DISCONTINUOUS DNA SYNTHESIS IN MAMMALIAN CELLS

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ABSTRACT

Many of the techniques used to analyze mammalian DNA on sucrose gradients give artifactual results. When a cell lysate is treated with alkali and sedimented through an alkaline sucrose gradient without purifying the DNA, an artifactual peak of acid precipitable radioactivity appears in the Okazaki peak region. Several methods used to purify DNA before centrifugation remove this artifact, but also cause breakdown of the bulk DNA; while some methods do not even remove the artifact. Two procedures were found that seem to remove the artifactual peak without degrading the DNA.

Treating the DNA in a way that does not produce any of these artifactual results allows observation of the course of DNA synthesis in mammalian cells. It seems that the DNA on both sides of the replication fork is first synthesized in the form of very short fragments ($\sim$ 100 nucleotides long), equivalent to the Okazaki fragments of bacteria. These fragments are joined to growing replicons which show up on alkaline sucrose gradients as an "intermediate" peak of DNA. The replicons, when completed, join together to form "bulk" DNA.

Inhibitors of protein synthesis severely reduce the incorporation of (^{3}H) thymidine without causing much change in the shapes of the sedimentation profiles. Nascent DNA seems to move from the Okazaki peak to the intermediate peak at the same rate whether or not these inhibitors are present. Various explanations of this result are proposed, and the implications of each scheme are considered.

Inhibitors of DNA synthesis cause dramatic changes in the sedimentation profiles of pulse labeled DNA. Hydroxyurea causes a build-up of small fragments and the disappearance of the intermediate peak. A constant percentage of the acid-precipitable radioactivity appears in the Okazaki peak over a wide range of pulse-times. Total incorporation decreases to about 2% of the normal rate.

At first, Ara C also causes a build-up of small fragments, but an intermediate peak eventually appears. The pattern of DNA synthesis appears similar to that of normal synthesis, but on a greatly expanded time scale. Incubating with FUdR also causes a build-up of small fragments, but no intermediate peak appears. Pulse labeling these cells with thymidine (instead of deoxycytidine) produces very unusual sedimentation profiles, not at all similar to the DNA sedimentation profiles of uninhibited cells.

Rationalizations for all these findings are offered and the possible implications of the results obtained by FUdR inhibition are considered.

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To my parents

INTRODUCTION

I. Discontinuous DNA synthesis

Discontinuous DNA synthesis in bacteria was first demonstrated in bacteria by Sakabe and Okazaki (1966), who showed that short strands of DNA (about 1000 nucleotides long) are rapidly synthesized and then apparently converted to longer strands by a joining process. Sakabe and Okazaki suggested that these short strands could explain the apparent discrepancy between the fact that one strand of the DNA helix must grow in the 3'⟶5' direction and the fact that known DNA polymerases synthesize DNA only in the 5'⟶3' direction. They proposed that these pieces are made on at least one strand in the 5'⟶3' direction and then joined together in an overall sequence of 3'⟶5'.

A. Bacterial DNA synthesis

1. Okazaki fragments

Since then discontinuous DNA synthesis has been repeatedly demonstrated in bacteria (Okazaki et al., 1968a,b; Yudelevich et al., 1968; Geider & Hoffman-Berling, 1971; Wang & Sternglanz, 1972; Dingman et al., 1974) and in phage (Sadowski et al., 1968; Polsinelli et al., 1969; Sugino & Okazaki, 1972), and evidence was obtained showing that all the Okazaki pieces are made in the 5'⟶3' direction (Sugino & Okazaki, 1972). Only Werner and coworkers disagree with this scheme,

claiming that Okazaki fragments are made through a process of random "crystallization" of nucleotides on the parental template rather than sequentially in a 5'⟶3' direction (Diaz et al., 1975; Diaz & Werner, 1975). However, their arguments seem very unclear and it is not at all certain that their pulse times were short enough to insure that most of the nascent Okazaki pieces were not labeled along their whole length.

2. Enzymes needed for joining

Bacterial Okazaki fragments chase into bulk-sized DNA (Sadowski et al., 1968), and some work has been done on elucidating the enzymes involved. DNA ligase is considered to be necessary for joining the fragments together (Hosoda & Mathews, 1968; Newman & Hanawalt, 1968a,b; Okazaki et al., 1968a; Sugimoto et al., 1968; Nozawa & Nizuno, 1969; Pauling & Hamm, 1969; Olivera & Lundquist, 1971; Sugino & Okazaki, 1972; Gottesman et al., 1973a,b). There have been reports that the peak of acid-precipitable radioactivity appearing near the top of the gradient when the DNA is sedimented through alkaline sucrose seems to slowly grow larger even when the ligase is supposedly absent or inactivated. However, in these experiments, there is probably some residual ligase activity or, in the case of phage, the bacterial ligase may be doing the joining (Newman & Hanawalt, 1968; Sugimoto et al., 1968; Pauling & Hamm, 1969; Olivera & Lundquist, 1971). There is a report that small sized fragments seen

at low concentrations of deoxynucleotides (see below) do not require the NAD (nicotinamide-adenine dinucleotide)-dependent ligase to join into larger pieces (Hess et al., 1973), but there is probably some other ligase that joins these pieces.

DNA polymerase I is apparently also required for joining (Sadowski et al., 1968; Yudelevich et al., 1968; Kuempel & Veomet, 1970; Okazaki et al., 1971), and Konrad & Lehman (1974) have demonstrated that a necessary activity is that of the 5'⟶3' exonuclease associated with the polymerase. Pol A^- cells also seem to be able to slowly join Okazaki fragments into long DNA in vitro (Geider & Hoffman-Berling, 1971; Okazaki et al., 1973), but probably in such cases there is residual enzyme activity.

Recently a new mutant of E. coli has been obtained which makes fragments much smaller than normal-sized Okazaki fragments (Konrad & Lehman, 1975). One implication of this sof (small Okazaki fragments) mutation is that the first intermediate in E. coli DNA synthesis may be smaller (4-5S) than originally detected (10S), and the mutation may cause some ligase deficiency that makes these pieces observable.

B. Okazaki fragments in other organisms

When I first started this project, there were several researchers who did not find Okazaki fragments in higher organisms (Tsukada et al., 1968; Lehmann & Ormerod, 1969, 1970; Habener et al., 1969b; Berger & Irvin, 1970; Hyodo et al., 1970; M. S. Horwitz, 1971). Now, however,

there is general agreement that Okazaki fragments are made by mammalian cells in vivo (Painter, 1968; Mueller, 1969; Painter & Schaefer, 1969b; Schandl & Taylor, 1969, 1971; Ensminger & Tamm, 1970; Nuzzo et al., 1970; Sato et al., 1970; Taylor et al., 1970, 1973a; Hyodo et al., 1971; Probst et al., 1971; Cheevers et al., 1972; Fox et al., 1973; Goldstein & Rutman, 1973; Berger & Huang, 1974; Friedman et al., 1975; Gautschi & Clarkson, 1975; Mendelsohn et al., 1975; Tseng & Goulian, 1975a) and in vitro (Kidwell & Mueller, 1969; Fox et al., 1973; Friedman, 1974; Hershey & Taylor, 1974; Tseng & Goulian, 1975a). They are also seen in other eukaryotes: sea urchins (Baker, 1971), Drosophila melanogaster (Kriegstein & Hogness, 1974) and Physarum polycephalum (Waqar & Huberman, 1975a); in higher plants (Sakamaki et al., 1975); and in viruses: CELO (Bellet & Younghusband, 1972), Adenovirus (Vlak et al., 1975), Polyoma (Cheevers et al., 1972; Magnusson, 1973; Pigiet et al., 1973, 1974; Hunter & Francke, 1974; Francke & Hunter, 1974a,b; Otto & Reichard, 1975) and SV40 (Fareed & Salzman, 1972; Fareed et al., 1973; Salzman & Thoren, 1973; Salzman et al., 1973; Qasba, 1974a,b; DePamphilis & Berg, 1975). In this thesis I present evidence confirming the existence of Okazaki fragments in mammalian cells.

C. Size of Okazaki fragments

The Okazaki fragments made by bacteria and phage are generally assumed to be between 1000 and 2000 nucleotides in length (7-11S) (Okazaki et al., 1968a; Yudelevich et al., 1968; Polsinelli et al., 1969),

and there are some reports of much smaller fragments (Geider & Hoffman-Berling, 1971; Wang & Sternglanz, 1972, 1974; Hess et al., 1973; Konrad & Lehman, 1975; Lark & Wechsler, 1975) that may or may not be precursors to the 7-11S pieces.

For the Okazaki fragments of other organisms there have been a wide range of sizes reported, ranging from 1000-2000 nucleotides (Kidwell & Mueller, 1969; Mueller, 1969; Painter & Schaefer, 1969; Schandl & Taylor, 1969, 1971; Taylor et al., 1970, 1973a; Baker, 1971; Bellett & Younghusband, 1972; Cheevers et al., 1972; Goldstein & Rutman, 1973; Friedman, 1974; Sakamaki et al., 1975; Vlak et al., 1975), to 300 nucleotides (Nuzzo et al., 1970) to 50-200 nucleotides (Hyodo et al., 1971; Fareed & Salzman & Thoren, 1973; Francke & Hunter, 1974a,b; Kriegstein & Hogness, 1974; Pigiet et al., 1974; Qasba, 1974b; Friedman et al., 1975; Gautschi & Clarkson, 1975; Mendelsohn et al., 1975; Tseng & Goulian, 1975a) to 10-20 nucleotides (Schandl & Taylor, 1969, 1971; Taylor et al., 1970; Schandl, 1972). In this thesis I present evidence that the Okazaki fragments of mammalian cells are about 100 nucleotides long, and I discuss why some researchers found other values.

D. Similarities between prokaryotic and eukaryotic fragments

1. Are the fragments single or double stranded?

Although there seems to be a large difference between the size of the Okazaki fragments in prokaryotes and eukaryotes, several of the other physical characteristics of Okazaki fragments appear similar.

The question of whether the fragments are single or double stranded before denaturation has not been decided in either case. Some report that they are mainly single stranded after lysis (Okazaki et al., 1968a; Painter & Schaefer, 1969; Sato et al., 1970; Cheevers et al., 1972; Mendelsohn et al., 1975) while others say they are not (Tsukada et al., 1968; Yudelevich et al., 1968; Fareed & Salzman, 1972; Gautschi & Clarkson, 1975). Probably the fragments are observed as single or double stranded on sucrose gradients depending on the severity of the procedures used to lyse the cells, and these experiments shed little light on the question of what state the fragments are in inside the living cells.

2. RNA primers

Evidence that Okazaki fragments are primed by stretches of RNA has been obtained in a wide variety of organisms. The existence of such RNA primers would resolve the difficulty presented by the fact that no known DNA polymerase can function without a primer. RNA covalently attached to Okazaki fragments has been detected in E. coli (Sugino et al., 1972; Hirose et al., 1973; Okazaki et al., 1973, 1975; Sugino & Okazaki, 1973), colicinogenic Factor E1 (Blair et al., 1972), phage (Brutlag et al., 1971; Miller, 1972; Schekman et al., 1972; Wickner et al., 1972), Physarum (Waqar & Huberman, 1973, 1975a), polyoma (Magnusson et al., 1973; Sadoff & Cheevers, 1973; Hunter & Francke, 1974b; Pigiet et al., 1974; Reichard et al., 1974), SV40 (Qasba, 1974b),

and mammalian cells (Sato et al., 1972; Fox et al., 1973; Taylor et al., 1973b; Neubort & Bases, 1974; Tseng & Goulian, 1975b; Qaqar & Huberman, 1975b). Evidence for RNA has been found either by Cs_2SO_4 equilibrium density gradient centrifugation or by nearest neighbor analysis. However, it has been shown that even non-covalently bound RNA can cause a density shift in Cs_2SO_4, perhaps due to the formation of an association of RNA and DNA based on non-specific aggregation or limited reannealing after a denaturation step (Mendelsohn et al., 1975). A few groups do not find RNA, but speculate that their pulse times may have been too long to observe attached RNA or that RNA may have been removed during treatment of the DNA samples (Berger and Huang, 1974; Gautschi and Clarkson, 1975).

3. Are both strands made discontinuously?

Having only one strand of the DNA helix made discontinuously is sufficient to resolve the problem posed by the inability of polymerases to synthesize DNA in the 3'⟶5' direction. Whether the other strand is also made discontinuously is still very much an open question. On the basis of the assumption that more than 50% of acid-precipitable radioactivity in small pieces after a short pulse-label indicates that both strands are being synthesized discontinuously, many groups have claimed to observe totally discontinuous synthesis (Okazaki et al., 1968a, b, 1973; Sadowski et al., 1968; Yudelevich et al., 1968; Sugino & Okazaki, 1972; Fareed et al., 1973; Goldstein & Rutman, 1973; Gottesman et al., 1973a; Hess et al., 1973; Kohn et al., 1974;

Gautschi & Clarkson, 1975; Kurosawa & Okazaki, 1975; Mendelsohn et al., 1975; Tseng & Goulian, 1975a). Others observe less than 50% of the radioactivity in the small pieces even after short pulses, and conclude that only one strand is made discontinuously (Painter & Schaefer, 1969; Hyodo et al., 1970, 1971; Iyer and Lark, 1970; Eisenberg & Dernhardt, 1974; Francke & Hunter, 1974a; Friedman, 1974; Hershey & Taylor, 1974; Louarn & Bird, 1974).

Even when this question is examined in light of the annealing properties of the Okazaki fragments, contradictory conclusions arise. Examining either the ability of the fragments to self-anneal or the extent of their annealing to one or both of the parent DNA strands, some groups conclude that both sides of the helix replicate discontinuously (Tomizawa & Ogawa, 1968; Okazaki & Okazaki, 1969; Polsinelli et al., 1969; Sugimoto et al., 1969; Ginsberg & Hurwitz, 1970; Fareed & Salzman, 1972; Sugino & Okazaki, 1972; Fareed et al., 1973; Laipis & Levine, 1973; Pigiet et al., 1973; Vlak et al., 1975). However, since some of the bacteria and phage studied replicate their DNA bi-directionally, only the self-annealing experiments can be considered as strong evidence. Another group finds that even this criterion indicates only semi-discontinuous synthesis (Francke & Hunter, 1974a; Francke & Vogt, 1975). As I will point out in the Discussion section, this is not an easy question to resolve. It is made even more difficult by the fact that the 5'$\longrightarrow$3' strand may be replicated more or less

discontinuously depending on mutations (Lark, 1972; Louarn & Bird, 1974) or on other conditions affecting the rate of synthesis (Herrmann et al., 1972; Olivera & Bonhoeffer, 1972).

E. Eukaryotic Okazaki fragments seem to chase into larger DNA

Proof that eukaryotic Okazaki fragments chase into larger DNA is not as easily obtainable as it is in the case of bacteria, both because of the relatively long time required for added nucleosides to equilibrate in the intracellular pool and because of the large amount of additional incorporation that usually occurs during in vivo chases. However, many groups have obtained evidence that eukaryotic Okazaki fragments also chase into bulk DNA. Such evidence has been obtained both in in vivo studies (Painter & Schaefer, 1969; Schandl & Taylor, 1969; Baker, 1971; Cheevers et al., 1972; Fareed & Salzman, 1972; Laipis & Levine, 1973; Magnusson, 1973a; Salzman & Thoren, 1973; Salzman et al., 1973; Berger & Huang, 1974; Qasba, 1974b; Gautschi & Clarkson, 1975; Mendelsohn et al., 1975; Tseng & Goulian, 1975a; Vlak et al., 1975) and in more rigorous in vitro experiments (Kidwell & Mueller, 1969; Mueller, 1969; Magnusson, 1973; Magnusson et al., 1973; Francke & Hunter, 1974a, b; Qasba, 1974a; Hunter & Francke, 1975; Otto & Reichard, 1975; Tseng & Goulian, 1975a; Fraser, unpublished).

F. Intermediate-sized DNA

Although many aspects of the pattern of DNA synthesis in

bacteria and mammalian cells are similar, there are several differences. One difference is that nascent DNA in mammalian cells is sometimes found both as Okazaki fragments and in a size class larger than Okazaki fragments but smaller than non-replicating bulk DNA. Several groups have reported a peak of nascent DNA sedimenting at a position between the Okazaki and bulk peaks (Painter, 1968; Taylor & Miner, 1968; Habener et al., 1969b; Kidwell & Mueller, 1969; Mueller, 1969; Schandl & Taylor, 1969; Berger & Irvin, 1970; Hyodo et al., 1970, 1971; Lehmann & Ormerod, 1971; Chiu & Rauth, 1972, Gautschi et al., 1973; Goldstein & Rutman, 1973; Berger & Huang, 1974; Kohn et al., 1974; Friedman et al., 1975; Rajalakshmi & Sarma, 1975; Sakamaki et al., 1975). This peak will be referred to as an "intermediate peak". Other groups do not see a discrete peak of intermediate DNA, but do observe some DNA smeared across that region, even though they sometimes do not discuss it (Friedman & Mueller, 1969; Nuzzo et al., 1970; Sato et al., 1970; Cheevers et al., 1972; Friedman, 1974; Gautschi & Clarkson, 1975; Mendelsohn et al., 1975). In spite of these reports the exact nature of what is going on is not clear. There are reports of no intermediate DNA being seen (Painter & Schaefer, 1969; Gautschi & Kern, 1973), of peaks being reported and then disclaimed (Hyodo et al., 1970, 1971), of intermediate peaks being seen that are actually only single fractions in an alkaline sucrose gradient (Friedman et al., 1975), of graphs purporting to show

peaks that really don't exist (Fukiwara, 1972), of peaks that appear *in vivo* but not *in vitro* (Tseng & Goulian, 1975a), of intermediate peaks that appear only when the bulk peak doesn't (Ensminger & Tamm, 1970), and of peaks that are made up of small double-stranded pieces joining together while the single-stranded pieces move directly from the Okazaki peak to the bulk peak (Baker, 1971). In this thesis I will demonstrate that the intermediate peak is reproducible and provide an explanation for it.

II. Inhibitors

In the past few years many studies have been done on the effect of inhibitors of protein or DNA synthesis on DNA replication. Such experiments are conducted with the hope of elucidating the exact process that is being affected by the inhibitors and thereby examining the relationships among the processes that are involved in DNA synthesis.

A. Inhibition of protein synthesis

1. Effect on DNA synthesis

In bacteria, protein synthesis is required for initiation of DNA replication but is not needed to support replication in progress (Lark, 1969; Brown *et al.*, 1970). Yeast seem to be affected in the same way: Protein synthesis is needed for the initiation, but not continuation, of DNA synthesis (Hereford & Hartwell, 1973; Williamson, 1973; Slater, 1974). In *Physarum*, protein synthesis seems to be required for the continuation of DNA synthesis as well

(Muldoon et al., 1971; Bersier & Braun, 1974), but it has been suggested that it is actually needed at only 10 discrete time points during S phase to allow the initiation of replicative units which begin at those 10 points (Muldoon et al., 1971).

Protein synthesis is required during G1 for vertebrate cells to enter S phase (Taylor, 1965; Terasima & Yasukawa, 1966; Schneiderman et al., 1971; Highfield & Dewey, 1972) and the inhibition of protein synthesis also inhibits DNA synthesis in progress in Chlorella (Wanka & Moors, 1970), Tetrahymena (Gale et al., 1972), polyoma (Yu et al., 1975), chick red blood cells (Weintraub & Holtzer, 1972) and mammalian cells (Taylor, 1965; Young, 1966; Brega et al., 1968; Mueller, 1969; Weiss, 1969; Brown et al., 1970; Ensminger & Tamm, 1970; Chung & Coffee, 1971; Hand & Tamm, 1972, 1973; Gautschi & Kern, 1973; Hori and Lark, 1973; Gautschi, 1974).

In many instances, when protein synthesis is reduced by 95% or more, DNA synthesis is quickly reduced to less than 20% of the normal amount (Brega et al., 1968; Ensminger & Tamm, 1970; Gautschi & Kern, 1973; Gautschi, 1974), but in other cases it is reduced only to between 50% and 20% of the control (Ennis & Lubin, 1964; Young, 1966; Grollman, 1969; Chung & Coffee, 1969; Hyodo et al., 1971; Seale & Simpson, 1975). Weintraub and Holtzer (1972) have suggested that DNA synthesis seems to be immediately reduced by 50% and later undergoes further exponential decline.

2. Mechanism of action

The mechanisms of action of both the protein and DNA inhibitors have been examined at the molecular level. Cycloheximide apparently inhibits translocation of peptidyl-tRNA from the ribosome A site to the P site. It thus prevents the movement of ribosomes along mRNA but does not accelerate release of nascent polypeptide chains or cause polyribosome breakdown (Ennis & Lubin, 1964; Ensminger & Tamm, 1970; Gale et al., 1972). This movement is mediated by transfer factor II, which may be the site of action of cycloheximide. It has been shown that the cause of the drop in DNA synthesis is not the inhibition of deoxynucleotide kinase or DNA polymerase (Taylor, 1965; Wanka & Moors, 1970). There is some speculation that cycloheximide's inhibition of DNA synthesis is due to its effect on histone synthesis (Weintraub, 1972), on a protein that controls the initiation of new rounds of genome replication (Yu et al., 1975), or on a protein that acts on initiation of DNA replication at the membrane complex (Fugiwara, 1972).

Emetine resembles cycloheximide in its mode of action. It inhibits protein synthesis at the transfer level by inhibiting peptide chain elongation (Grollman, 1968; Gale et al., 1972).

Puromycin is a structural analog of aminoacyl-adenosine, the 3' terminus of aminoacyl-tRNA. It can therefore substitute for the aminoacyl-tRNA bound to the ribosome A site by taking part in

the ribosome peptide bond-forming reaction, and can then accept the nascent peptide chain. Since puromycin binds only weakly to ribosomes, the resultant peptide-puromycin molecule usually falls off the ribosome almost at once (Nathans, 1967; Grollman, 1968; Ensminger & Tamm, 1970; Gale et al., 1972). As a secondary consequence of releasing nascent peptides from ribosomes, puromycin causes degradation of polyribosomes both in vivo and in vitro (Grollman, 1968; Gale et al., 1972).

B. Inhibitors of DNA synthesis

The inhibitors of DNA synthesis act much more directly on steps involved in DNA replication. 5-fluorouracil deoxyriboside (FUdR) seems to act at the level of thymidylate synthetase, blocking the conversion of dUMP to dTMP (Cleaver, 1969; Hand & Tamm, 1973; Manteuil & Girard, 1974). Apparently in vivo it is converted to 5-fluorodeoxy-UMP, which is a strong and specific inhibitor of thymidylate synthetase (Gale et al., 1972). It has been suggested that a polymerase used to fill in the gaps between Okazaki fragments is preferentially inhibited by this reduced level of [dTTP].

Hydroxyurea has been reported to act by inhibiting ribonucleotide diphosphate reductase (Frenkel et al., 1964; Neuhard, 1967; Bjursell & Reichard, 1973; Hand & Tamm, 1973). This inhibition is not accomplished by competition with the substrate (Krakoff et al., 1968).

but may be due to an inactivation of protein B2 of the reductase complex (Krakoff et al., 1968), and may be based on the ability of hydroxyurea to chelate metal ions (Moore, 1969). There is one report that the inhibition caused by hydroxyurea is due to a lack of dGTP rather than a general lack of intranuclear dNTP pools (Skoog & Bjursell, 1974).

