \times 10^4$	4 S	Hubermann and Horwitz, 1973
Mouse (P-815)	$5 \times 10^4$	4 S	Gautschi and Clarkson, 1975
Drosophila	$5 \times 10^4$	4 S	Blumenthal and Clark, 1977
SV40	$5 \times 10^4$	4 S	Salzman and Thoren, 1973
Polyoma	$5 \times 10^4$	4 S	Pigiet et al., 1973

It is also interesting to speculate whether or not synthesis is semi -- or totally discontinuous in the CV-1 system. The results have shown that after a 30 second pulse, approximately 37 - 45% of  $^3\text{H}$  cpm migrates as low molecular weight DNA or the Okazaki fragment peak at the bottom of the gel, whereas the remaining  $^3\text{H}$  cpm are found in larger molecular weight DNA. The kinetic approach of determining whether synthesis at the replication fork is semi -- or totally discontinuous states, that there should be the same amount of label in both classes at short pulse times if synthesis is semi-discontinuous. A very tentative observation for our system, therefore, is that synthesis appears to be semi-discontinuous. Shorter pulse times or lower incubation temperatures at the time of pulse-labeling would help clarify this situation, since it would increase the likelihood of isolating Okazaki fragments and decrease the time available for ligation of small fragments. A more certain approach to this question would be to determine whether the Okazaki fragments can anneal to each other, annealing of the fragments from one fork would be proof that synthesis at that fork is totally discontinuous. These are both future considerations.

Lastly, it is valuable to consider how this system can be applied to or integrated with the work done by others in the field of DNA replication. Southern (1975) has described an interesting method for the detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. In this system DNA fragments are transferred from agarose gels to cellulose nitrate filters and then hybridized to radioactive RNA. The hybrids are detected by radioautography or fluorography. Similarly, Okazaki fragments isolated by agarose gel electrophoresis could be investigated for RNA primers using such a system.

### Conclusion

The results indicate that an agarose gel electrophoresis system is a useful alternative to alkaline sucrose gradients for the isolation and sizing of Okazaki fragments. The slab gels accurately display the typical pulse and pulse-chase characteristics of Okazaki fragments. They can be used to separate Okazaki fragments from bulk DNA following direct lysis of the cells. Also, the purified Okazaki fragments have similar mobility in gels to the fragments released by direct lysis. Lastly, Hind III digested SV40 resulted in six fragments whose log molecular weight versus mobility in gels gave a linear plot enabling accurate sizing of the Okazaki fragments.

In conclusion, this system is of value since it would permit better resolution and more precise sizing of Okazaki fragments than the conventional alkaline sucrose gradient. In gels, the low molecular weight fragments migrate to the gel bottom, whereas in gradients they are found at the top along with cell proteins, unincorporated radioactive precursors and other cell debris. The estimates of Okazaki fragment size, using this method, are  $2.3 \times 10^5$  and  $2.1 \times 10^5$  molecular weight for the crude lysate and purified DNA preparations, respectively.



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## APPENDIX I

The Use of Agarose Gel Electrophoresis to Measure the Size of DNA  
Molecules in Crude Cell Lysates

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[ABSTRACT]

We have used agarose gel electrophoresis to measure the size of DNA molecules taken directly from crude cell lysates. Pulse-labeled DNA from CV-1 cells that was denatured migrated as small molecules the size of Okazaki pieces when it was subjected to electrophoresis on 0.7% agarose gels under alkaline and neutral conditions. The gels also displayed the incorporation of the Okazaki pieces into bulk DNA when the DNA was labeled by pulse-chase techniques. The migration of the DNA from crude cell lysates was identical to DNA purified by chloroform-isoamyl alcohol extraction, and estimates of the size of the pulse-labeled molecules were similar by electrophoresis and by alkaline sucrose gradient centrifugation. These results indicate that, in these experimental conditions, agarose gel electrophoresis can be used in the same manner as alkaline sucrose gradient centrifugation to analyze replicating DNA from crude cell lysates.

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## [INTRODUCTION]

Agarose gel electrophoresis has been a valuable technique for sizing DNA molecules. It has been used to analyze Okazaki pieces (1) in experiments on DNA replication in mammalian cells and important observations on size and structure of the pieces have been made (2-4). In these analyses, the Okazaki pieces have been purified from cell lysates before they were subjected to electrophoresis. The extraction procedures aimed at purifying nascent DNA from mammalian cells cause some loss of small molecules and may also cause breakdown of large DNA molecules.