The mode of action of cytosine arabinoside (Ara C) has apparently been more difficult to determine. Some groups claim it blocks the conversion of CDP to dCDP (Evans et al., 1964; Cleaver, 1969; Manteuil & Girard, 1974), while others say it has no effect on the ribonucleotide reductase activity (Moore & Cohen, 1967; Skoog & Nordenskjöld, 1971). Momparler (1969) claimed that Ara C acts by causing chain termination and is found mainly at the 3'-hydroxyl terminus, while others say it can be incorporated internally into the DNA chain as a substitute for deoxycytidine and does not cause chain termination (Graham & Whitmore, 1970a, b; Hunter & Francke, 1975). It may be that the nucleotide added to a growing chain immediately after a molecule of Ara C is incorporated at a much slower rate, so when synthesis is stopped a disproportionately large percentage of the nascent chains have Ara C at the end position. This would give rise to the false assumption that Ara C causes chain termination.

C. Effect on inhibition

Besides looking at the site of or the molecular bases for inhibition, many investigators have examined the apparent gross effect of inhibitors of protein or DNA synthesis. They have focused primarily on the question of whether it is a form of DNA initiation or of DNA chain elongation that is primarily responsible for the overall reduction in DNA synthesis. This question has been raised mainly in relation to inhibition of protein synthesis, but there are some reports that both FUdR and hydroxyurea markedly reduce the rate of DNA chain elongation (Hand & Tamm, 1973) while 2-4 dinitrophenol (an uncoupler of oxidative phosphorylation) acts by reducing the number of operating replicons without affecting the average rate of chain elongation (Gautschi et al., 1973).

Most investigators report that cycloheximide does not inhibit the initiation of new replicons, but rather severely reduces the rate of chain elongation (Weintraub, 1972b; Weintraub & Holtzer, 1972; Gautschi & Kern, 1973; Gautschi et al., 1973; Hand & Tamm, 1973). Others report that the rate of elongation is not changed (Ensminger & Tamm, 1970; Fujiwara, 1972).

When puromycin is used as the inhibitor of protein synthesis, the effect on DNA synthesis is much less clear. Some claim that, as with cycloheximide, the rate of chain elongation is significantly reduced without any change in the pattern of initiation (Gautschi, 1974). Others

say that the elongation rate is affected, but not enough to account for the drop in overall DNA synthesis (Hand & Tamm, 1972, 1973). Still other groups say that the rate of elongation is not reduced at all, so the inhibition of protein synthesis must be stopping initiation of new replicons (Ensminger & Tamm, 1970; Hori & Lark, 1973).

Although many of the above studies were carried out using DNA autoradiography as the main investigative tool, sucrose gradient analysis has also begun to provide much useful information. The most spectacular effect of inhibition seems to be that the DNA inhibitors cause a build-up of small pieces that may or may not be the same size as Okazaki fragments (Graham & Whitmore, 1970b; Laipis & Levine, 1973; Magnusson, 1973; Magnusson et al., 1973; Salzman & Thoren, 1973; Berger & Huang, 1974; Manteuil & Girard, 1974; Hunter & Francke, 1975; Vlak et al., 1975). With inhibition of protein synthesis a build-up in the percentage of smaller-than-normal-sized pieces has also been reported (Gautschi & Kern, 1973; Weiner et al., 1974). However, other reports indicate it is mainly the growth of intermediate-sized DNA into molecules of bulk length that is slowed down (Hyodo et al., 1971; Gautschi, 1974; Seale & Simpson, 1975) and one group even sees a reduction in the amount of small pieces in proportion to the other size classes (Fugiwara, 1972). In this thesis I will provide further insight into the mechanism of action of these inhibitors on DNA synthesis.

MATERIALS & METHODS

I. Materials

Chinese hamster ovary cells were obtained from David Baltimore. Joklik-modified minimum essential medium and non-essential amino acids came from Grand Island Biological Co. Fetal calf serum came from Microbiological Associates.

(^{14}C) thymidine (30 mCi/mmole), (^{3}H) thymidine (40-60 Ci/mmole), and (^{3}H) deoxycytidine (30 Ci/mmole) were purchased from New England Nuclear. The unlabeled thymidine and deoxycytidine were purchased from Calbiochem.

Pronase was obtained from Calbiochem, proteinase K from EM Laboratories, Inc., NP-40 from Shell Chemicals and Angio-CONRAY (sodium iothalamate, 80% (w/v) solution) from Mallinckrodt Chemical Works.

Emetine, puromycin, cycloheximide, hydroxyurea, and cytosine arabinoside (Ara C) came from Sigma Chemical Co. FUdR came from Hoffman-La Roche.

The short (^{32}P)-labeled DNA markers were generous gifts from Dr. Tom Maniatis. The P22 DNA marker was a gift from Michael Mulholland and the phage λ DNA marker was a gift from David Bottstein. The small (~300 nucleotides) (^{14}C)-labeled DNA marker was made by labeling CHO cells overnight with (^{14}C) thymidine, extracting and purifying the DNA, dissolving it in SSC and sonicating it.

II. Solutions

Buffer A (Gross-Bellard et al., 1973): 10 mM tris-HCl, 10 mM EDTA, 10 mM NaCl, 0.5% sodium dodecylsulfate, pH 8.

SSC: 0.15 M NaCl, 0.015 M Na Citrate.

TD: 0.137 M NaCl, 0.005 M KCl, 0.007 M NaH_2PO_4, 0.025 M Tris, pH 7.4.

III. Methods

A. Growing the cells

CHO cells were maintained in spinner bottles and grown on plastic petri dishes (60 x 15mm, Falcon) in Joklik-modified MEM supplemented with non-essential amino acids and 7% fetal calf serum. Cells were grown in a 5% CO_2 atmosphere at 37°C.

B. Pulse and pulse-chase labeling

For labeling of bulk DNA, (^{14}C) thymidine was added overnight at 0.025 μCi/ml. Pulse labeling was done by pouring off the medium in which the cells had been growing, mixing it 1:1 with fresh medium, putting back a reduced volume (1.5 ml) of this mixture and allowing the cells to grow for another 1/2 hour at either 37°C or room temperature (22-26°C). At the end of this time a syringe was used to add (^{3}H) thymidine at 100-300 μCi/ml.

Chases were done by pouring off the label-containing medium, washing the cells twice in 5 ml volumes of the medium mixture which had been made 0.7 mM in cold thymidine and 10 μM in deoxycytidine,

and then letting the cells continue to incubate in this medium for the indicated times.

Pulses or pulse-chases were terminated in any one of several ways, depending on the subsequent treatment of the lysate (see below).

C. Inhibitors

If pulse labeling was to be done in the presence of inhibitors of protein or DNA synthesis, the inhibitor was added to the conditioned medium on the plate, and the cells were incubated for 90 minutes at 37°C. The medium was then poured off and 1.5 ml of a mixture of new and conditioned medium containing the same concentration of inhibitor was added. The plates were further incubated in a 5% CO_2 atmosphere at room temperature for 30 minutes and then pulsed.

D. Stopping the pulse and preparing the DNA for centrifugation

Three main methods were used to lyse the cells and treat the lysate:

1) Total lysate method. Pulses were stopped by pouring off the medium and pouring on 1 ml of either 0.2 N NaOH or a low concentration (0.25-1.0%) of SDS or sarkosyl. In either case the solution was 10 mM in EDTA. If SDS or sarkosyl was used the lysate was made 0.2 N in NaOH. The lysate was then heated at 50°C for 45 minutes to an hour, cooled to room temperature, layered on gradients and analyzed by sucrose gradient centrifugation.

2) Proteinase K - chloroform - ethanol method. Pulses were stopped by pouring off the medium and pouring on 3 ml of Buffer A containing 150 μg of proteinase K. Before being used, the proteinase K was autodigested overnight at 37°C in buffer A to inactivate the nucleases. After the lysate was incubated at 37°C for one hour, it was rolled for half an hour with an equal volume of chloroform containing 4% isoamyl alcohol. After centrifuging the resulting suspension the aqueous layer was removed. Two volumes of ice-cold ethanol were added, the mixture was gently shaken, stored at -20°C for half an hour, and centrifuged. The resulting pellet of DNA was washed three times in 70% ethanol and dissolved in 0.2 N NaOH. The solution was then heated at 50°C for 45 minutes to an hour, cooled to room temperature, layered on gradients and analyzed by sucrose gradient centrifugation.

3) Nuclear isolation method. Pulses were stopped by pouring off the medium and pouring on 3 ml of ice cold TD containing 0.65% Nonidet P-40. The resulting nuclei were scraped off the dish with a rubber policeman and after 10 minutes were pelleted, washed once with TD, and suspended in 1 ml of TD which was then made 0.2 N in NaOH. The solution was then heated for 45 minutes - one hour at 50°C, cooled to room temperature, layered on gradients and analyzed by sucrose gradient centrifugation.

E. Sucrose gradient centrifugation

5-20% sucrose gradients were formed over a shelf of 20% (w/v)

sucrose in Angio-CONRAY (Bottstein, 1968). For the SW27 rotor, gradients were 34 ml of 5-20% sucrose solution over a 2 ml shelf; for the SW41, 10.8 ml of solution over a 0.5 ml shelf, and for the SW50.1, 4.2 ml of solution over a 0.3 ml shelf. Gradients were 0.9 M in NaCl and 1 mM in EDTA. If alkaline gradients were required the pH was adjusted to 12.2 - 12.3 with NaOH. All the lysate from one plate was layered onto one gradient. Gradients were centrifuged under conditions described in the figure legends. Fractions were collected by punching a hole in the bottom of the tube. In most cases, fractions of 0.2 ml were collected directly on filter discs (Schleicher & Schuell, Inc.). The papers were dried, washed three times in cold 1 M HCl and twice in ethanol, dried, and counted in a liquid scintillation counter. When certain fractions were to be analyzed further, or when gradients were run in the SW27 rotor, fractions were collected in tubes and 0.1 ml aliquots were dried onto filter papers which were then processed as described above.

F. CsCl equilibrium centrifugation

CsCl was added to the sample, the solution was made 0.01 M in EDTA and the pH was adjusted to >12.1. Final density was about 1.730-1.750. The solution (2.5 ml for the SW50.1 rotor and 5 ml for the 50 rotor) was put in a cellulose nitrate tube which was then filled to the top with mineral oil. Gradients were spun in an SW50.1 rotor or a 50 rotor under conditions described in the figure legends.

Gradients were collected and counted as discussed for sucrose gradients.

G. Polyacrylamide gel electrophoresis

All gels were made and run in the laboratory of Dr. Tom Maniatis. 5% gels were made by deionizing 100 ml of formamide with 5 grams of mixed bed ion exchange resin (20-50 mesh, Bio Rad AG 501-X8) for 1 hour, filtering it through a Millipore filter and then mixing 50-60 ml of it with 3.1875 grams of acrylamide, 0.5625 of bis-acrylamide, and 0.18 grams of tris. The pH was adjusted to 9.0 with NaOH, the volume was brought up to 74 ml with formamide, the pH was adjusted again, and the solution was filtered through a Whatman #1 filter. 150 μg of TEMED (N,N,N',N'-tetramethyl ethylene diamine) was added and the solution was heated at 37°C. 100 mg of ammonium persulfate dissolved in 1 ml of water was added to the solution and stirring was begun immediately. When the solution was thoroughly mixed, it was poured into a slab gel, 2mm thick x 160mm long.

The DNA pellet, which had been ethanol precipitated and washed several times with 70% ethanol, was dried, taken up in 50 μl of deionized formamide and layered on the gel. DNA pieces of known size were layered onto wells of the slab gel and the gel was run at 200 volts and 8 milliamps for about 10 hours at room temperature. The gel was sliced into 2mm slices and the fractions were put into a 1 ml 9:1 NCS:water solution and heated at 37°C for 3 hours. After

cooling, 15 ml of toluene containing PPO and POPOP was added and the samples were counted.

H. Electron microscopy

Electron microscopy was done by Howard J. Edenberg. Samples were taken from the alkaline solutions that had been heated for various times, and allowed to cool to room temperature. Within 20-30 minutes, 20 μl samples (in 0.2 or 0.5 M NaOH) were diluted with a mix to give 100 μl of solution containing 0.5 M ammonium acetate, 0.2 M tris (pH 8.5) and 10 μg of cytochrome C, and spread on a hypophase of 0.2 M ammonium acetate. The grids were stained with a fresh solution of 5×10^{-5} M uranyl acetate in 95% ethanol and rotary shadowed (10:1) angle with 80% Pt/20% Pd. Photographs were taken at 10,000X in a JEOL microscope and printed at a 2X enlargement.

RESULTS

I. Artifacts

A. Aggregation and breakdown

Since there were so many discrepancies in the published reports and there was disagreement even as to the basic question of discontinuous synthesis, it was very important to choose experimental techniques that would alter the DNA under observation as little as possible.

As examples of the difficulties encountered while using different DNA purification techniques, Figure 1 shows two different problems that arise when a cell lysate is sedimented through a neutral sucrose gradient and the peak fractions are then denatured and sedimented through alkaline sucrose gradients. Figures 1A and 1C show the sedimentation profiles of DNA from CHO cells pulse labeled for short times at 37°C and run on neutral sucrose gradients. In both cases the pulse labeled and bulk labeled DNA sedimented together, and the peak fractions showed considerable DNA aggregation. (Because of this aggregation, the DNA peak position is not reproducible.) In Fig. 1B the aggregated DNA was sedimented through alkaline sucrose after being denatured by raising the pH to 13. The DNA was still clumped and the pulse labeled DNA ran together with the bulk DNA to the bottom of the gradient. In Fig. 1D the DNA run through neutral sucrose was manipulated by hand and vortexed to break up the clumping before being centrifuged again. As a result, the bulk labeled DNA was

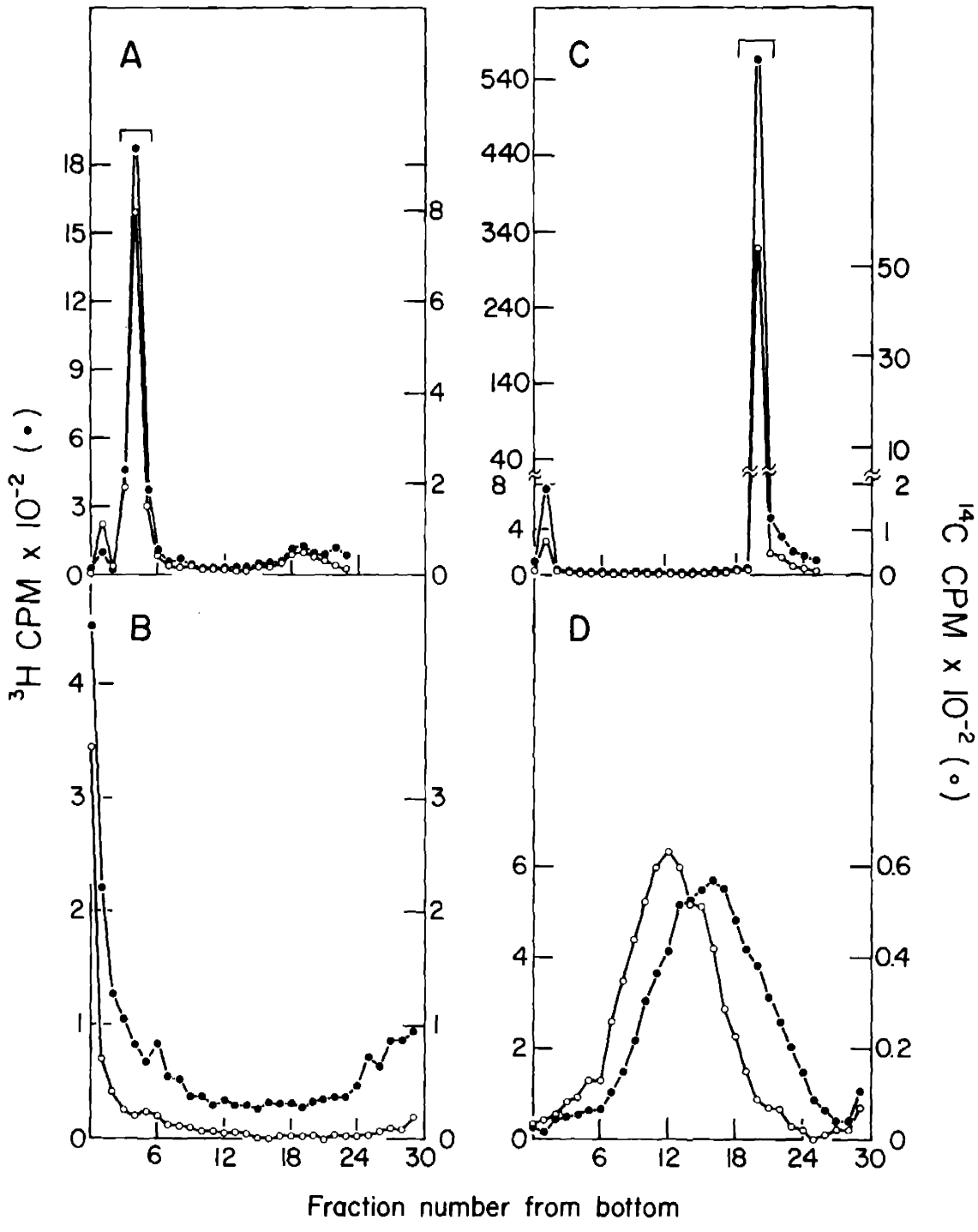
Figure 1. Profiles of DNA sedimented through neutral and alkaline sucrose gradients. CHO cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled with (^{3}H) thymidine, lysed and centrifuged through a neutral sucrose gradient. The peak fractions were pooled, denatured with NaOH, and centrifuged through an alkaline sucrose gradient. In one case (D) an attempt was made to break up the aggregated DNA before it was sedimented through the alkaline sucrose (see text). (A) and (C) are the neutral sucrose gradients, centrifuged in an SW27 rotor at 20,000 rpm for 80 minutes at 10°C. (B) and (D) are the alkaline gradients, centrifuged in an SW27 rotor at 22,500 rpm for 14 hours at 10°C.

(A) 75 second pulse, neutral sucrose gradient.

(B) 75 second pulse, alkaline sucrose gradient.

(C) 5 minute pulse, neutral sucrose gradient.

(D) 5 minute pulse, alkaline sucrose gradient.


A
B
C
D
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom
0
3
6
9
12
15
18
2
4
6
8
1
2
3
4
40
140
240
340
440
540
10
30
50
0.2
0.4
0.6
6
12
18
24
30

severely broken down and no valid comparisons could be made between it and the pulse labeled DNA.

B. Determination of acid-precipitable radioactivity

1. Leakage through filters

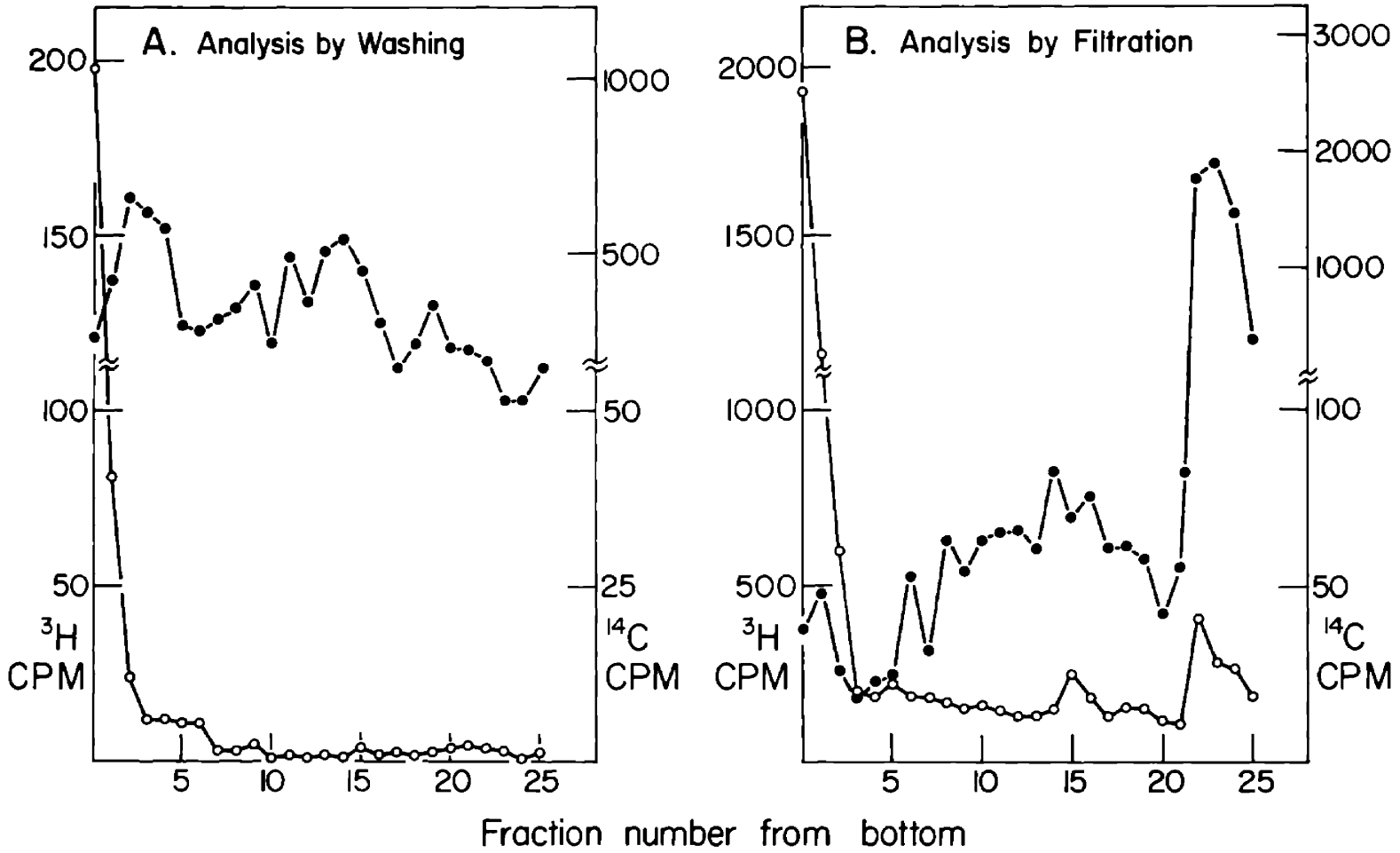
The above are examples of artifacts which cause DNA to sediment either more rapidly or more slowly than it should on the basis of its molecular weight. Serious problems also arise with techniques of analysis that cause a peak of radioactivity to appear where there is actually either no more than a background level of DNA or no DNA at all. An example of this is shown in Figure 2. A culture of CHO cells, labeled overnight with ^{14}C thymidine, was lysed with 1% sarkosyl, denatured, and sedimented through an alkaline sucrose gradient. The gradient was then collected in 1.5 ml fractions. An equal amount of (^{3}H)-lambda DNA was added to each fraction and each fraction was thoroughly mixed. 0.1 ml aliquots from each fraction were put on filter papers which were then dried, washed with 1 N HCl and then ethanol and dried again. Then 200 μg of bovine serum albumen (BSA) were added to each tube as a carrier. The tubes were mixed, made 5% in TCA, mixed again and allowed to settle for 10 minutes at 0°C. The contents of each tube were then poured through a glass fiber filter which was then washed with TCA and ethanol and allowed to dry. Both sets of filters were then counted. The filters containing aliquots (Fig. 2A) appear as expected; there seems to be about

Figure 2. Profiles of sucrose gradients analyzed in different ways. Cells prelabeled overnight with (^{14}C) thymidine were lysed with 1% sarkosyl (10 mM in EDTA), denatured and centrifuged through an alkaline sucrose gradient in an SW27 rotor at 20,000 rpm for 16 hours at 10°C. The gradient was collected and (^{3}H)-labeled phageλ DNA was added to each fraction in equal amounts.