To avoid losses and to reduce the chance of breakdown of large molecules, many researchers lyse cells and denature the DNA directly atop alkaline sucrose gradients. This keeps manipulation of the molecules to a minimum. Subsequent analysis by velocity centrifugation gives reproducible sedimentation rates of the DNA molecules that can be used to estimate their molecular weights. Although useful, the procedure has some drawbacks. To prepare and analyze many gradients is tedious and time-consuming. Equipment used for the analyses, such as ultracentrifuges and scintillation counters, are expensive. Compared to ultracentrifugation, gel electrophoresis is simple, inexpensive and fast.

In the experiments in this paper, we examined the possibility of using agarose gel electrophoresis to analyze DNA from crude lysates of mammalian cells. Our results show that it can be substituted for alkaline sucrose gradients in the experimental conditions we have used.

## MATERIALS AND METHODS

Cells. CV-1 cells, a line derived from African green monkey kidney cells, were grown and maintained in minimal essential medium (MEM)<sup>1</sup> with 5% fetal calf serum (FCS) as described previously (5). The cells were grown in 75 cm<sup>2</sup> cell culture flasks at 37°C until confluent, at which point  $2 \times 10^5$  cells were transferred to new flasks.

[<sup>3</sup>H]thymidine labeling and cell lysis. Cells that had been seeded the day before at  $6 \times 10^5$  cells per 60 mm plastic culture dish were labeled with [<sup>14</sup>C]thymidine (0.1  $\mu$ Ci/ml; 50 mCi/mmol), for approximately 24 h. The radioactive medium was then removed and the cells were washed and incubated in fresh MEM for 2 h. FdUrd ( $2 \times 10^{-6}$  M) was then added for 1 h, after which the cells were either pulse-labeled with [<sup>3</sup>H]thymidine (100  $\mu$ Ci/ml; 50 Ci/mmol) for 30 sec at 37°C, or pulse-labeled and chased for 1 h in medium containing  $2 \times 10^{-5}$  M nonradioactive thymidine. The FdUrd was used to allow rapid labeling of the Okazaki pieces by exogenous [<sup>3</sup>H]thymidine and to increase the incorporation of the isotope. After the cells were washed with cold phosphate-buffered saline (PBS), they were trypsinized immediately and suspended in cold PBS. They were then lysed in 0.3 M NaCl, 0.015 M sodium citrate and 1% sarcosyl for 1 h at room temperature, and the lysates were lyophilized and dissolved in small volumes of electrophoresis buffer. The NaCl concentration of the resuspended lysate was 2.0 M. These preparations, which had been concentrated five-fold by lyophilization, served as the crude lysates of DNA for subsequent electrophoresis and gradient centrifugation experiments. Crude lysates were stored for not longer than 24 h at -20°C so as to keep the degradation of the DNA to a minimum.

Preparation of purified DNA. This was a modification of the method described by Gautschi and Clarkson (6). The concentration of NaCl in the cell lysate was adjusted to 1 M and the lysate was left overnight at 4°C. The precipitate was removed by centrifugation. The supernatants were incubated for 30 min at 37°C with pronase and the remaining protein was removed by 2 rounds of extraction with chloroform-isoamyl alcohol (24:1 v/v). The deproteinated sample was then precipitated with ethanol and prepared for electrophoresis.

Agarose gel electrophoresis. A horizontal slab gel system for electrophoresis (7) was used in these experiments. The buffer for neutral gel electrophoresis was 0.04 M Tris, pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA. To prepare gels, 1.4% melted agarose (SeaKem, Marine Colloids Inc.) in distilled water was mixed with an equal volume of twice-concentrated neutral electrophoresis buffer to give a final agarose concentration of 0.7%. Samples of lysate derived from  $10^5$  cells in a volume of 25  $\mu$ l were heated for 5 min at 100°C and applied to the gel. Bromophenol blue (BPB) was run in a separate well as a marker dye. Electrophoresis was carried out at room temperature until the BPB had migrated to a distance of 5-8 cm, generally a period of 5 h at 40 millivolts. After electrophoresis, the gels were soaked in 10% trichloroacetic acid — 0.5% sodium pyrophosphate for 1 h to precipitate the DNA and to complex unincorporated  $^3\text{H}$ . The gel columns were cut from the slabs and sliced into 1 mm fractions using stacked razor blades. Slices were analyzed for radioactivity by liquid scintillation counting.