(A) 0.1 ml aliquots from each fraction were put on filter papers, which were then processed as described in the text.

(B) 100 μg of BSA were added to the remaining contents of each tube and the fractions were processed by filtration as described in the text.

-o-o-, ^{3}H; -o-o-, ^{14}C.


A. Analysis by Washing
B. Analysis by Filtration
200
150
100
50
1000
500
50
25
2000
1500
1000
500
3000
2000
1000
100
50
^{3}H CPM
^{14}C CPM
5
10
15
20
25
Fraction number from bottom

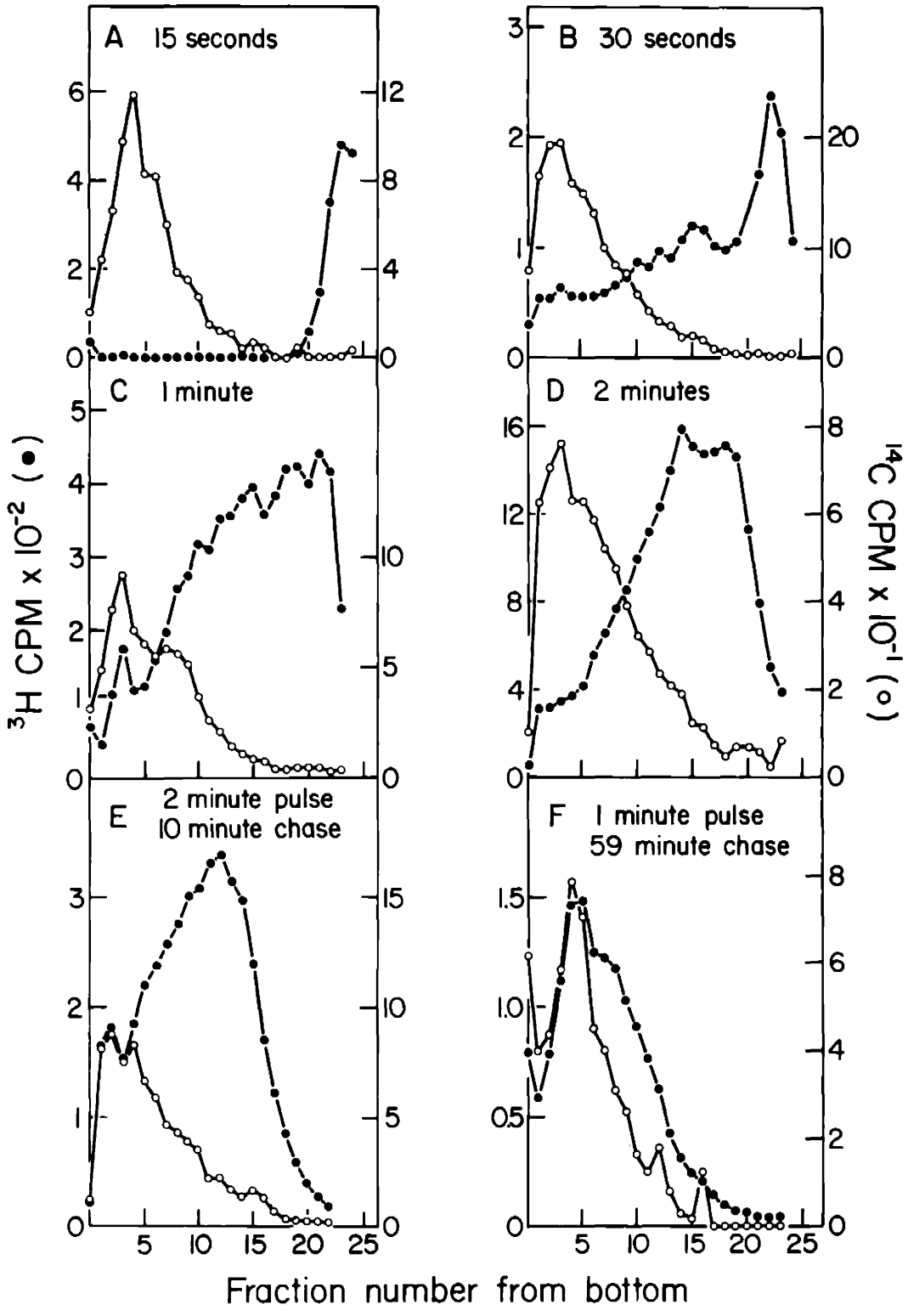
the same amount of tritium-labeled DNA in each fraction, with the downward slope probably due to quenching caused by the cell debris found near the top of the gradient.

However, the filters through which the fractions had been poured (Fig. 2B) show a sharp peak near the top of the gradient. Since these filters should have 10-15 times as many counts as the ones containing aliquots, it can be assumed that across a large part of the gradient most of the DNA was pulled through the filters, but at the top the cell debris trapped the DNA onto the filters.

2. Adsorption of nucleosides or nucleotides to cellular components

Finally a method was chosen which appeared at first to give reliable results. This is the method of Nuzzo et al. (1970) in which the cells are lysed with 0.2M NaOH containing 10 mM EDTA. The lysate is heated at 50°C for 30-45 minutes in order to thoroughly denature the DNA, gently layered onto an alkaline sucrose gradient and centrifuged under the conditions described in the figure legends. Figure 3 shows the sedimentation profile of acid-precipitable radioactivity from cells pulse labeled for short times at 37°C as well as cells in which the pulse was chased for periods up to one hour. After the shortest pulse (Fig. 3A) all the nascent DNA seems to be in the form of very short strands comparable to the Okazaki fragments of bacteria. As the pulse time increases (Figs. 3B, C, D) label gradually appears in longer strands so that a peak is formed in the

Figure 3. Sucrose gradient profiles of pulse labeled DNA prepared by NaOH lysis. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for varying lengths of time with (^{3}H) thymidine at 37°C and lysed with 0.2 M NaOH containing 10 mM EDTA (see Methods, D). The lysate was heated at 50°C for 30-45 minutes and then centrifuged through an alkaline sucrose gradient in an SW27 rotor at 25,000 rpm for 16 hours at 0°C. Chases were done in the presence of nonradioactive thymidine.

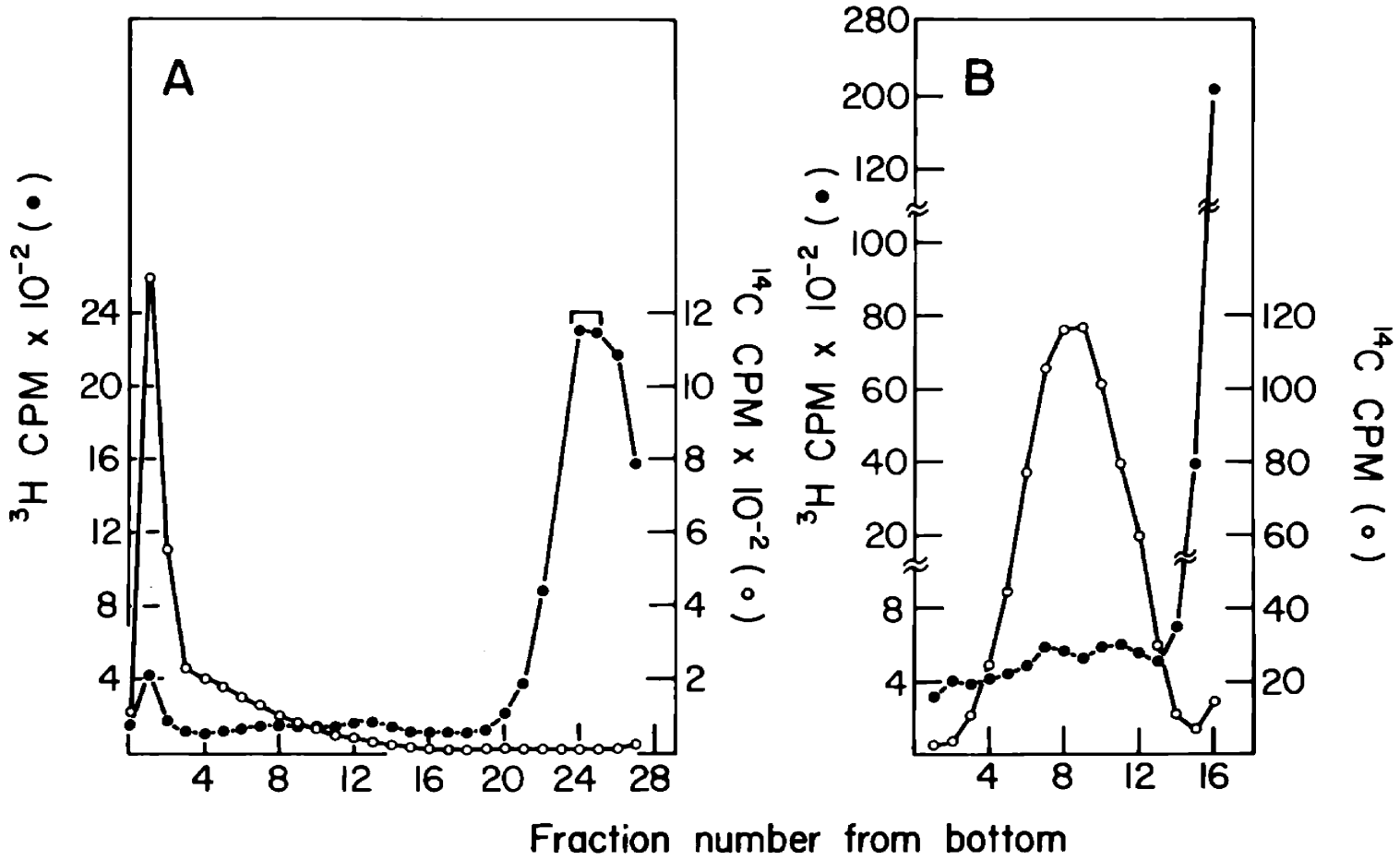

A 15 seconds
B 30 seconds
C 1 minute
D 2 minutes
E 2 minute pulse
10 minute chase
F 1 minute pulse
59 minute chase
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-1} (○)
Fraction number from bottom

middle of the gradient. After a 30 second pulse the peak of putative short strands is still clearly visible, while after 1 minute it is no longer distinguishable as a separate peak and after 2 minutes it is completely obscured by a peak of larger strands. (This latter peak will be referred to as the intermediate peak.) As the pulse is chased with medium containing an excess of cold thymidine the intermediate peak moves down the gradient (Fig. 3E) and after 1 hour the pulse-labeled DNA sediments almost as rapidly as the bulk DNA (Fig. 3F).

These results led to the assumption that a pattern of discontinuous DNA synthesis in mammalian cells had been confirmed (Huberman & Horwitz, 1973). However, it turned out that the acid-precipitable radioactivity in the "Okazaki peak" did not band at the density of DNA in cesium chloride gradients (Fig. 4). CHO cells were pulse-labeled for 2 minutes at 25°C and sedimented through an alkaline sucrose gradient (Fig. 4A). The peak fractions (#24-25) were then centrifuged to equilibrium in a CsCl buoyant density gradient. More than 75% of the acid-precipitable counts went to the top of the gradient (Fig. 4B). This suggests that the radioactivity in the Okazaki peak does not represent short DNA fragments, but rather may simply be a small fraction of the cells' (^{3}H) thymidine pool adhering to some cellular component that floats in CsCl.

Further confirmation of this hypothesis was obtained by running control lysates containing CHO cells lysed before the addition of

Figure 4. Sucrose and CsCl gradient profiles of DNA prepared by total lysate method. Cells were prelabeled overnight with (^{14}C) thymidine and pulse labeled for 2 minutes with (^{3}H)-thymidine at 25°C. The total cell lysate was centrifuged on an alkaline sucrose gradient (A) in an SW27 rotor spun at 25,000 rpm for 16 hours at 0°C. The peak fractions (#24-25) were centrifuged to equilibrium in an alkaline CsCl gradient (B) to which short (300 nucleotides) pieces of (^{14}C)-labeled DNA had been added as a marker. The gradient was spun in an SW50.1 rotor at 31,000 rpm for 48 hours at 20°C.


A
B
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (○)
^{14}C CPM (○)
Fraction number from bottom

radioactivity. Figure 5 shows the results of such an experiment. In one case (Fig. 5A) CHO cells were pulse-labeled with (^{3}H) thymidine for 15 seconds at 37°C. For the controls, cells were first lysed by the addition of 0.2 M NaOH and then (^{3}H) thymidine was added to one lysate (Fig. 5B) and (^{32}P) TTP to the other lysate (Fig. 5C). All three lysates were sedimented through sucrose gradients and all the sedimentation profiles look the same, even though neither of the controls (Fig. 5B,C) could have incorporated the radioactive label into DNA. Thus it appeared that either thymidine or TTP was adsorbing to some cellular component that moved only slowly into the sucrose gradient. A similar artifact appears if the cells are lysed with 0.5% SDS instead of NaOH. Figure 6 shows that the "Okazaki peak" observed under such conditions floats at the top of a CsCl gradient even if the gradient's density is adjusted in such a way that the DNA marker sediments to the bottom.

It should be noted that although the "total lysate" method of analysis described above gives an artifactual "Okazaki peak," its intermediate peak is partially real, as demonstrated in Figure 7. Cells were pulse labeled for 5 minutes, lysed with NaOH and sedimented through an alkaline sucrose gradient (Fig. 7A). Pooled fractions from the intermediate peak of 7A were then centrifuged to equilibrium in a CsCl buoyant density gradient (Fig. 7B). Although some of the radioactivity bands at the density of DNA, almost one-half of the

Figure 5. Sucrose gradient profiles of DNA prepared by NaOH lysis, when radioactivity was added before or after lysis. Cells were lysed with 0.2 M NaOH, heated and centrifuged on an alkaline sucrose gradient in an SW50.1 rotor spun at 45,000 rpm for 19.5 hours at 20°C. (Note that under these conditions the Okazaki peak should sediment near the middle of the gradient.)

(A) ^{3}(H) thymidine was added to the cells for 15 seconds at 37°C before lysis.

(B) (^{3}H) thymidine (200 μCi) was added after lysis with NaOH.

(C) (^{32}P) TTP (50 μCi) was added after lysis with NaOH.

-●-●-●-, (^{3}H) - total counts; -●-●-●-, (^{3}H) - acid precipitable counts; - Δ-Δ-Δ-, (^{32}P) - total counts; -▲-▲-▲-, (^{32}P) - acid precipitable counts.

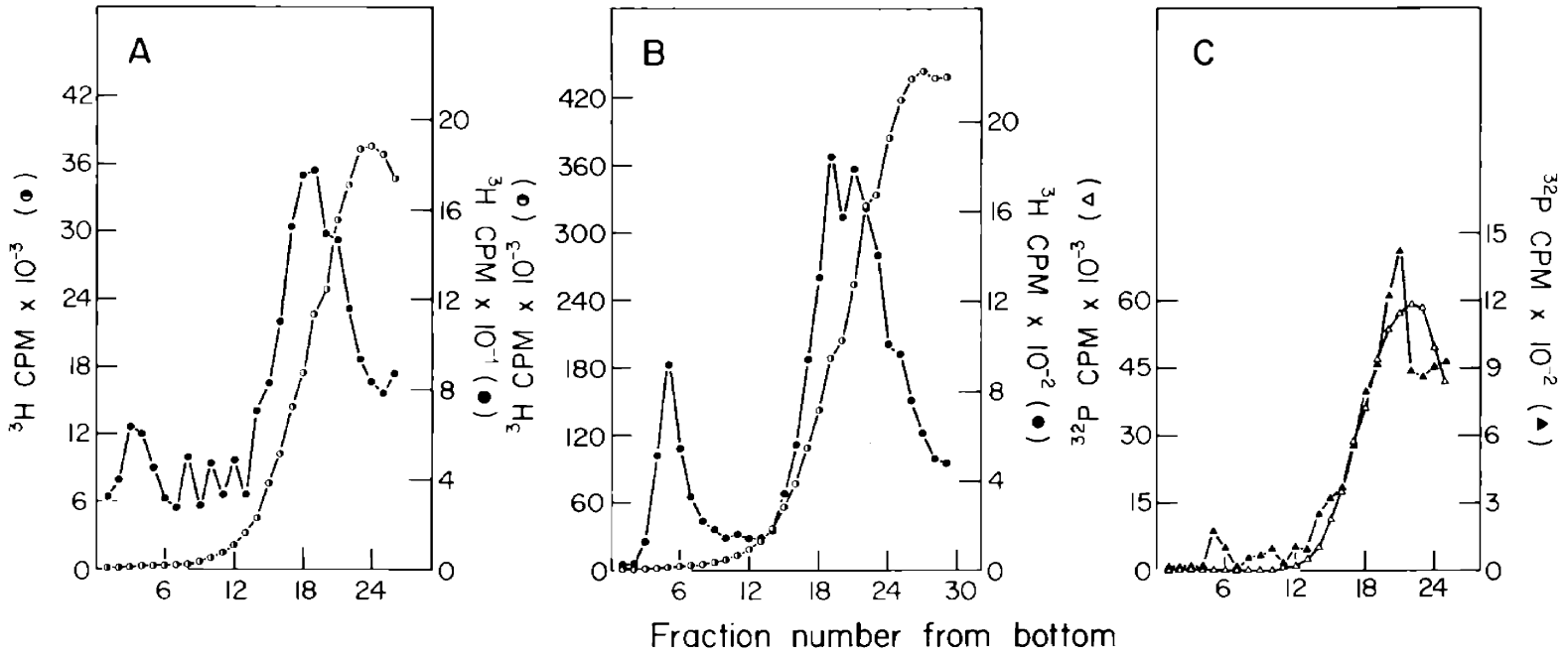

A
B
C
^{3}H CPM x 10^{-3} (●)
^{3}H CPM x 10^{-1} (●)
^{3}H CPM x 10^{-3} (●)
^{3}H CPM x 10^{-2} (●)
^{32}P CPM x 10^{-3} (△)
^{32}P CPM x 10^{-2} (▲)
Fraction number from bottom

Figure 6. Sucrose and CsCl gradient profiles of DNA prepared by lysis with SDS. Cells were pulse labeled with (^{3}H) thymidine for 20 seconds at 37°C and lysed with 0.5% SDS. After adding NaOH to a final concentration of 0.2 M and heating the lysate at 50°C for 45 minutes, the sample was centrifuged through an alkaline sucrose gradient.

(A) in an SW50.1 rotor at 45,000 rpm for 10 hours at 20°C. The peak fractions (#17-22) were collected and centrifuged to equilibrium in an alkaline CsCl gradient.

(B) The gradient was spun in an SW50.1 rotor at 30,000 rpm for 48 hours at 20°C, and (^{14}C) labeled bulk DNA was added as marker.

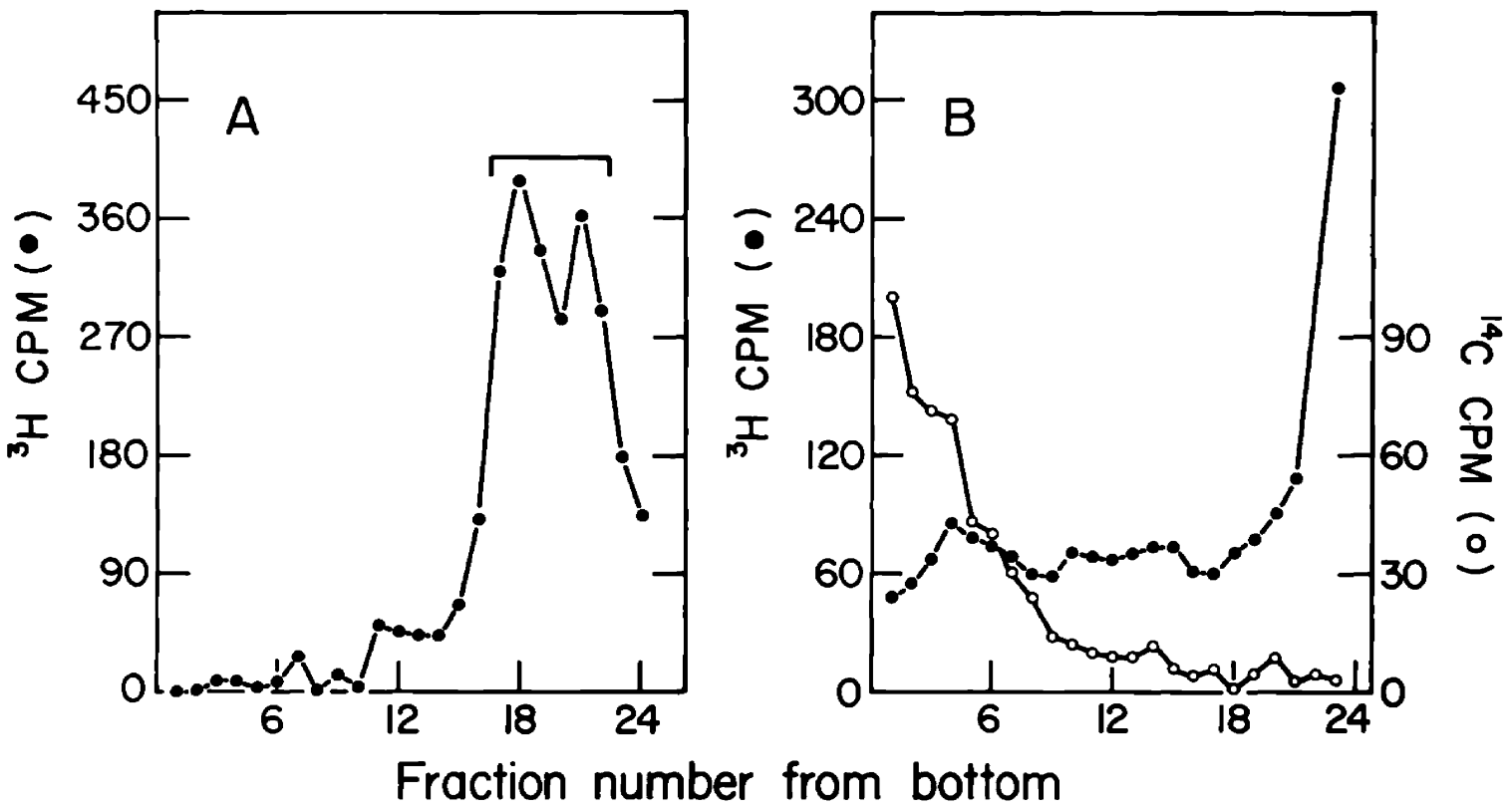
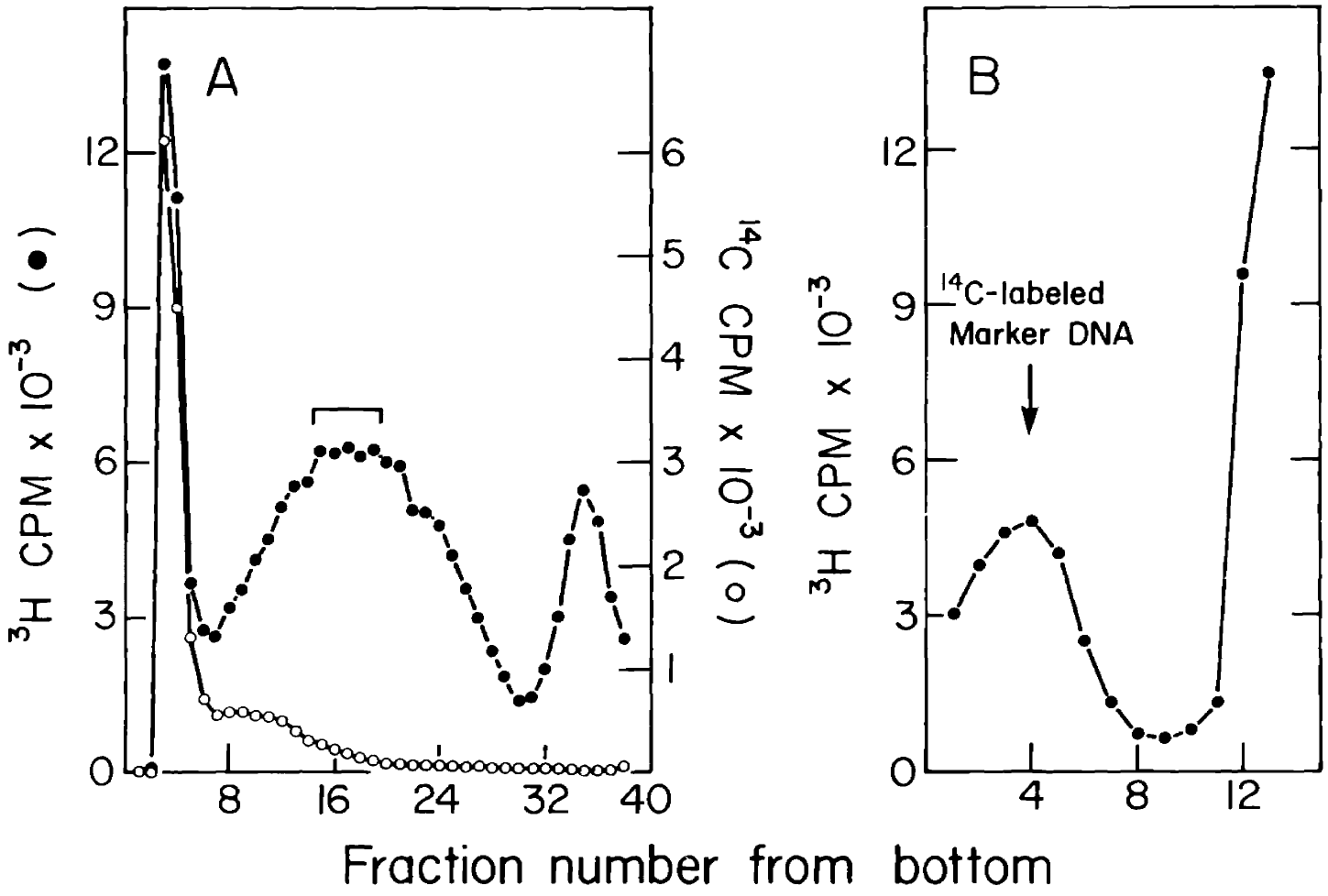