For alkaline gel electrophoresis, the gel buffer consisted of 30 mM NaOH and 2 mM EDTA, pH 12.2 (7). The denatured samples were dissolved in neutral electrophoresis buffer rather than in alkali. We used a neutral

buffer here to reduce the chance of breakdown of the small DNA fragments, since these samples were also heated for 5 min at 100°C to match the conditions we used for sample preparation for neutral gels. Electrophoresis and subsequent processing of the alkaline gels were the same as outlined above. With both neutral and alkaline gels, more than 75% of the  $^3\text{H}$  counts loaded onto the gels were recovered after electrophoresis.

Velocity sedimentation in alkaline sucrose gradients. Gradients of 5 ml of 5-20% sucrose in alkali (pH 12.2) were formed over a cushion of 70% sucrose (8) and centrifugation was carried out as described by Zannis-Hadjopoulos et al. (9).  $^3\text{H}$ -labeled SV40 DNA form II was used as a marker.

Preparation of SV40 DNA and its cleavage by Hind III restriction endonuclease. Viral DNA was prepared from CV-1 cells infected with SV40. The Hirt supernatant (10) was purified and subjected to CsCl-ethidium bromide centrifugation. Form I DNA was digested with Hind III restriction endonuclease as described by Danna et al. (11).



## RESULTS AND DISCUSSION

Pulse-labeled and pulse-chase labeled DNA from crude lysates of CV-1 cells was subjected to agarose gel electrophoresis. For technical reasons, we could not lyse the cells directly on the gels. We were unable to load sufficient amounts of radioactivity in the required small volumes on the gels to allow analysis. We resorted to lysing the cells in neutral buffer containing sarkosyl and then lyophilizing the lysate to concentrate it. We chose lyophilization as the method for concentrating our samples since the purpose of these experiments was to examine whether DNA from unpurified samples could be analyzed by electrophoresis. Other concentration procedures such as ethanol precipitation might have introduced some degree of purification. The dried lysate was resuspended in electrophoresis buffer and the DNA was denatured by heating it to 100°C immediately before it was applied to the gel. The patterns displayed on electrophoresis by pulse-labeled and pulse-chase labeled DNA prepared in this way are shown in Fig. 1.

On gels prepared and run in alkaline buffer (Fig. 1a, b), 60% of the pulse-labeled DNA migrated faster than PhiX 174 DNA replicative form. The rest of the pulse-labeled DNA migrated more slowly and its distribution overlapped the distribution of bulk DNA that had been uniformly labeled with [ $^{14}\text{C}$ ]thymidine. The pulse-chase-labeled DNA migrated exclusively as large DNA molecules the size of bulk DNA. If the gels were prepared and run under neutral conditions, identical results were obtained except that small discrete peaks in the middle of the gels were found with pulse-labeled, pulse-chase-labeled and uniformly labeled DNA (Fig. 1c, d). These intermediate peaks contain high molecular weight DNA. We do not know the reason for the multiple peaks of high-molecular-weight material

in the neutral gels, but whatever the cause of these artifacts, they seem to be less evident in alkaline gels. We obtained identical patterns of migration when purified DNA was subjected to electrophoresis (data not shown). Thus, gel electrophoresis gives qualitatively the same sort of analysis as alkaline sucrose gradient centrifugation. Furthermore, the denatured DNA displays essentially the same migration pattern in alkaline and neutral gels. Apparently, the DNA can be maintained in the denatured state during electrophoresis despite neutral pH. The gels prepared in neutral buffer were much easier to slice and prepare for scintillation counting.

To examine further whether electrophoresis could be substituted for alkaline sucrose gradient centrifugation, we directly compared pulse-labeled DNA samples subjected to the two procedures. As shown in Fig. 2, crude and purified pulse-labeled DNA migrated as low-molecular weight species on gels and gradients. In order to obtain a more accurate estimate of the molecular weight of the pulse-labeled DNA, we compared its migration to that of marker DNAs prepared by digesting purified SV40 DNA with restriction enzyme Hind III. The markers were run on the same gel and the positions of the 4 largest SV40 fragments are plotted in Fig. 2a. The range of molecular weights is large. From fractions 36 to 63 (that is, those fractions within the peak that have at least 50% of the counts of the peak fraction), the molecular weights range from  $1 \times 10^5$  (323 nucleotides) to  $6.8 \times 10^5$  (2200 nucleotides). The peak fractions, from 44 to 56, containing 60% of the counts, range in molecular weight from  $1.6 \times 10^5$  (516 nucleotides) to  $3.1 \times 10^5$  (1000 nucleotides). The range of molecular weights for the purified Okazaki pieces is similar. These molecular weights are three to six times larger than those obtained when