A
B
450
360
270
180
90
0
300
240
180
120
60
0
90
60
30
0
6
12
18
24
^{3}H CPM (●)
^{14}C CPM (○)
Fraction number from bottom

Figure 7. Sucrose and CsCl gradient profiles of pulse labeled DNA lysed with NaOH. Cells, pre-labeled overnight with (^{14}C) thymidine, were pulse labeled for 5 minutes at 37°C with (^{3}H)-thymidine, lysed with NaOH, heated and centrifuged on an alkaline sucrose gradient

(A) in an SW4 rotor at 40,000 rpm for 3.5 hours at 23°C. The "intermediate" peak fractions (#15-19) were pooled and centrifuged to equilibrium in a CsCl gradient

(B) in an SW50.1 rotor at 35,000 rpm for 48 hours at 20°C.


A
B
^{3}H CPM x 10^{-3} (●)
^{14}C CPM x 10^{-3} (○)
^{3}H CPM x 10^{-3}
^{14}C-labeled
Marker DNA
Fraction number from bottom

acid-precipitable counts are found at the top of the gradient. If the fractions are dialyzed before being run in the CsCl gradient, all the remaining radioactivity bands in CsCl at the density of DNA. This indicates that the "intermediate peak" shown in Fig. 7A is really a result of both DNA strands and free nucleotides.

3. Elimination of artifactual adsorbtion

a. Unsuccessful methods

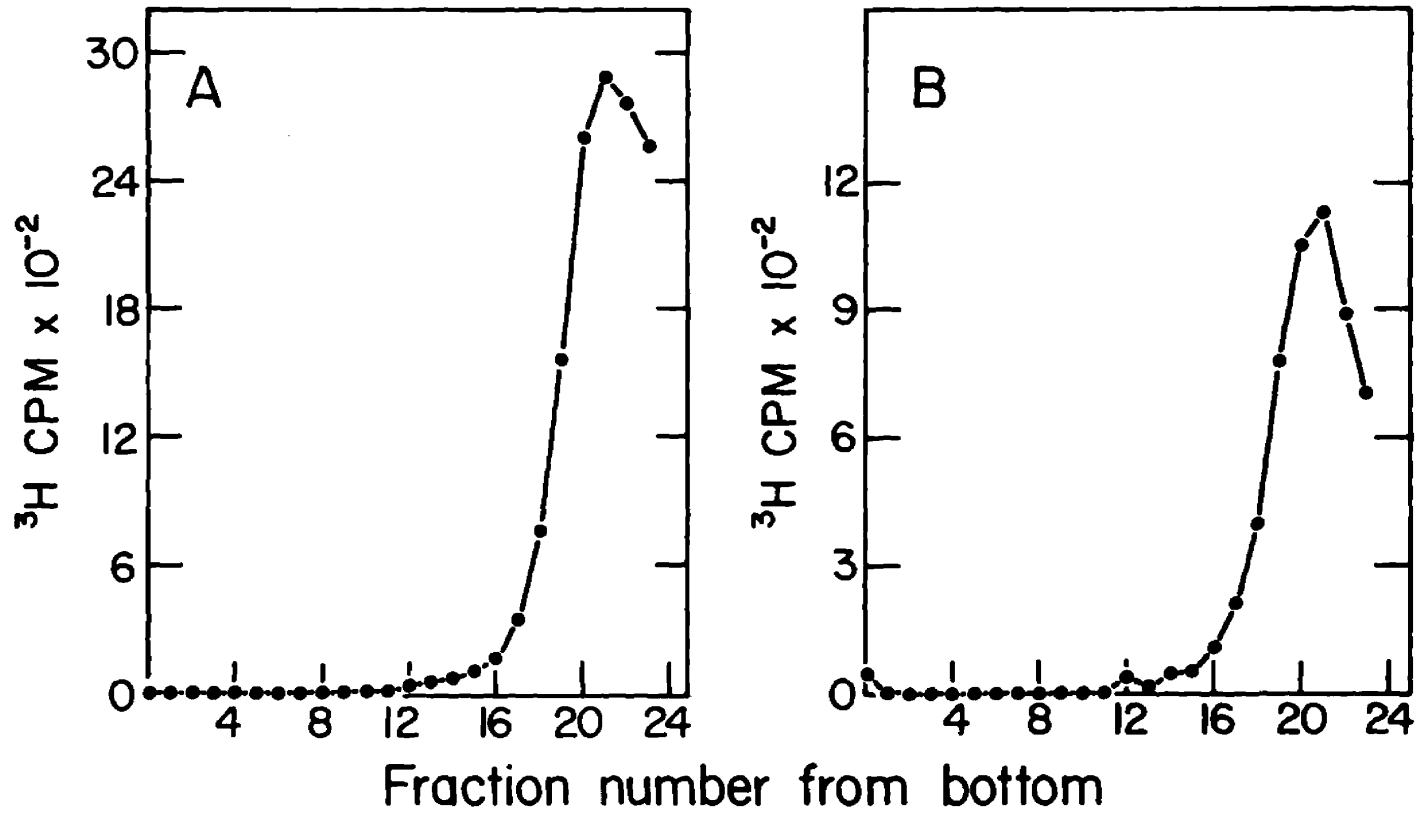
Since it was obvious that the "Okazaki peak" that had been observed was at least to a large extent an artifact, the artifact had to be eliminated to allow determination of the type of DNA synthesis actually taking place during short pulse labeling with thymidine. On the assumption that this artifact was caused by (^{3}H) thymidine adhering to some cellular component that was acid precipitable, attempts were made to eliminate this adhesion. After lysing CHO cells and adding (^{3}H) thymidine to the lysate, a 100-fold excess of cold thymidine was immediately added. The lysate was then sedimented through an alkaline sucrose gradient. This treatment did not remove the artifact (Fig. 8A) even if the filter papers containing the DNA were put into HCl before the DNA had dried (Fig. 8B).

Since untreated cell lysates seem to produce an artifactual Okazaki peak, several methods of purifying the DNA before sedimenting it were examined in the hope of finding some method that would eliminate the artifact without breaking down the larger pieces of DNA. Dialysis

Figure 8. Sucrose gradient profiles of DNA from cells lysed before the addition of radioactivity. Unlabeled CHO cells were lysed with NaOH. (^{3}H) thymidine (200 μCi) was added to the lysate, followed immediately by a 100-fold excess of cold thymidine. The lysate was then heated and centrifuged on an alkaline sucrose gradient in an SW27 rotor at 25,000 rpm for 16 hours at 0°C.

(A) Aliquots were put on filter papers, which were then treated as in Figure 2A;

(B) Aliquots were put on filter papers, which were immediately put into 1 N HCl and then treated as in (A).


A
B
^{3}H CPM x 10^{-2}
30
24
18
12
6
0
12
9
6
3
0
4
8
12
16
20
24
Fraction number from bottom

was the first method tried. CHO cells were pulse labeled for 15 seconds at 37°C, lysed with NaOH and dialyzed overnight against 1/10 SSC. The cell lysate was then sedimented through an alkaline sucrose gradient (Fig. 9A) and the peak fractions (#13-19) were banded in a CsCl buoyant density gradient (Fig. 9B). Although the number of total tritium counts was considerably reduced by the dialysis (from 75,612 to 1,748 counts per aliquot), the number of acid precipitable counts was not affected (519 per aliquot before dialysis and 489 after) and the "Okazaki peak" did not band in the CsCl gradient.

Banding the dialyzed cell lysate in a CsCl buoyant density gradient was then attempted as a means of purifying the DNA. This method seemed to eliminate the artifact, as can be seen in Figure 10. After CHO cells were lysed with 1% Sarkosyl, (^{3}H) thymidine was added to the lysate and part of it was run on an alkaline sucrose gradient, producing the sedimentation pattern shown in Figure 10A. Another part of the lysate was dialyzed overnight against 1/10 SSC containing 0.1% Sarkosyl; then centrifuged in a CsCl gradient (Fig. 10B). The peak fractions (#2-6) were pooled, dialyzed against 1/10 SSC and sedimented through an alkaline sucrose gradient (Fig. 10C). Most of the tritium counts disappeared and the few remaining are probably due to spill-over from the (^{14}C) thymidine-labeled marker. However, while this method does seem to eliminate the artifact, it also causes

Figure 9. Sucrose and CsCl gradient profiles of pulse labeled DNA purified by dialysis. Cells were pulse labeled for 15 seconds at 37°C with (^{3}H) thymidine, lysed with NaOH and dialyzed overnight against 1/10 SSC. The cell lysate was then centrifuged on an alkaline sucrose gradient spun in an SW50. 1 rotor at 45,000 rpm for 19 hours at 20°C. The "Okazaki peak" fractions (#13-19) were pooled, the (^{14}C)-labeled DNA used in Figure 4 was added as a marker, and the sample was centrifuged to equilibrium in a CsCl gradient spun in an SW50. 1 rotor at 34,000 rpm for 45 hours at 20°C.

(A) Alkaline sucrose gradient. -●-●-●-, (^{3}H) - total counts; -◐-◐-◐-, (^{3}H) - acid precipitable counts

(B) CsCl gradients. -●-●-●-, (^{3}H) - acid precipitable counts; -o-o-o-, (^{14}C).

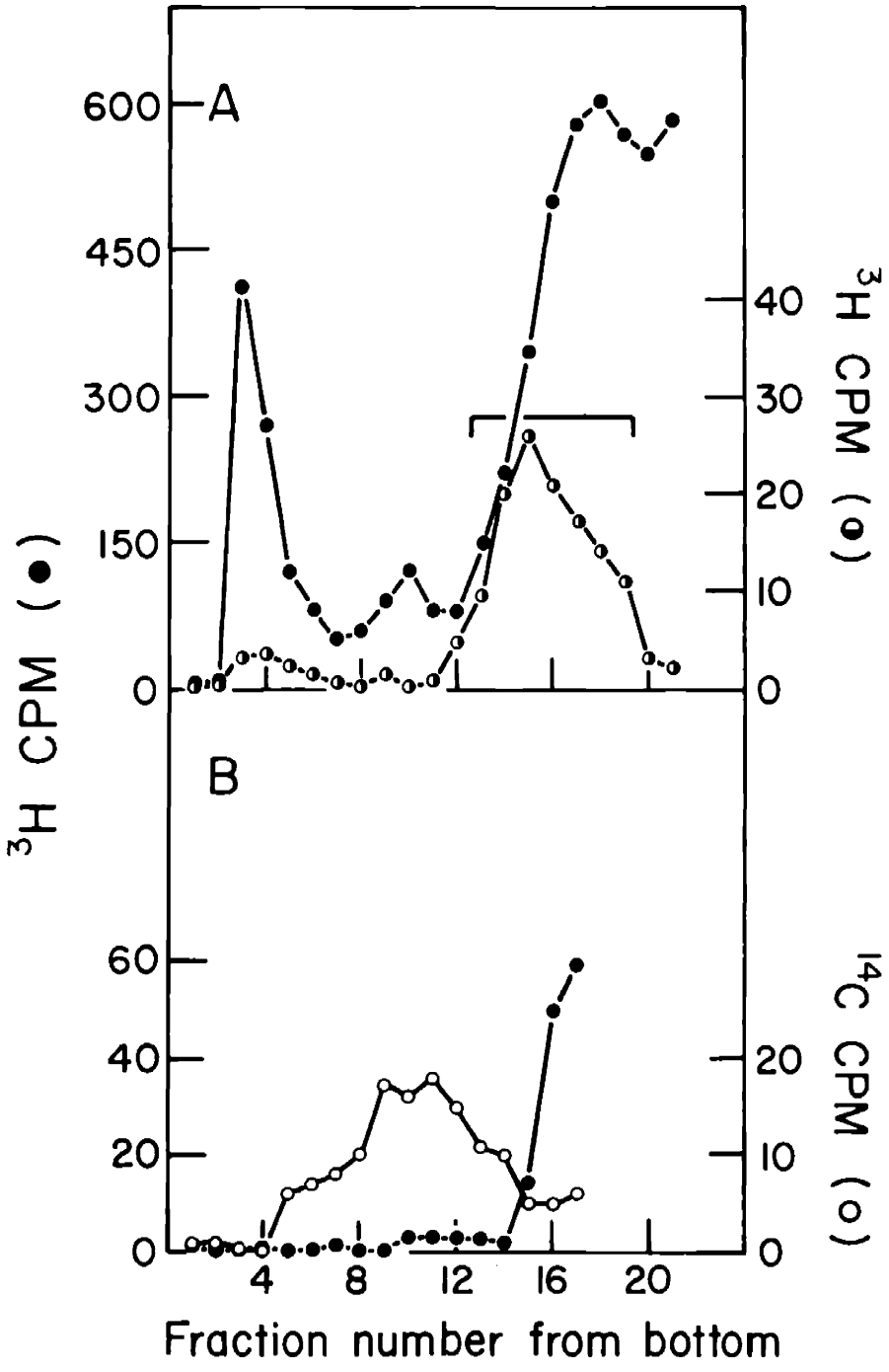
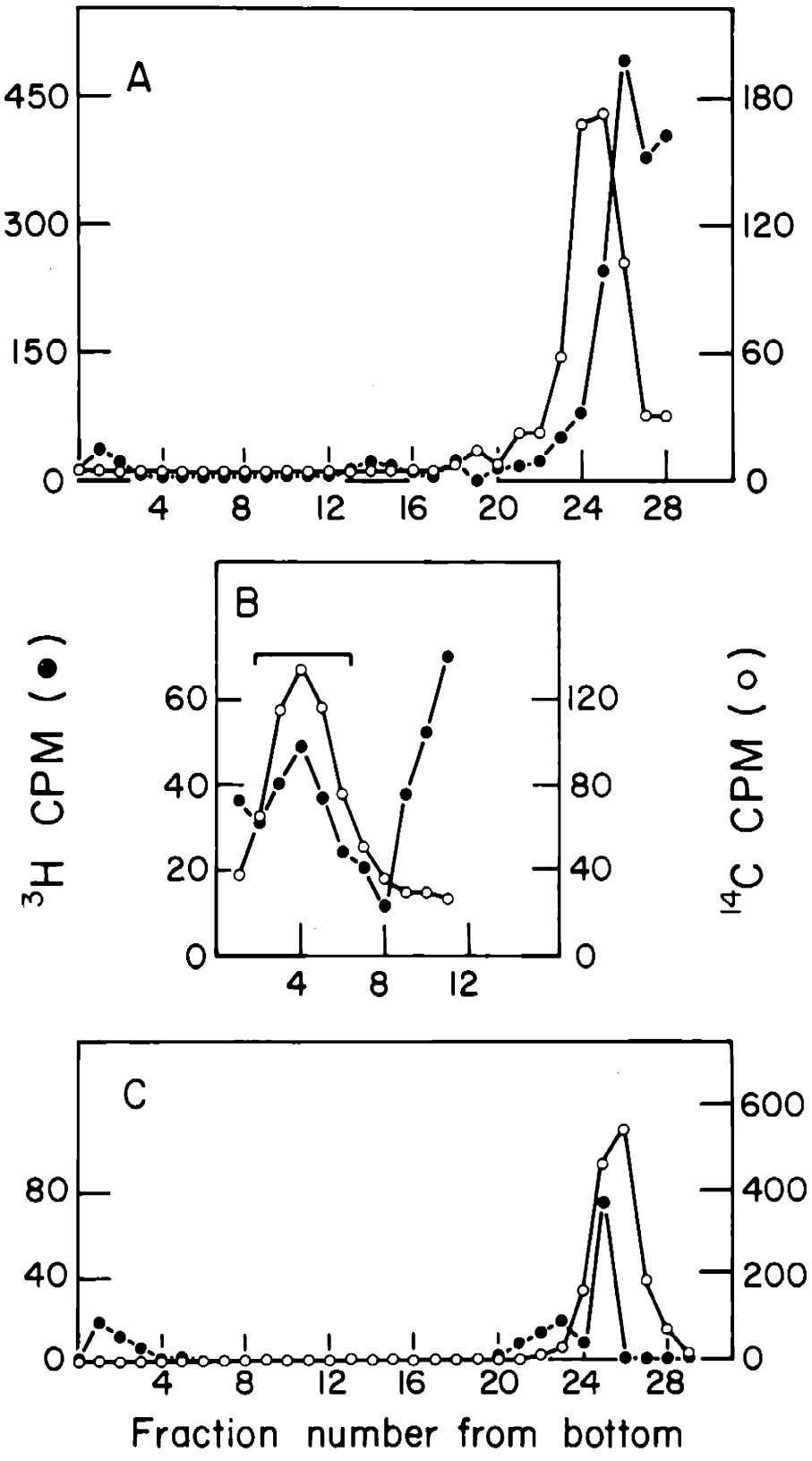

A
B
600
450
300
150
0
40
30
20
10
0
60
40
20
0
20
10
0
4
8
12
16
20
^{3}H CPM (●)
^{3}H CPM (o)
^{14}C CPM (o)
Fraction number from bottom

Figure 10. Sucrose and CsCl gradient profiles of DNA from cells lysed before the addition of radioactivity and purified by dialysis and CsCl centrifugation. Cells were lysed with 1% sarkosyl and (^{3}H)-thymidine (200 μCi) was added to the lysate. Some (^{14}C)-labeled small DNA (Fig. 4) was added to part of the lysate, which was then heated and centrifuged on an alkaline sucrose gradient

(A) in an SW27 rotor at 25,000 rpm for 16 hours at 0°C. Another part of the lysate was dialyzed overnight against 1/10 SSC. Some (^{14}C)-labeled small DNA was added to the lysate which was then made 0.2 N in NaOH, heated and centrifuged to equilibrium in a CsCl gradient.

(B) in a 50 rotor at 42,000 rpm for 41 hours at 20°C. The peak fractions (#2-6) were pooled and dialyzed against 1/10 SSC for 4 hours and sedimented through an alkaline sucrose gradient.

(C) in an SW27 rotor at 25,000 rpm for 16 hours at 0°C.


A
450
300
150
0
180
120
60
0
4
8
12
16
20
24
28
B
60
40
20
0
120
80
40
0
4
8
12
^{3}H CPM (●)
^{14}C CPM (○)
C
80
40
0
600
400
200
0
4
8
12
16
20
24
28
Fraction number from bottom

severe breakdown of the bulk DNA. In Figure 11 we see the results of a similar experiment carried out on cells pulse labeled for 3 minutes at 37°C. It is quite obvious (Fig. 11C) that the bulk DNA is considerably smaller than it was before the dialysis and CsCl centrifugation (Fig. 11A).

Although dialysis by itself did not remove the artifact, it did remove most of the free labeled nucleotides (see above). It was therefore possible that the combination of dialysis plus some treatment to break down and remove proteins would be sufficient to remove the artifact. To examine this possibility some CHO cells were labeled for 1 minute at room temperature and then lysed with 0.25% sarkosyl, while other cells were first lysed with sarkosyl and then received some radioactive thymidine. After an overnight incubation with pronase at 37°C, both lysates were chloroform extracted and dialyzed overnight against 1/10 SSC containing 0.1% sarkosyl. Both lysates were then run on sucrose gradients. The sedimentation profiles show an Okazaki peak for the pulsed cells (Fig. 12B) and no such peak for the control (Fig. 12A). Also the Okazaki peak fractions band in a CsCl gradient (results not shown) so they are probably DNA.

Unfortunately this purification scheme also causes bulk breakdown, as can be seen by pulse labeling cells that had been labeled overnight with (^{14}C) thymidine. After a 20 minute pulse and the purification described above, the sedimentation pattern shows clearly that there

Figure 11. Sucrose and CsCl gradient profiles of pulse labeled DNA purified by dialysis and CsCl centrifugation. Cells prelabeled overnight with (^{14}C) thymidine were pulse labeled at 37°C for 3 minutes with (^{3}H) thymidine. The lysate was divided and treated as in Figure 10.

(A) Alkaline sucrose gradient of cells lysed with 1% sarkosyl;

(B) CsCl gradient of dialyzed lysate;

(C) alkaline sucrose gradient of DNA from peak fractions of CsCl gradient.

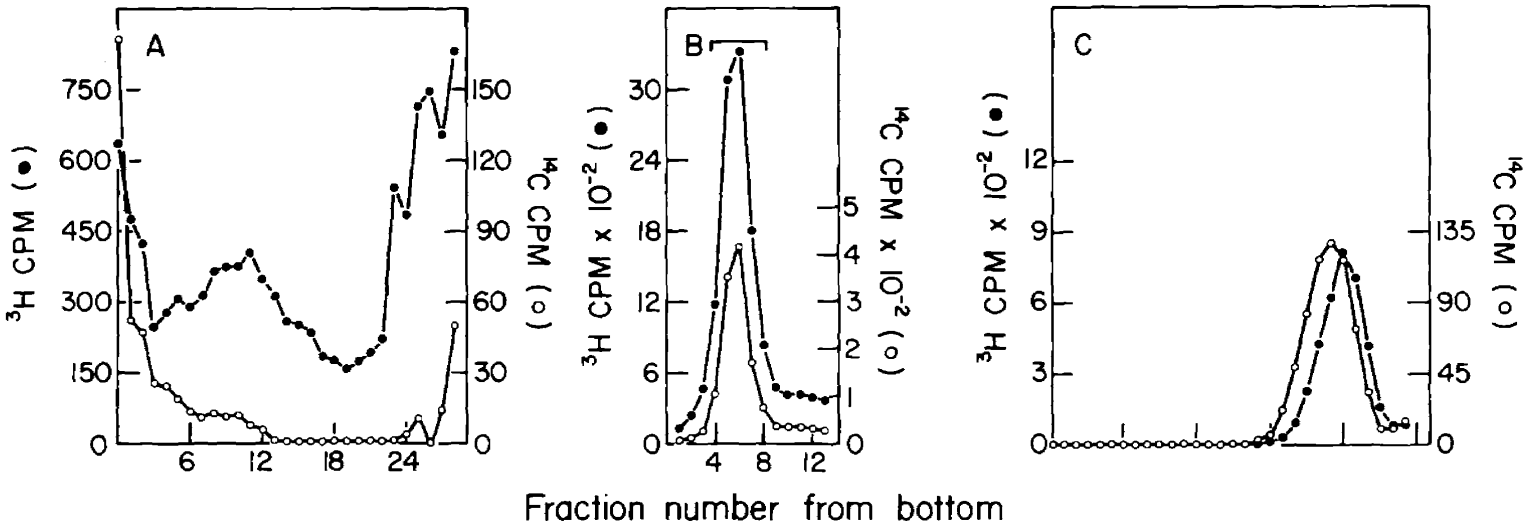
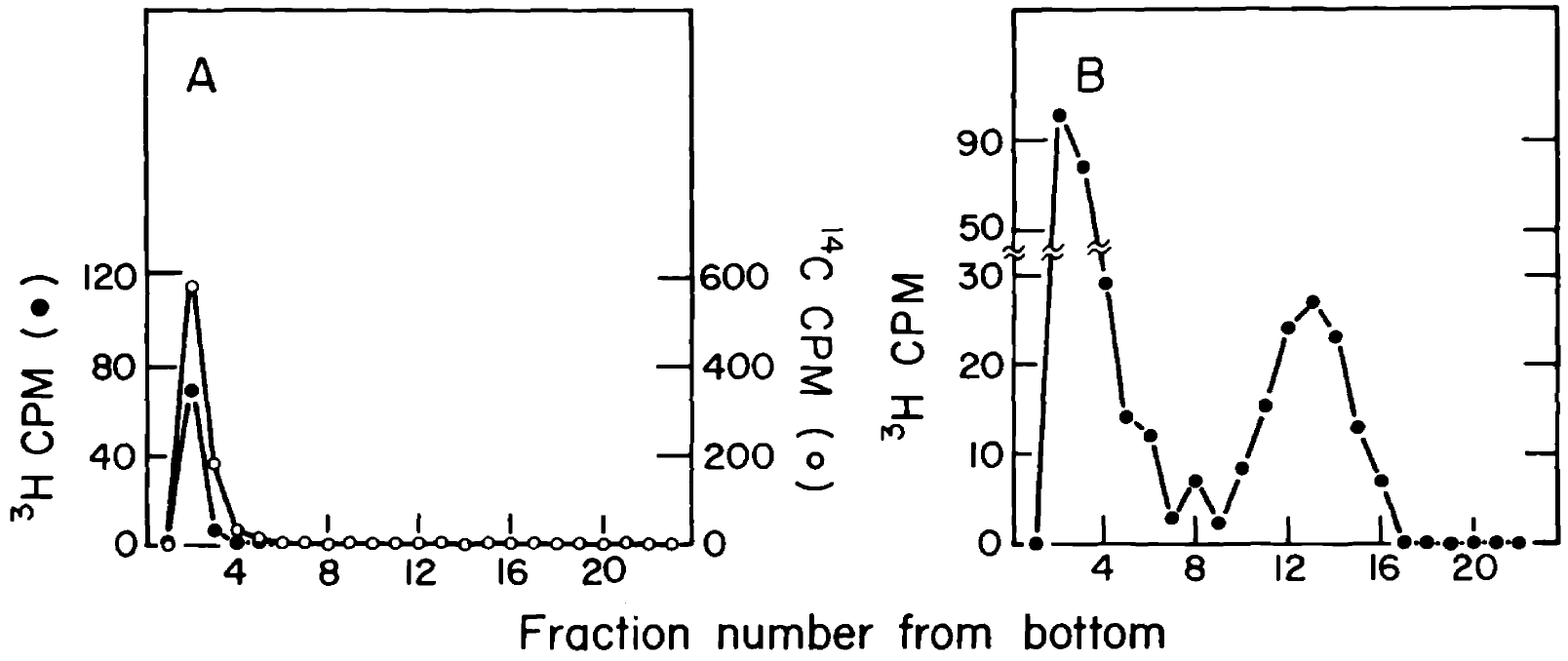

A
B
C
^{3}H CPM (●)
^{14}C CPM (○)
^{3}H CPM x 10^{-2} (●)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom

Figure 12. Sucrose gradient profiles of DNA purified by pronase, chloroform extraction and dialysis.

(A) Cells were prelabeled overnight with (^{14}C) thymidine and (^{3}H) thymidine was added after lysis with 0.25% sarkosyl.

(B) Cells with no prelabel were pulse labeled for 1 minute at room temperature and lysed with 0.25% sarkosyl.

Both samples were incubated overnight with pronase at 37°C, chloroform extracted and dialyzed overnight against 1/10 SSC containing 0.1% sarkosyl. Both lysates were then centrifuged through alkaline sucrose gradients spun in an SW50.1 rotor at 35,000 rpm for 16 hours at 20°C.


A
B
^{3}H CPM (●)
^{14}C CPM (○)
120
80
40
0
600
400
200
0
4
8
12
16
20
90
50
30
20
10
0
Fraction number from bottom

has been large scale breakdown of the bulk DNA (Fig. 13A). This breakdown is not primarily caused by the chloroform extraction, since a similar sedimentation pattern is observed even if the chloroform step is eliminated (Fig. 13B). Even if the cells were incubated with enzyme (in this case proteinase K) for only 1 hour instead of overnight, the overnight dialysis was enough to cause DNA breakdown (Fig. 13C). On the other hand, overnight incubation with enzyme caused some breakdown even without the dialysis step (Fig. 13D), although the breakdown is much less severe. Apparently any treatment that causes the DNA to remain overnight before being run on the sucrose gradients will cause at least some breakdown.

b. Proteinase K-chloroform-ethanol method

To avoid having the DNA sit overnight, ethanol precipitation and washing was substituted for dialysis, and the incubation with proteinase K was limited to 1 hour. This method of purification seems to work, as can be seen in Fig. 14. The Okazaki peak produced (#34-36) by pulse labeling cells for 30 seconds at room temperature, purifying the DNA and sedimenting it through a sucrose gradient (Fig. 14A) banded at the density of DNA in a CsCl gradient. 100% of the counts put into the CsCl gradient were recovered and there was no radioactivity floating on the top (Fig. 14B).

That the chloroform extraction is necessary can be demonstrated by examining the sedimentation profile of DNA from cells lysed before

Figure 13. Sucrose gradient profiles of pulse labeled DNA purified by enzyme digestion and dialysis, with and without chloroform extraction. Cells were labeled overnight with (^{14}C) thymidine, lysed with 0.25% sarkosyl, purified in various ways and centrifuged on alkaline sucrose gradients.

(A) The cells were pulsed for 20 minutes at room temperature before lysis. After lysis they were incubated overnight with pronase at 37°C, chloroform extracted, dialyzed overnight against 1/10 SSC containing 0.1% sarkosyl, denatured and spun in an SW27 rotor at 25,000 rpm for 16 hours at 0°C.

(B) The cells were treated and in (A) except that the chloroform extraction was eliminated.

(C) After lysis the cells were purified as in (B) except that they were incubated with proteinase K for 1 hour instead of with pronase overnight. After purification the DNA was spun in an SW50.1 rotor at 35,000 rpm for 2.5 hours at 23°C.

(D) After lysis the cells were purified as in (B) except that the dialysis step was eliminated and proteinase K was substituted for pronase. After purification the DNA was spun in an SW41 rotor at 40,000 rpm for 4 hours at 23°C.

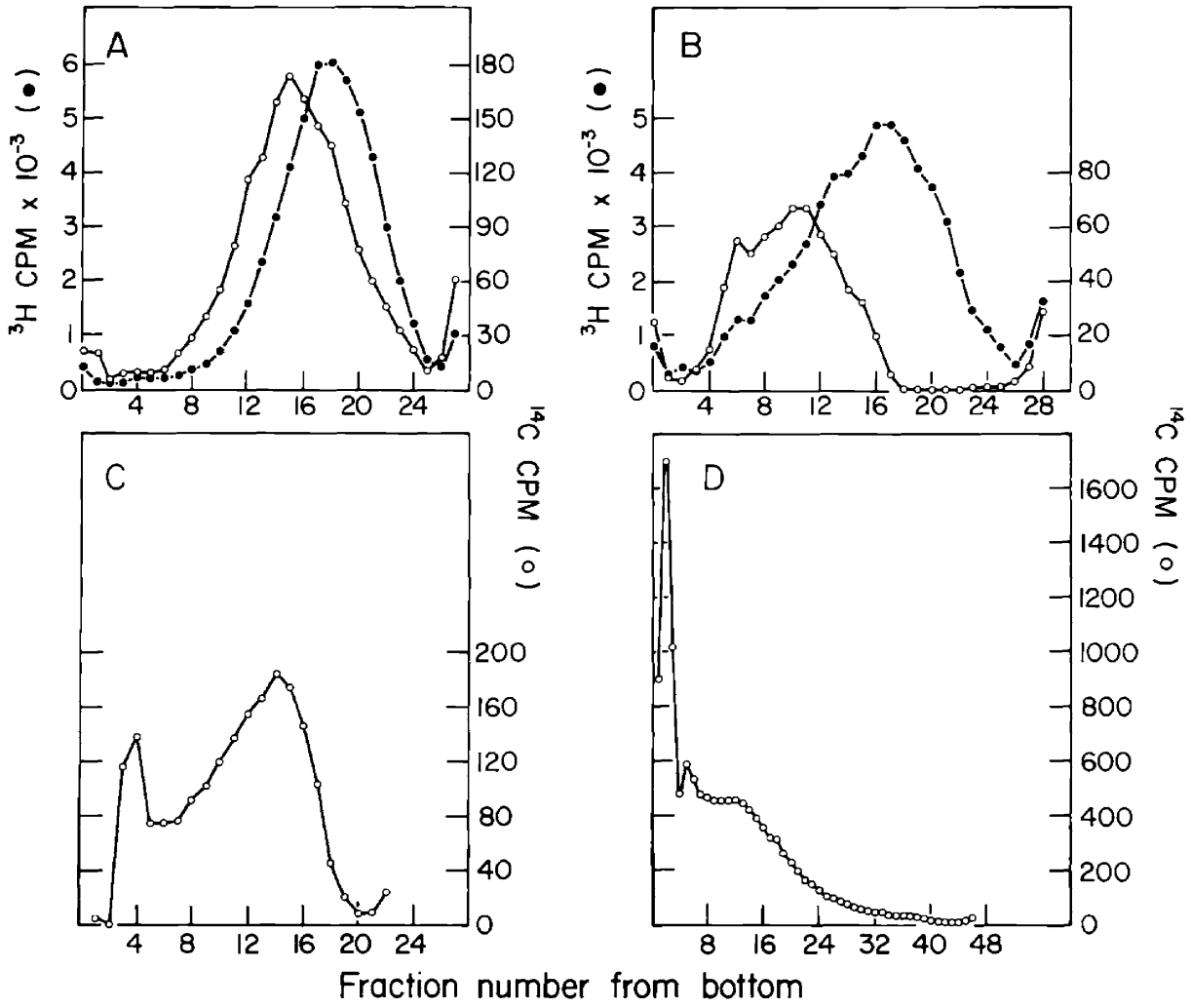
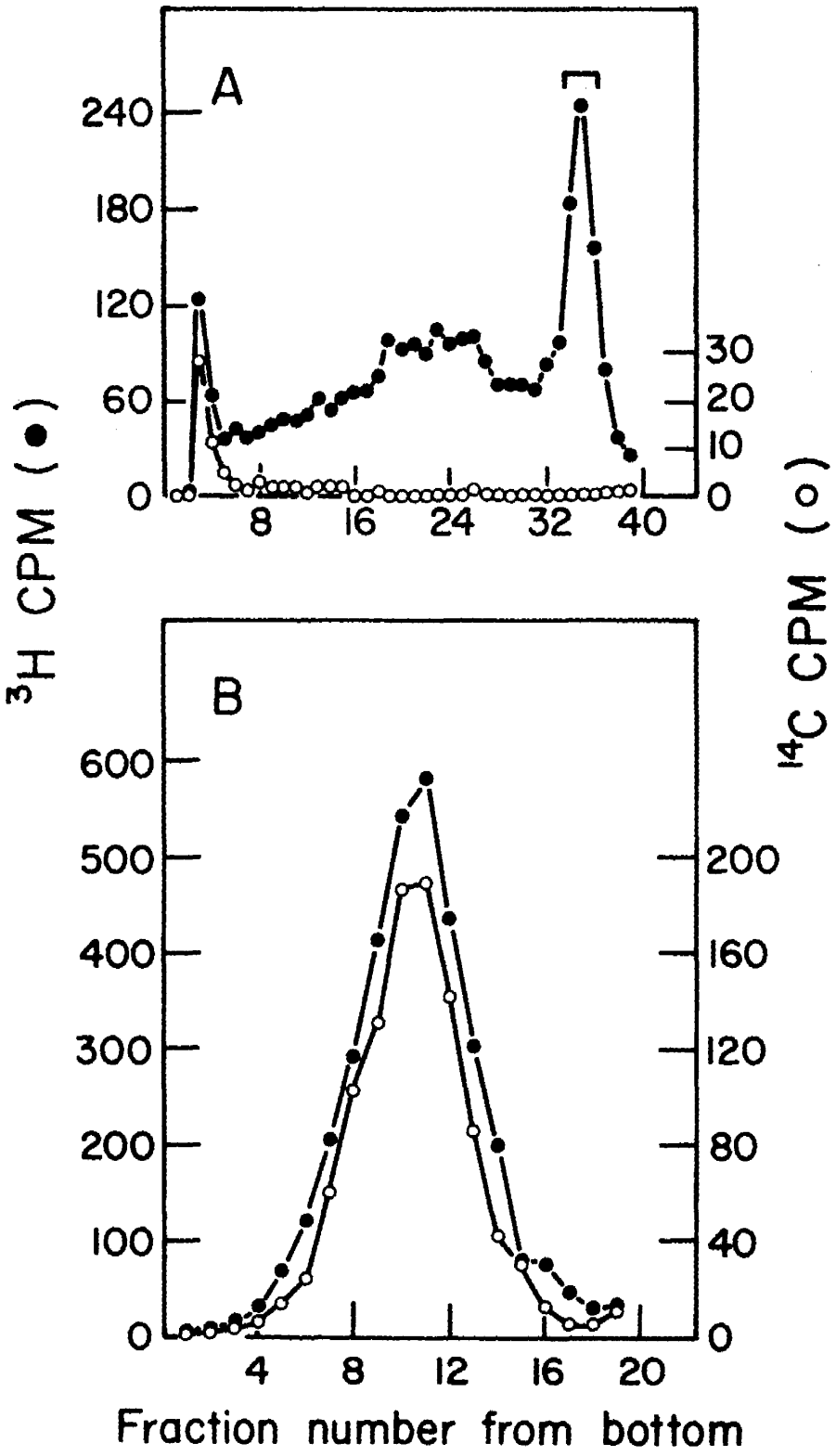

A
B
C
D
^{3}H CPM x 10^{-3} (●)
^{14}C CPM (○)
Fraction number from bottom

Figure 14. Sucrose and CsCl gradient profiles of pulse labeled DNA purified by proteinase K, chloroform extraction and ethanol precipitation. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 30 seconds at room temperature. The cells were lysed with buffer A containing proteinase K, incubated for 1 hour at 37°C, chloroform extracted and ethanol precipitated. The precipitated DNA was then washed 3 times with 70% ethanol, made 0.2 N in NaOH, heated at 50°C for 45 minutes and centrifuged on an alkaline sucrose gradient (A) in an SW41 rotor at 40,000 rpm for 4 hours at 23°C. The peak fractions (#34-36) were pooled, (^{14}C)-labeled small DNA was added and the sample was centrifuged to equilibrium in a CsCl gradient (B) in an SW50.1 rotor at 33,000 rpm for 67 hours at 20°C.


A
B
240
180
120
60
0
30
20
10
0
8
16
24
32
40
600
500
400
300
200
100
0
200
160
120
80
40
0
4
8
12
16
20
^{3}H CPM (●)
^{14}C CPM (○)
Fraction number from bottom

the addition of (^{3}H) thymidine, treated with proteinase K and ethanol precipitated (Fig. 15). When the chloroform extraction is eliminated, a substantial artifactual Okazaki peak appears.

It is also obvious that the ethanol precipitation and subsequent washings with 70% ethanol are necessary for removal of the artifact. The sedimentation pattern produced by cells lysed before the addition of (^{3}H) thymidine, treated with proteinase K and chloroform extracted (Fig. 16B) shows that the ethanol treatment is necessary for removal of the artifact. When both the chloroform and ethanol steps are done, a 3 hour incubation with proteinase K is as effective as an overnight incubation in removing the artifact (Figs. 16C,D).

c. Nuclear isolation method

Since it is obvious that some cellular component is contributing to the creation of the artifact, purifying nuclei away from cytoplasmic debris might also eliminate the artifact. This is indeed the case. If cells are pulse labeled and the pulse is stopped by the addition of NP40 (Pearson and Hanawalt, 1971), whether the resulting nuclei are lysed with SDS and incubated with proteinase K (Fig. 17A) or whether they are merely lysed with NaOH (results not shown), the resulting Okazaki peak bands completely in a CsCl gradient (Fig. 17B). Thus simply isolating nuclei and washing them once is sufficient to completely eliminate the artifact.

Figure 15. Sucrose gradient profile of pulse labeled DNA from cells lysed before the addition of radioactivity, purified by proteinase K and ethanol precipitation. Cells were lysed with buffer A and pronase, and (^{3}H) thymidine (200 μCi) was added to the lysate. The lysate was treated as in Figure 14, except that the chloroform extraction was eliminated. The purified DNA was centrifuged in an alkaline sucrose gradient in an SW41 rotor at 40,000 rpm for 3.5 hours at 23°C.

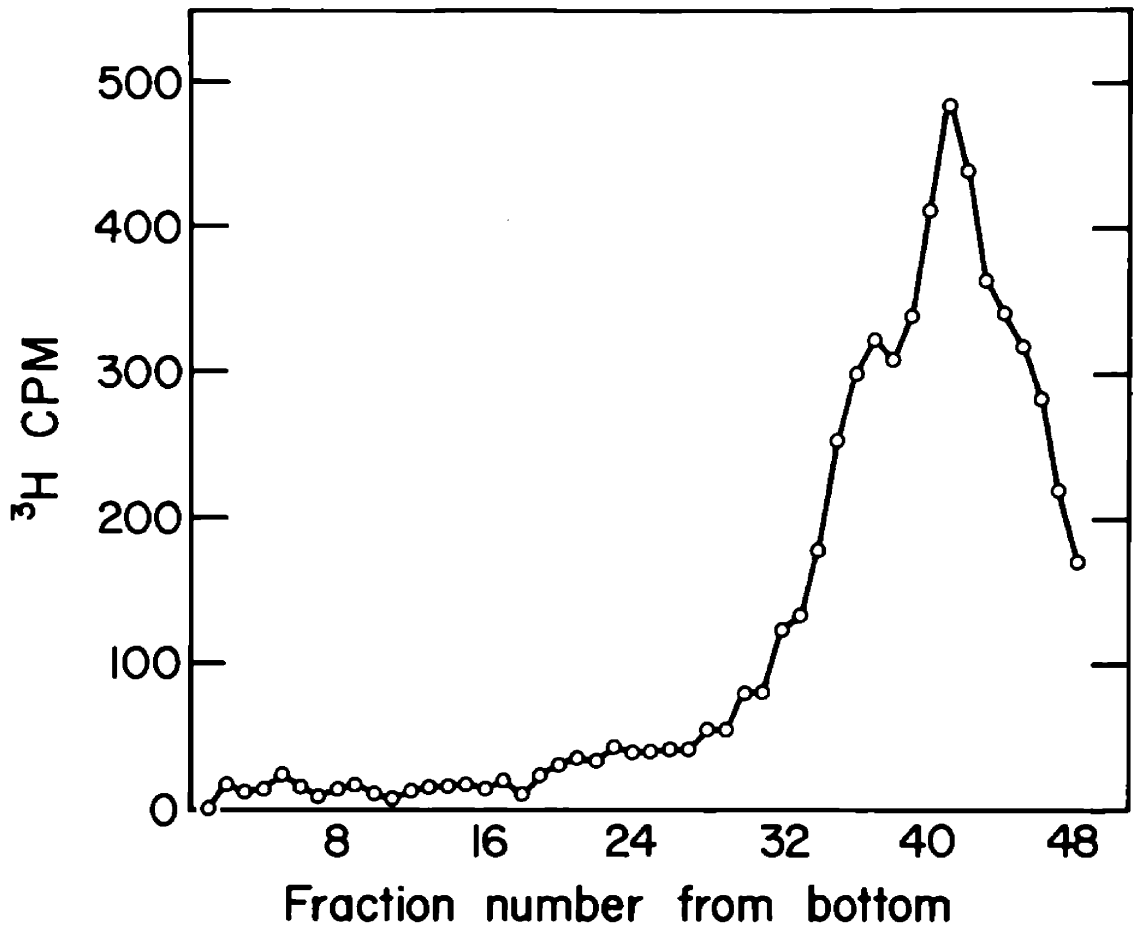

500
400
300
200
100
0
^{3}H CPM
8
16
24
32
40
48
Fraction number from bottom

Figure 16. Sucrose gradient profiles of DNA purified by enzyme and chloroform, with or without ethanol precipitation. Cells were prelabeled overnight with (^{14}C) thymidine and lysed as in Figure 14 either before or after the addition of 200 μCi of (^{3}H) thymidine. Cells were treated as in Figure 14, except that in (B) the ethanol precipitation and washings were eliminated and in (D) the lysate was incubated in proteinase K overnight instead of for only one hour. The purified lysates were centrifuged in an alkaline sucrose gradient in an SW50. 1 rotor at 35,000 rpm for 18 hours at 20°C.

(A) Cells were pulse labeled with (^{3}H) thymidine for 1 minute at room temperature before lysis (B), (C) and (D) The (^{3}H) thymidine was added to the cells after lysis.