purified Okazaki fragments synthesized in vitro are sized by gel electrophoresis (4). Because the DNA was labeled in vivo, ligation of the Okazaki pieces was unimpaired, and we may be looking at DNA fragments composed of three to six ligated Okazaki pieces. On the other hand, we cannot completely exclude the possibility that Okazaki pieces synthesized in vivo in mammalian cells are larger than those synthesized on papovavirus DNA templates in vitro (4) or in vivo (2). The DNA molecules are replication intermediates since, as shown in Fig. 1, they can be chased into bulk DNA. The molecules most probably did not arise as a result of incorporation and subsequent excision of uracil-containing nucleotides, since this mechanism of generating small DNA molecules is not significant in mammalian cells (12, 13). In our hands, the small DNA molecules from crude lysates had the same size distribution as purified Okazaki pieces. This indicates that purification is not necessary for size determination, and that the losses in yield from purification may be avoided, as they are in analyses of these molecules on alkaline sucrose gradients. Electrophoresis offers several advantages over gradient centrifugation. Multiple samples may be run under identical conditions in the same gel and markers can be incorporated into the gel. This can give a marked increase in accuracy of molecular weight determinations and also allow precise comparisons of DNA subjected to different experimental manipulations. The speed and simplicity of electrophoresis has already been alluded to. In general, the resolution of electrophoresis is higher than that of gradient centrifugation, but we have not examined that here.

We used DNA labeled with both  $^{14}\text{C}$  and  $^3\text{H}$  in these experiments in order to examine the size of Okazaki pieces and bulk DNA in the same samples. In future experiments, it should be possible to label the

Okazaki pieces with a single isotope such as  $^3\text{H}$ . The distribution of bulk DNA could be determined by its fluorescence after staining with a dye such as Hoechst 33258. After destaining, the gel could be subjected to fluorography to analyze the distribution of the Okazaki pieces.

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[FIGURE LEGENDS]

Fig. 1. Gel electrophoresis of denatured DNA from lysates of CV-1 cells. The DNA was uniformly labeled with [ $^{14}\text{C}$ ]thymidine, and then pulse-labeled with [ $^3\text{H}$ ]thymidine for 30 sec (a, c) or pulse-labeled for 30 sec and then subjected to chase conditions with unlabeled thymidine for 1 h (b, d). The labeled cells were lysed and lyophilized. The crude lysates from  $10^5$  cells were placed in electrophoresis buffer and heated to  $100^\circ\text{C}$  for 5 minutes. The heated preparations were applied to alkaline agarose gels (a, b) or neutral gels (c, d) and subjected to electrophoresis. (●), [ $^3\text{H}$ ]DNA; (○), [ $^{14}\text{C}$ ]DNA. The arrow in each panel marks the position of PhiX 174 DNA run in the same slab gel as a marker.

Fig. 2. Comparison of gel electrophoresis and alkaline sucrose gradient analysis of DNA. Cellular DNA was uniformly labeled with [ $^{14}\text{C}$ ]thymidine and subjected to pulse labeling with [ $^3\text{H}$ ]thymidine for 30 sec. The cells were lysed and the extracts analyzed directly after lyophilization (a, c) or the DNA from the extracts was purified (b, d). The samples were denatured by heating and subjected to electrophoresis on neutral agarose gels (a, b) or layered directly on alkaline sucrose gradients and centrifuged (c, d). (●), [ $^3\text{H}$ ]DNA; (○), [ $^{14}\text{C}$ ]DNA; (▲), molecular weight markers derived by digestion of SV40 DNA with restriction enzyme Hind III. The ordinate on the right in panel a indicates the  $\log_{10}$  molecular weight of these markers and the positions of the 4 largest SV40 fragments are shown. The arrows in panels c and d show the position of SV40 DNA centrifuged at the same time in a separate gradient. Sedimentation is from right to left in the gradients.

## [FOOTNOTES]

<sup>1</sup> Abbreviations used: MEM, minimal essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TNE, Tris 0.05 M, NaCl 0.15 M, EDTA 0.05 M, pH 8; BPB, bromophenol blue; SV40, simian virus 40.