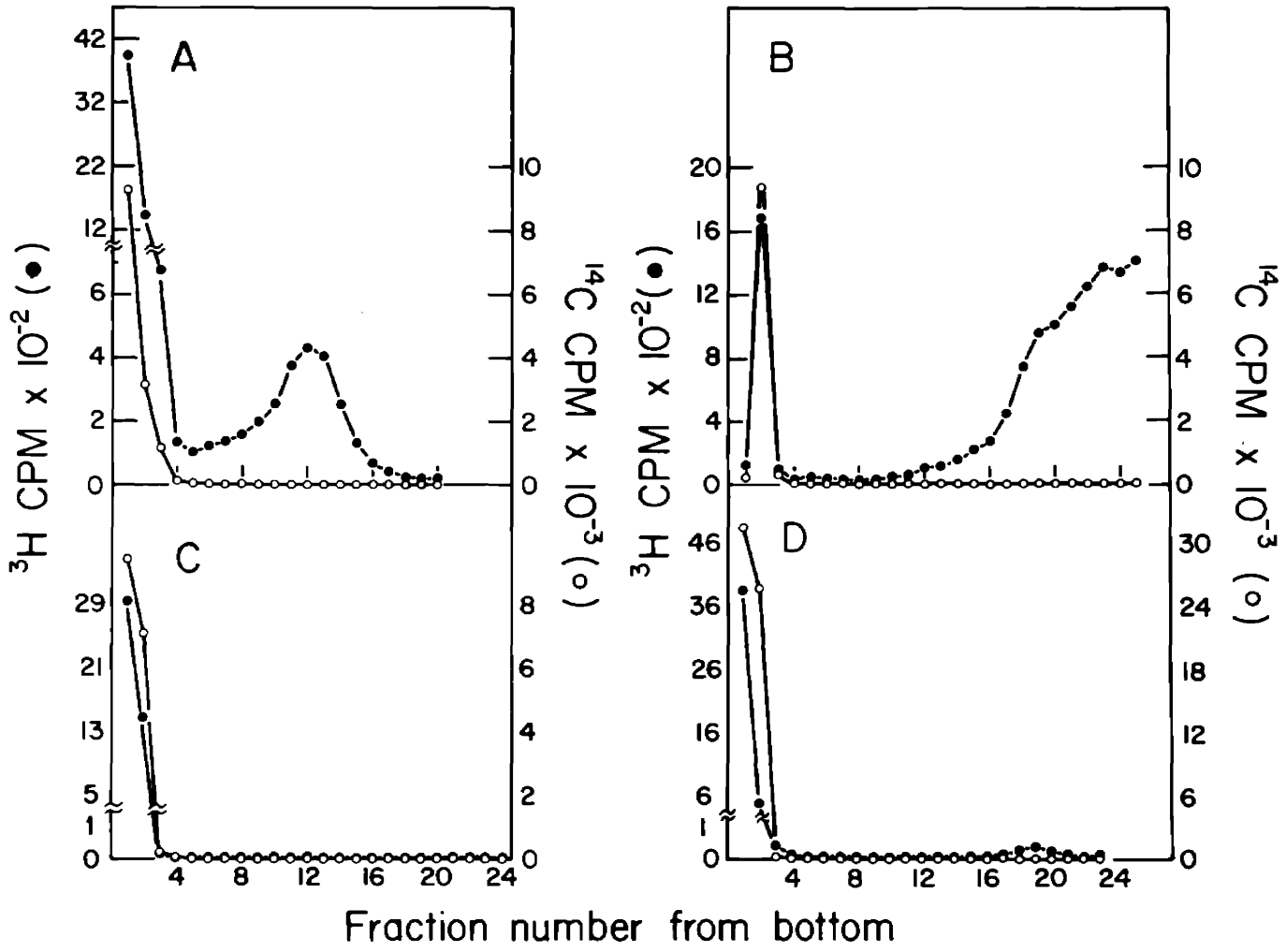
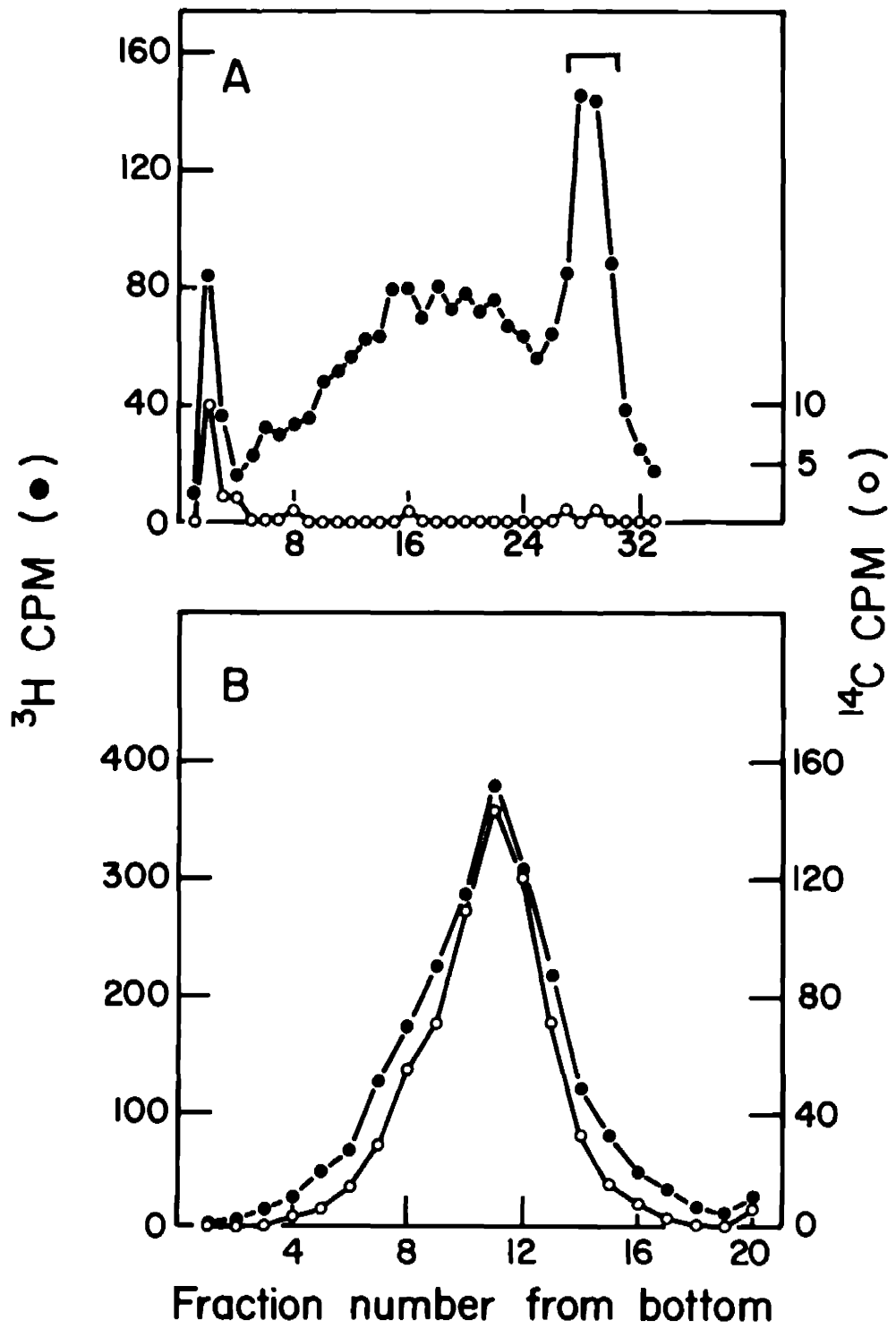

A
B
C
D
^{3}H CPM x 10^{-2} (●)
^{14}C CPM x 10^{-3} (○)
Fraction number from bottom

Figure 17. Sucrose and CsCl gradient profiles of pulse labeled DNA purified by nuclear isolation. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 30 seconds at 37°C with (^{3}H)-thymidine and the pulse was stopped by the addition of 0. 65% NP40. The resulting nuclei were scraped into a tube with a rubber policeman and, after a 10 minute incubation, spun down, washed once in TD and lysed in 0. 2 N NaOH. This solution was heated at 50°C for 45 minutes and centrifuged in an alkaline sucrose gradient

(A) in an SW41 totor at 40, 000 rpm for 3. 5 hours at 23°C. The Okazaki peak fractions (#28-30) were pooled,(^{14}C) labeled small DNA was added as a marker and the sample was centrifuged to equilibrium in a CsCl gradient.

(B) in an SW50. 1 rotor at 33, 000 rpm for 67 hours at 20°C.


A
B
160
120
80
40
0
10
5
8
16
24
32
400
300
200
100
0
160
120
80
40
0
4
8
12
16
20
^{3}H CPM (●)
^{14}C CPM (○)
Fraction number from bottom

C. Sedimentation conditions

1. Amount of DNA

Although the above purification methods seem to have eliminated artifacts due to radioactivity not in DNA, artifacts may also arise if the DNA is improperly denatured or because of sedimentation conditions. Since an analysis of the sequence of events during pulse labeling depends primarily on analyzing sedimentation profiles, it is necessary to ascertain that within the limits of the conditions used nothing was done to the cells after lysis that could produce artifactual results.

To make sure that the number of cells being pulse labeled or the amount of DNA being sedimented does not cause variations in the sedimentation profiles, plates containing widely differing numbers of cells were pulse labeled and sedimented through alkaline sucrose gradients. Figure 18 shows the sedimentation patterns of all the DNA from plates containing 7×10^5 cells (Fig. 18A) or 4×10^6 cells (Fig. 18B) which were pulse-labeled for 1 minute at 37°C. There are obviously no significant differences between the two sedimentation patterns.

However, when the gradients are centrifuged under conditions that do not bring the bulk DNA to the bottom of the gradient, the bulk DNA sedimentation profile is affected by the amount of DNA being analyzed. When the DNA from plates varying in cell number from 2×10^5 (Fig. 19A) to 1×10^7 (Fig. 19C) is run on gradients, the

Figure 18. Sucrose gradient profiles of differeing amounts of pulse labeled DNA. Cells, pre-labeled overnight with (^{14}C) thymidine, were pulse labeled for 1 minute at 37°C with (^{3}H) thymidine. Cells were then treated and centrifuged as in Figure 17.

(A) 7×10^5 cells were pulse labeled and centrifuged.

(B) 4×10^6 cells were pulse labeled and centrifuged.

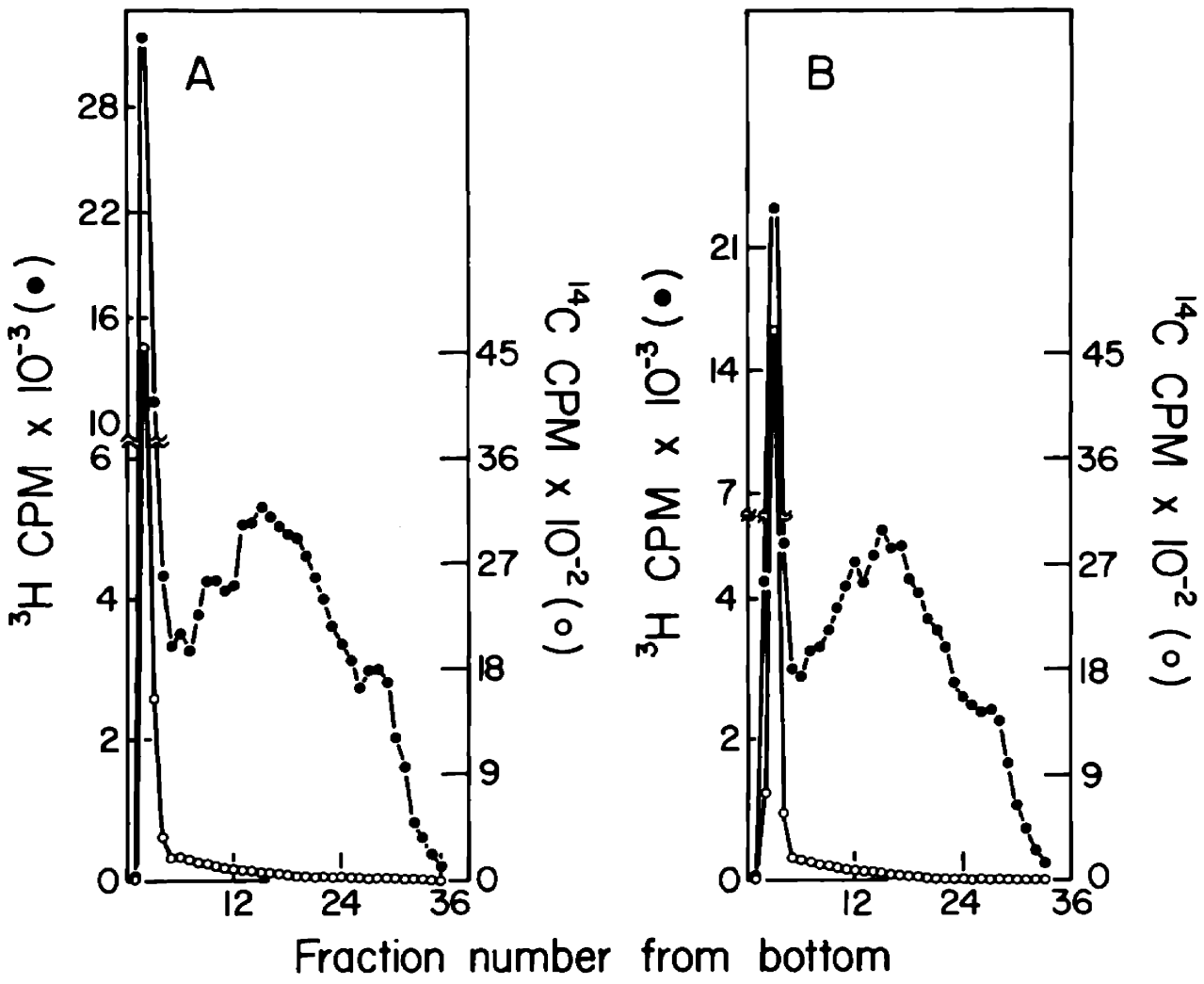
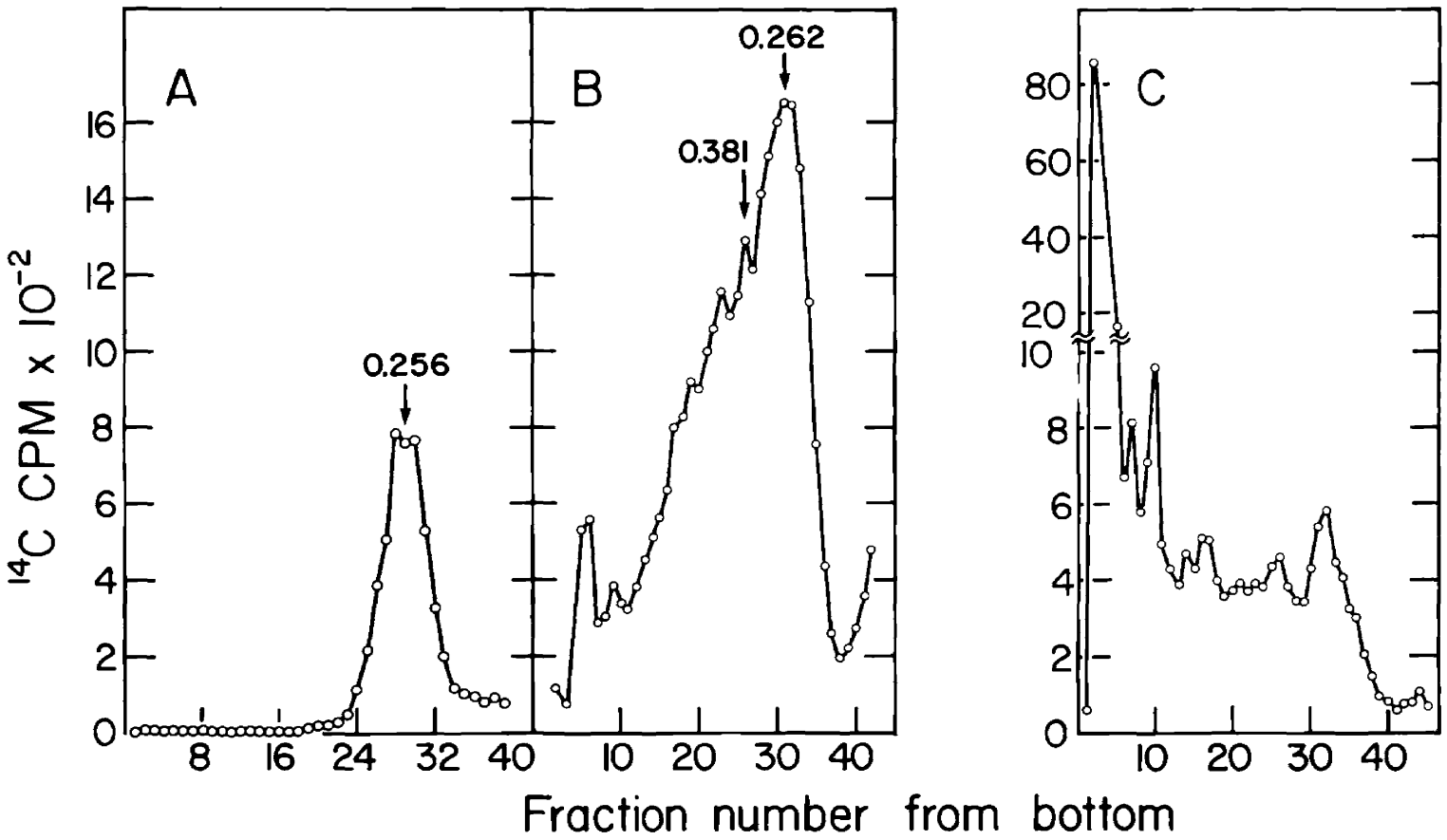

A
B
^{3}H CPM x 10^{-3} (•)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom

Figure 19. Sucrose gradient profiles of differing amounts of DNA. Cells were labeled overnight with (^{14}C) thymidine, lysed and treated as in Figure 17. The DNA was centrifuged on an alkaline sucrose gradient in an SW41 rotor at 20,000 rpm for 3.5 hours at 23°C. The numbers above the arrows indicate the fraction of the distance down the gradient traveled by the DNA at that point.

(A) DNA from 2×10^5 cells.

(B) DNA from 2×10^6 cells.

(C) DNA from 1×10^7 cells.


A
B
C
0.256
0.381
0.262
^{14}C CPM x 10^{-2}
Fraction number from bottom

resulting profiles make it quite obvious that the more DNA layered onto the gradient, the further the peak will travel. In fact, the sedimentation profiles of bulk DNA from 2×10^5 cells, 2×10^6 cells and 1×10^7 cells are quite different.

It should be remembered, however, that this concentration effect only applies when the bulk DNA is not sedimented all the way through the gradient. Once the bulk DNA is sedimented to the bottom the pulse-label pattern does not change within this concentration range (Fig. 18). All the experiments reported here were done at cell concentrations of about 2×10^6.

Also note that the amount of pulse labeled DNA at the bottom of the gradient is not necessarily indicative of the extent to which the pulse labeled DNA has been converted to strands of bulk length. In different experiments of the same pulse time, widely varying percentages of acid-precipitable radioactivity are found at the bottom of the gradient (Fig. 20) although the rest of the sedimentation pattern is fairly reproducible, suggesting that variable percentages of smaller DNA (intermediate and Okazaki size) can be aggregated with bulk DNA.

2. Speed of centrifugation

The speed of centrifugation does not seem to affect the profile of either bulk- or intermediate-sized DNA. Equal amounts of DNA prepared in the same manner and run on alkaline sucrose gradients either at 38,000 rpm for 2 hours (Fig. 21A) or at 13,500 rpm for 16 hours (Fig. 21B) give profiles of similar shape even though they don't sediment exactly the same distance down the gradients.

Figure 20. Sucrose gradient profiles of pulse labeled DNA. Two plates of cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 5 minutes at room temperature with (^{3}H) thymidine. The cells were lysed and treated as in Figure 14. Lysates were centrifuged on alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 3.5 hours at 23°C.

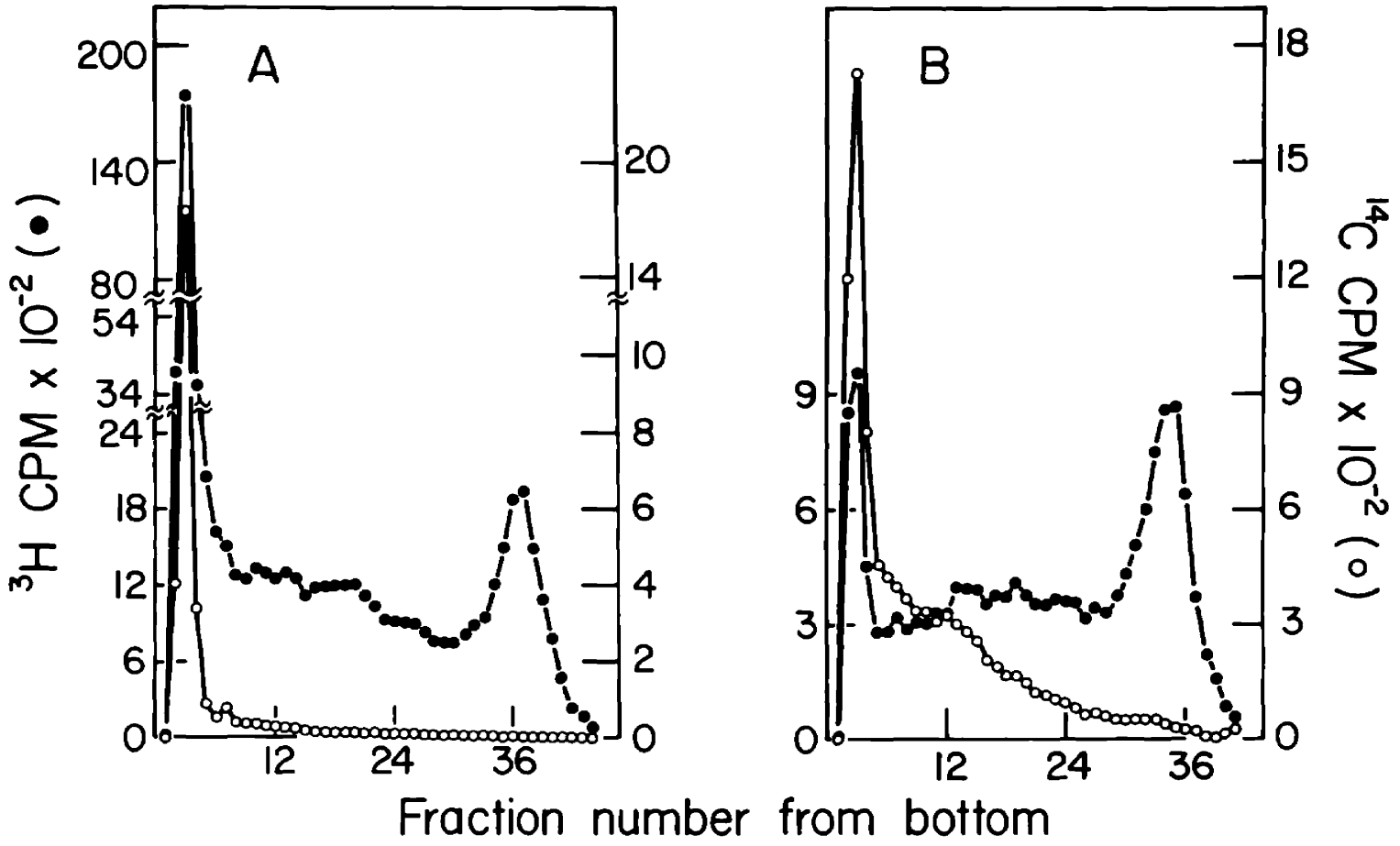
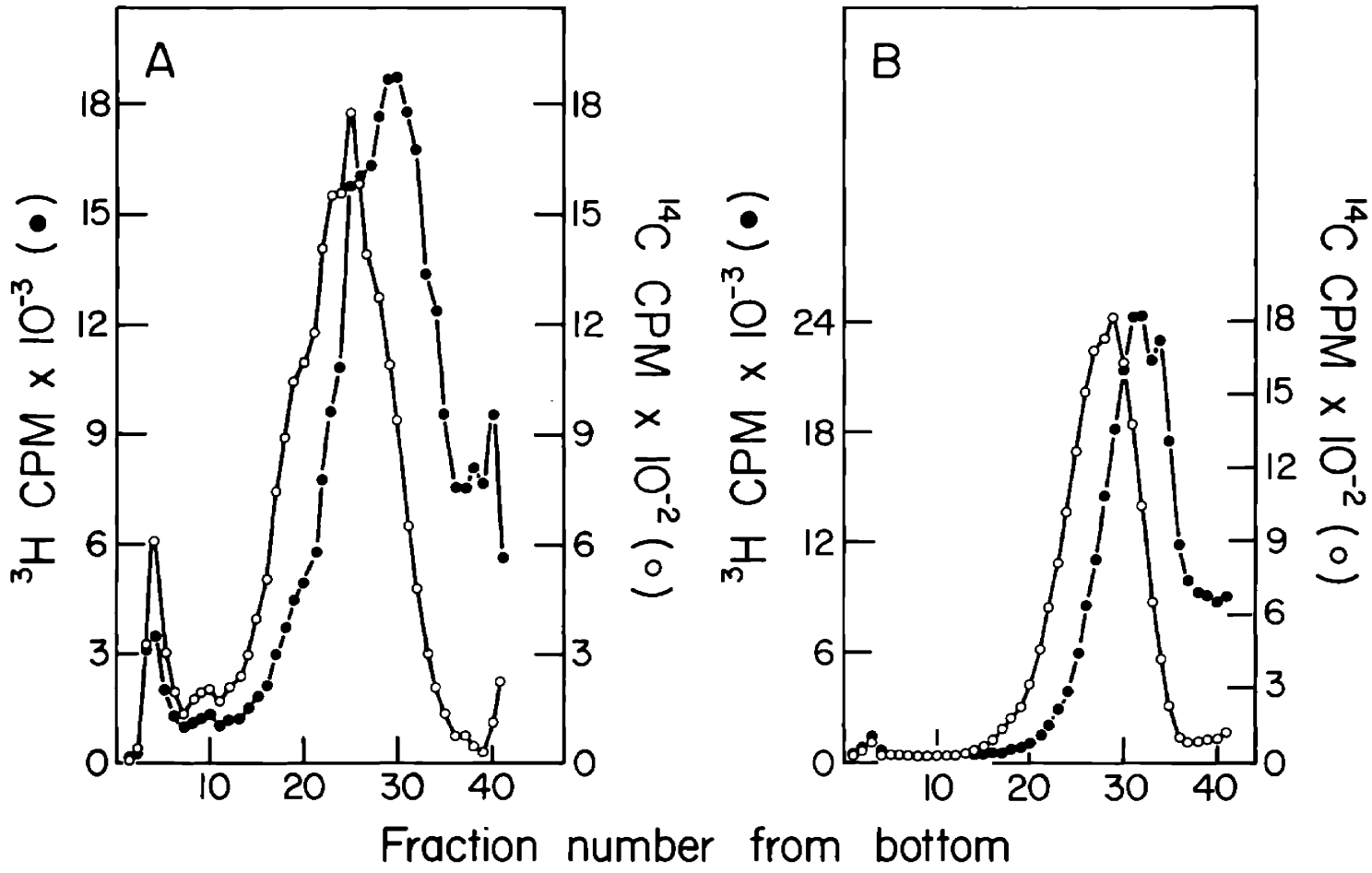

A
B
200
140
80
54
34
24
18
12
6
0
20
14
10
8
6
4
2
0
18
15
12
9
6
3
0
12
24
36
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom

Figure 21. Sucrose gradient profiles of DNA centrifuged at different speeds. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 20 minutes at 37°C with (^{3}H) thymidine and lysed by the addition of 0.2 M NaOH. The lysates were heated at 50°C for 45 minutes and centrifuged on alkaline sucrose gradients in an SW41 rotor.

(A) Gradient centrifuged at 38,000 rpm for 2 hours at 23°C.

(B) Gradient centrifuged at 13,500 rpm for 16 hours at 23°C.


A
B
^{3}H CPM x 10^{-3} (●)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom

3. pH

pH might affect the sedimentation profiles in one of two ways. Variations in the pH of the alkaline sucrose gradients may cause the profiles to change, and variations in the pH of the denaturing solution may have the same effect. Figures 22 and 23 show the results of varying the pH of the sucrose solutions from 12.1 to 12.8. Up to 12.5 there does not seem to be much difference in the profiles of bulk (Figs. 22A,B,C) or intermediate (Figs. 23A,B,C) DNA. (The slightly skewed peak in 22B is a random variation that is occasionally observed.) However, at pH 12.8 the bulk peak (Fig. 22D) loses its leading edge, and the intermediate peak (Fig. 23D) seems to get a little smaller.

D. Denaturing conditions

Increasing the NaOH concentration during denaturation also changes the sedimentation profile. Raising the NaOH concentration of the denaturing solution to 0.3 M causes some breakdown of both bulk and intermediate DNA (Figs. 24A,B). This result can also be achieved by raising the temperature to 60°C (Fig. 24C) or allowing the denaturation to continue for an additional 3 hours (Fig. 24D).

These degradative effects can be demonstrated through another type of analysis as well. Using electron microscopy to examine DNA after denaturing it under different conditions, it becomes obvious that DNA denatured at 70°C (Fig. 25) is significantly smaller than that denatured at 50°C (Fig. 26). DNA denatured for 2 hours or at 0.5 M NaOH shows the same effect, but to a lesser degree (results not shown).

Figure 22. Profiles of DNA sedimented through alkaline sucrose gradients of differing pH. Cells were labeled overnight with (^{14}C)-thymidine and treated as in Figure 17. The DNA was sedimented through alkaline sucrose gradients of differing pH. The gradients were spun in an SW41 rotor at 20,000 rpm for 3.5 hours at 23°C. Numbers above the arrows indicate the fraction of the distance down the gradient traveled by the DNA at that point.

(A) pH of sucrose solution, 12.1.

(B) pH of sucrose solution, 12.3.

(C) pH of sucrose solution, 12.5.

(D) pH of sucrose solution, 12.8.

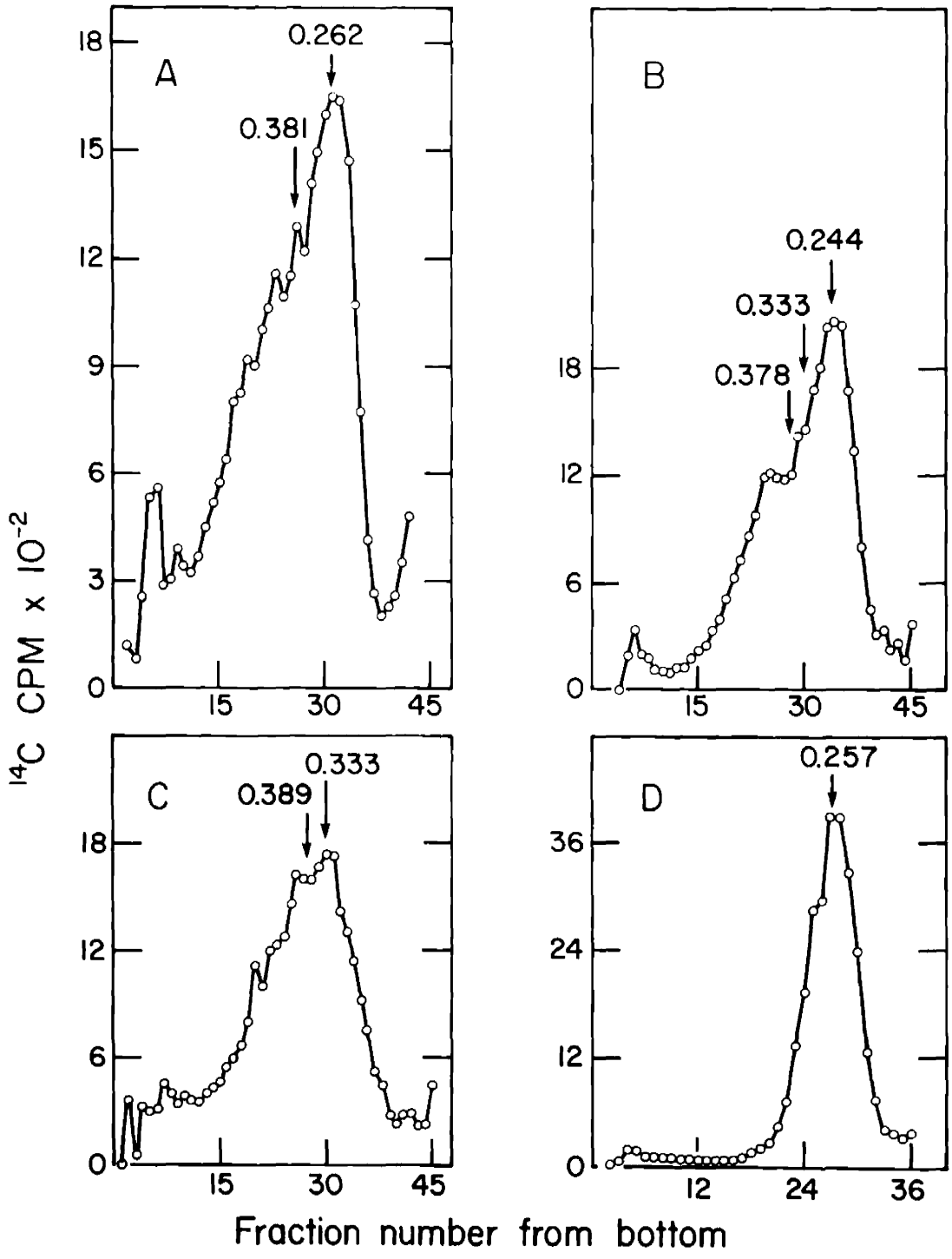

A
0.262
0.381
B
0.244
0.333
0.378
C
0.333
0.389
D
0.257
^{14}C CPM x 10^{-2}
Fraction number from bottom

Figure 23. Profiles of pulse labeled DNA sedimented through alkaline sucrose gradients of differing pH. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 5 minutes at 37°C with (^{3}H) thymidine. Cells were treated as in Figure 17 and sedimented through alkaline sucrose gradients of differing pHs. Gradients were centrifuged in an SW41 rotor at 40,000 rpm for 2.5 hours at 23°C.

(A) pH of sucrose solution, 12.1.

(B) pH of sucrose solution, 12.3.

(C) pH of sucrose solution, 12.5.

(D) pH of sucrose solution, 12.8.

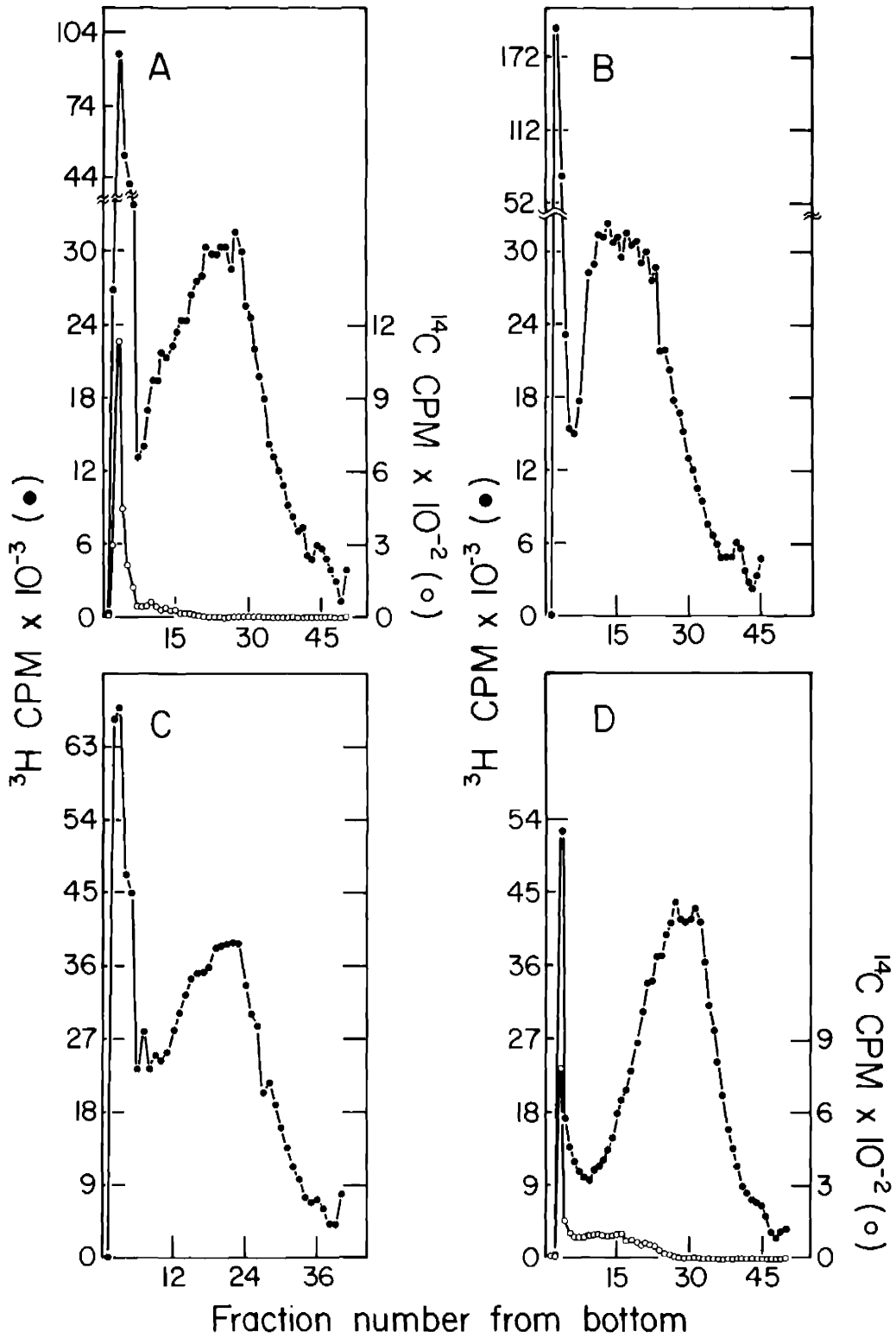

A
B
C
D
^{3}H CPM x 10^{-3} (●)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom

Figure 24. Sucrose gradient profiles of pulse labeled DNA denatured in various ways. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 20 minutes at room temperature with (^{3}H) thymidine. Cells were treated as in Figure 14 and the resulting DNA was denatured and heated in different ways. The DNA was then centrifuged through alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for only 2 hours at 23°C, so that the bulk DNA would not all sediment to the bottom. The numbers above the arrows indicate the fraction of the distance down the gradient traveled by the DNA at that point.

(A) DNA was denatured by 0.2 N NaOH and heated for 45 minutes at 50°C.

(B) DNA was denatured by 0.3 N NaOH and heated for 45 minutes at 50°C.

(C) DNA was denatured by 0.2 N NaOH and heated for 45 minutes at 60°C.

(D) DNA was denatured by 0.2 N NaOH and heated for 4 hours at 50°C.

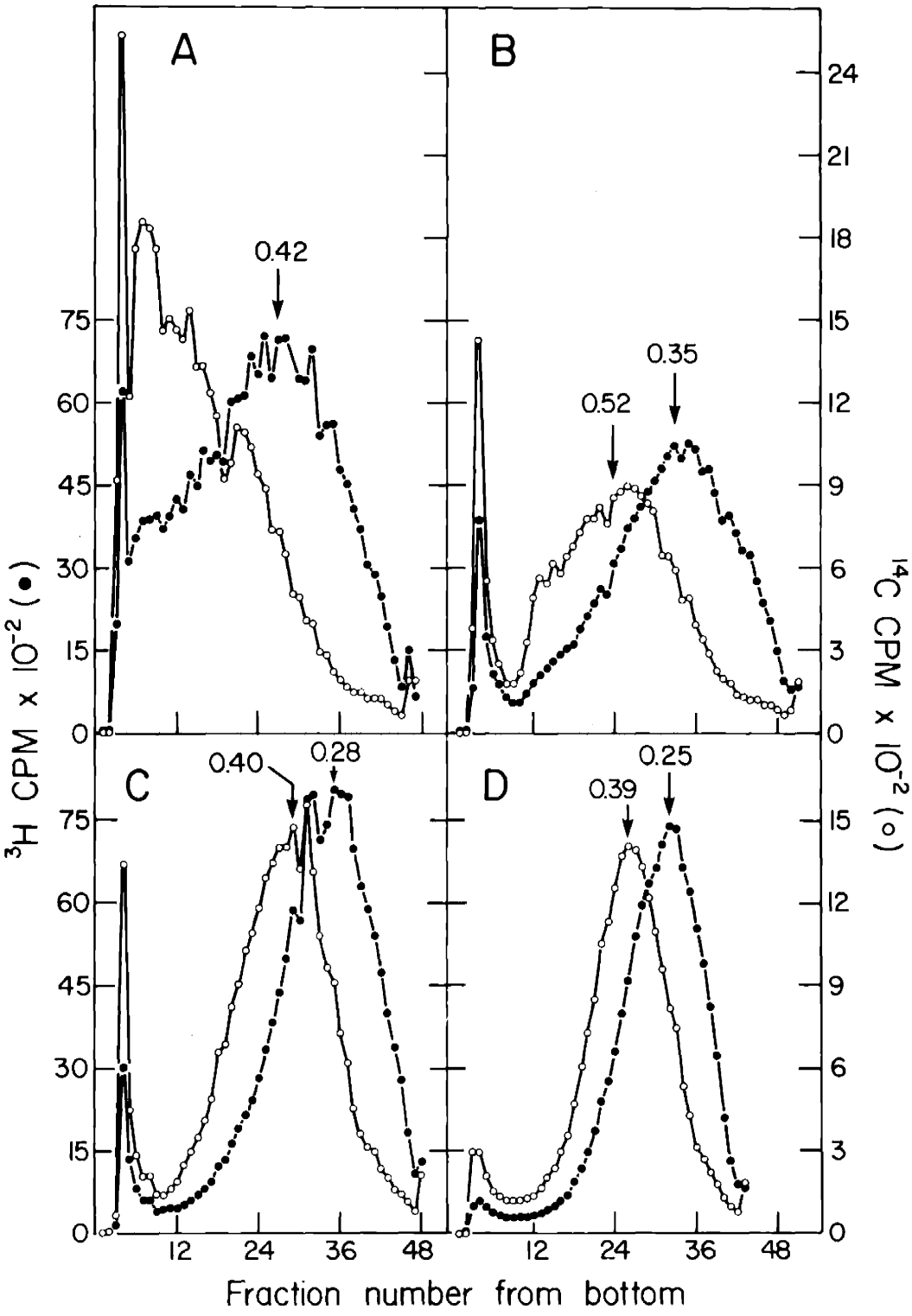

A
B
C
D
0.42
0.52
0.35
0.40
0.28
0.39
0.25
^{3}H CPM x 10^{-2} (●)
^{14}C CPM x 10^{-2} (○)
Fraction number from bottom
0
15
30
45
60
75
3
6
9
12
15
18
21
24
12
24
36
48

Figure 25. Electron micrograph of DNA denatured at 70°C. Cells were pulse labeled and treated as in Figure 24, and the resulting DNA was denatured with 0.2 N NaOH and heated at 70°C for 45 minutes. The DNA was then examined by electron microscopy. The DNA was analyzed by an aqueous Kleinschmidt spread. Magnification is 20,000X.

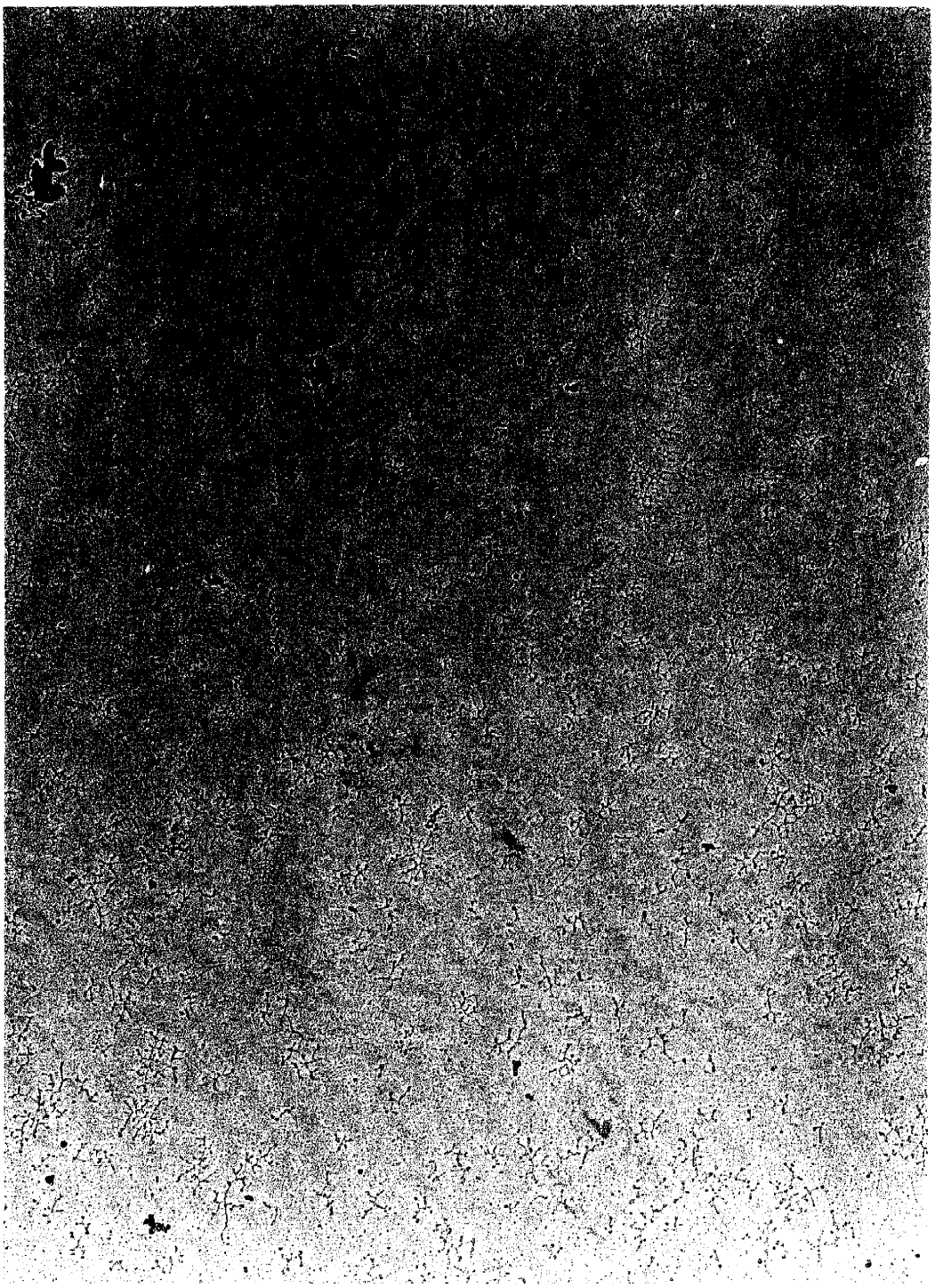
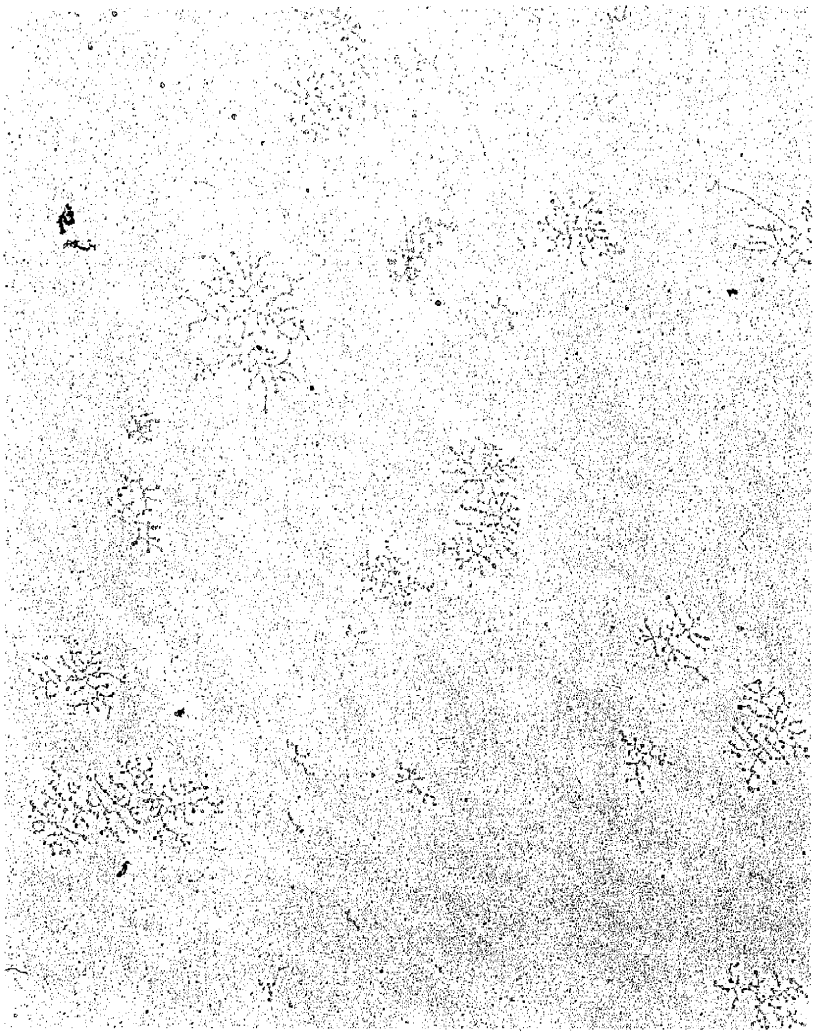


Figure 26. Electron migrograph of DNA denatured at 50°C. Experiment done exactly as in Figure 25, except the denatured DNA was heated at only 50°C. Magnification is 20,000X.



If the denaturing NaOH concentration is raised all the way to 2. 6 N, both the bulk and intermediate DNA are reduced to small strands (Fig. 27). Allowing the denaturation to proceed for more than one hour at this high pH causes the DNA strands to become even smaller (results not shown).

II. Discontinuous DNA synthesis

Once experimental methods had been devised which produce sedimentation profiles that are accurate reflections of DNA synthesis, the sequence of events during pulse-labeling of CHO cells could be examined again. Since the nuclear isolation method eliminates the artifact as well as does the proteinase K-chloroform-ethanol method and involves much less handling of the DNA prior to centrifugation (thus minimizing the chances of DNA breakdown), it was used for most of the following experiments. Some examples of experiments done with the other method are given to demonstrate that both methods give similar results.

A. Pulse labeling at 37°C

Cells pulse labeled for short times at 37°C give sedimentation profiles (Fig. 28) similar to those reported previously (Fig. 3). However, the proportion of radioactivity in the "Okazaki fragment" peak after the shortest pulses (8 sec - 30 sec, Figs. 28A, B) is greatly reduced (42%-28%) in comparison to those other pulses (15 sec-30 sec, Figs. 3A, B) which showed most of the radioactivity in the

Figure 27. Sucrose gradient profiles of pulse labeled DNA denatured at different concentrations of NaOH. Cells, prelabeled overnight with (^{14}C) thymidine, were pulse labeled for 20 minutes at 37 °C with (^{3}H)-thymidine, and lysed with differing concentrations of NaOH. The lysates were heated for 45 minutes at 50 °C and centrifuged on alkaline sucrose gradients in an SW27 rotor at 26, 000 rpm for 15 hours at 0 °C.

(A) NaOH concentration, 0. 2 N.

(B) NaOH concentration, 2. 6 N.

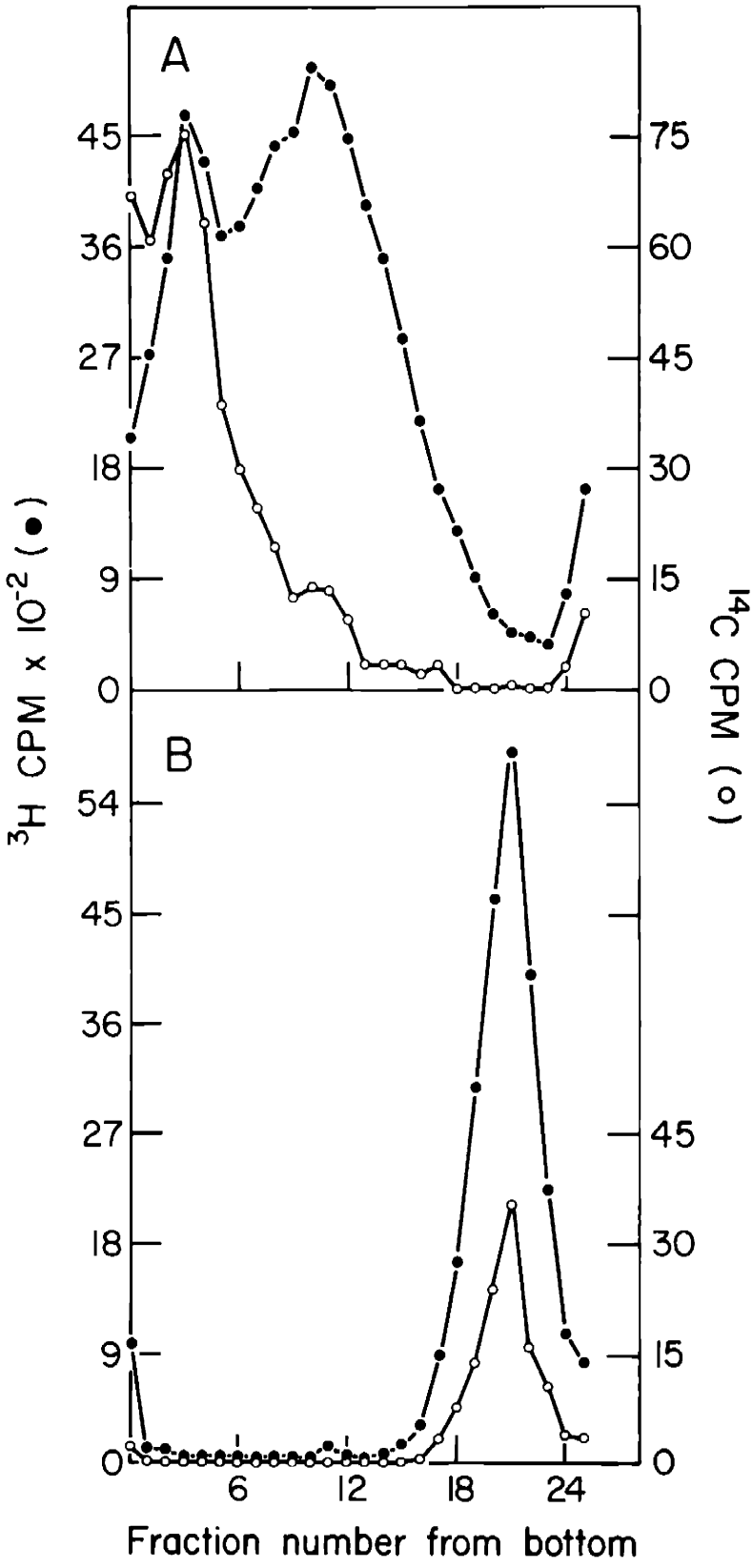
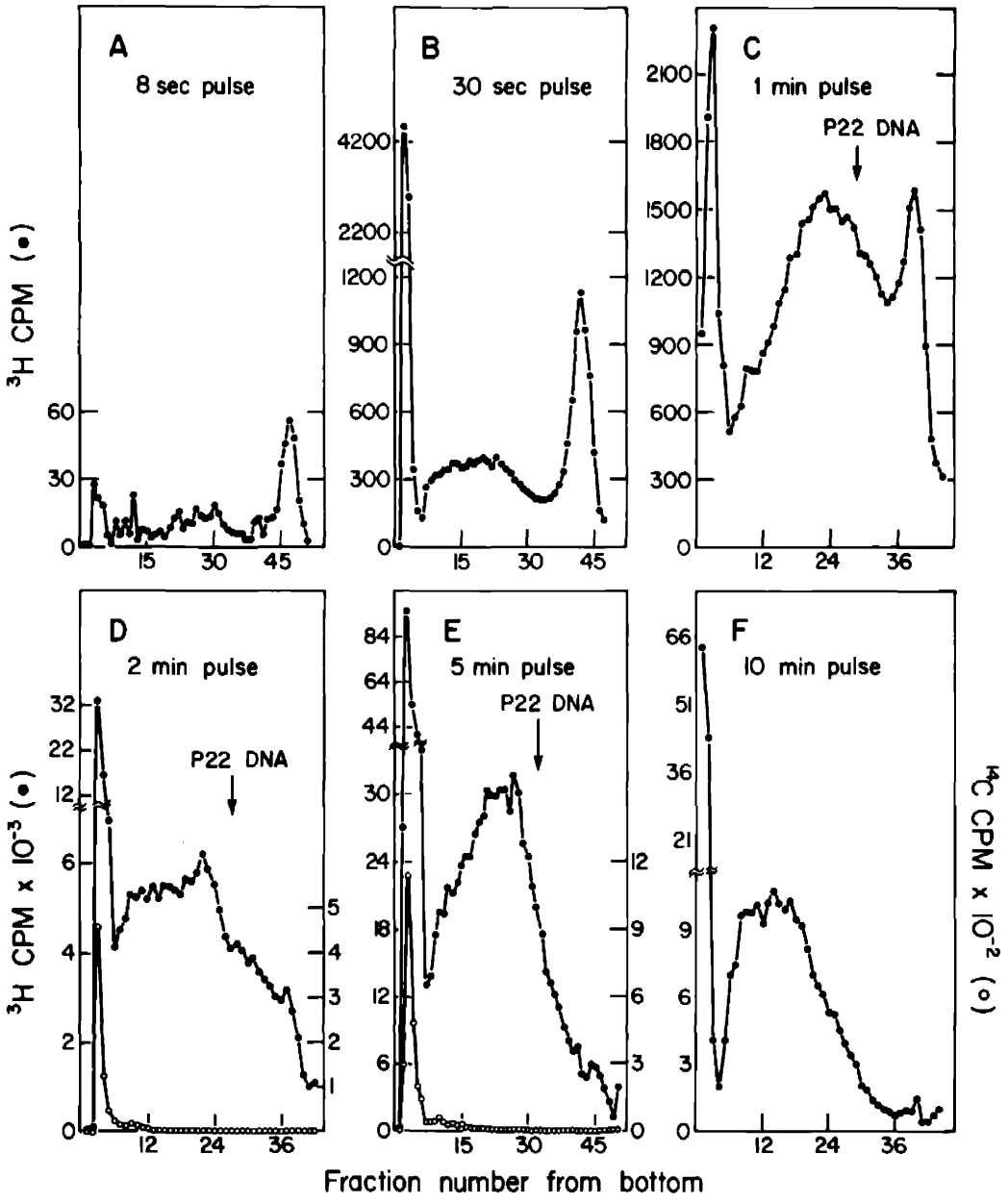

A
B
45
36
27
18
9
0
75
60
45
30
15
0
54
45
36
27
18
9
0
45
30
15
0
6
12
18
24
^{3}H CPM x 10^{-2} (●)
^{14}C CPM (○)
Fraction number from bottom

Figure 28. Sucrose gradient profiles of DNA pulse labeled at 37°C and purified by nuclear isolation. Cells, some of which were pre-labeled overnight with (^{14}C) thymidine, were pulse labeled for various times at 37°C with (^{3}H) thymidine, lysed, and treated as in Figure 17. Lysates were centrifuged on alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 2.5 hours at 23°C. The P22 DNA marker was added before heating the denatured DNA.


A
8 sec pulse
B
30 sec pulse
C
1 min pulse
P22 DNA
D
2 min pulse
P22 DNA
E
5 min pulse
P22 DNA
F
10 min pulse
^{3}H CPM (•)
^{3}H CPM x 10^{-3} (•)
^{14}C CPM x 10^{-2} (o)
Fraction number from bottom

"Okazaki fragment" peak (99%-44%). Also, the Okazaki and intermediate peaks are now more clearly resolved; this may be due to the elimination of artifactually adsorbed radioactivity from the region between the Okazaki and intermediate peaks or to the use of an SW41 rotor in the more recent experiments instead of the SW27 used earlier.

A similar sequence of events is observed when the DNA from pulse-labeled cells is purified by the proteinase K-chloroform-ethanol method (Fig. 29).

B. Pulse-chase labeling done at 37°C

When the cells are pulse labeled for 1 minute and chased for increasing time periods (Fig. 30) the intermediate peak can be seen to move across the gradient (Figs. 30A, 30B, 30C, 30D) until it is no longer a distinct peak (Fig. 30E) and finally sediments together with the bulk-labeled DNA (Fig. 30F). All of these sedimentation profiles, with the exception of the 1 minute pulse-24 minute chase, were produced by labeling 2×10^6 cells with 130 μCi/ml of (^{3}H)thymidine. Table I shows that the total amount of acid precipitable radioactivity increased between 3 and 4 times, but after 15 minutes there is almost no additional incorporation of (^{3}H) thymidine into DNA.

Not only does the intermediate peak move down the gradient, but its position after a pulse-chase is different than after a pulse of equivalent length. Figure 31 demonstrates that after a pulse of 1

Figure 29. Sucrose gradient profiles of DNA pulse labeled at 37°C and purified by proteinase K, chloroform extraction and ethanol precipitation. Cells, some of which were prelabeled with (^{14}C)-thymidine, were pulse labeled for various times at 37°C with (^{3}H)-thymidine, lysed and treated as in Figure 14. Lysates were centrifuged on alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 3.5 hours at 23°C.

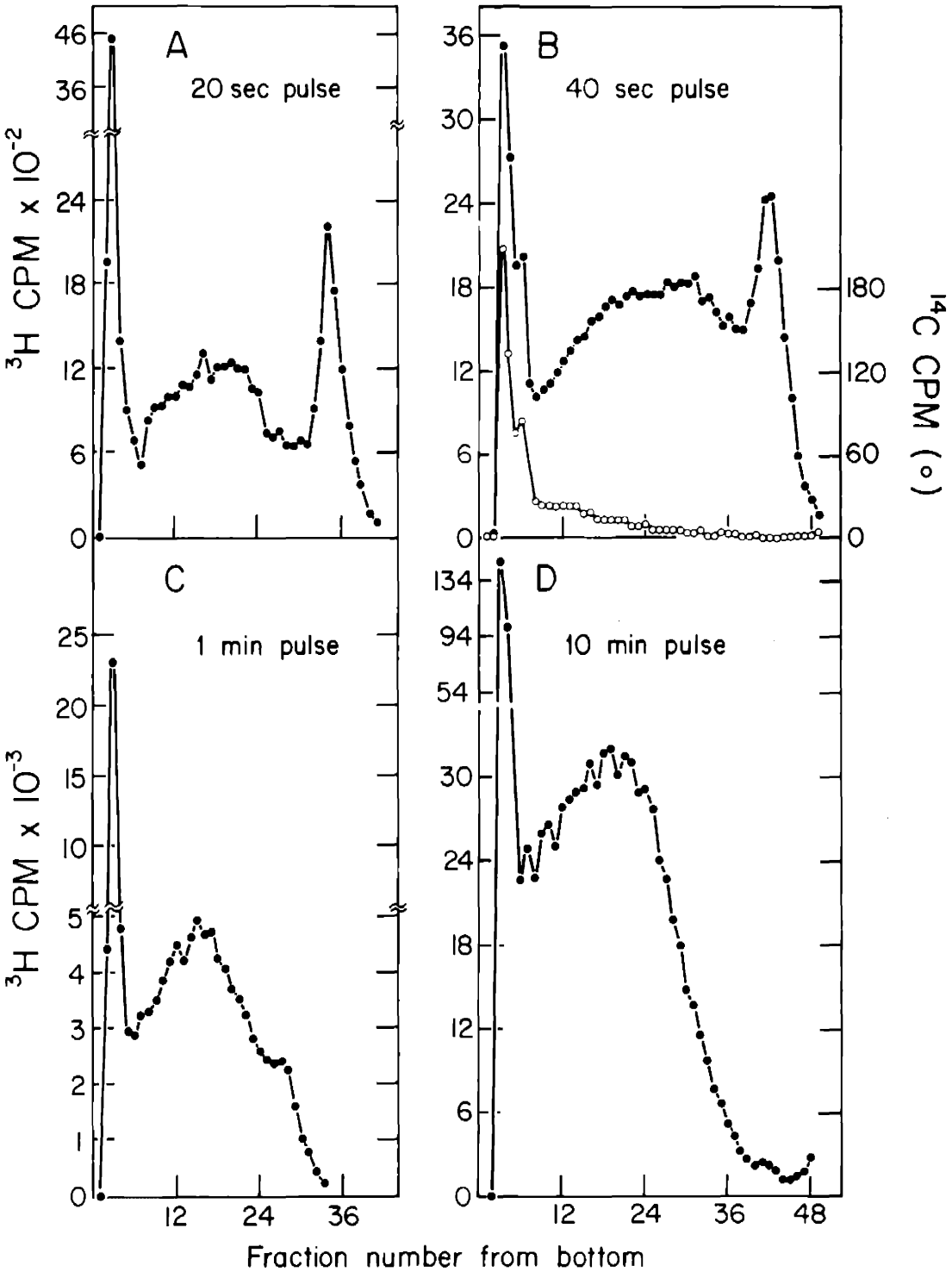
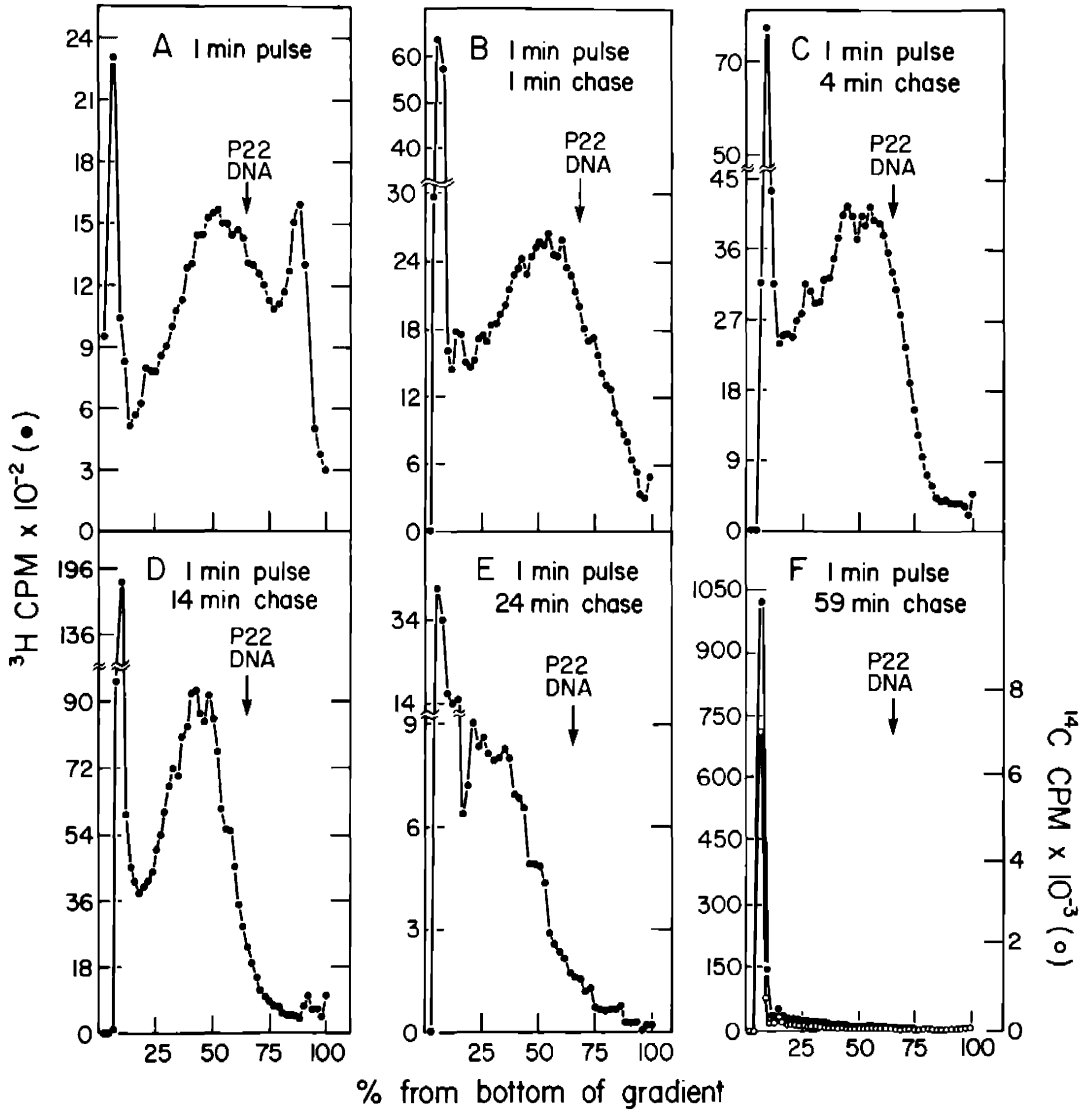

A
20 sec pulse
B
40 sec pulse
C
1 min pulse
D
10 min pulse
^{3}H CPM x 10^{-2}
^{3}H CPM x 10^{-3}
^{14}C CPM (○)
Fraction number from bottom

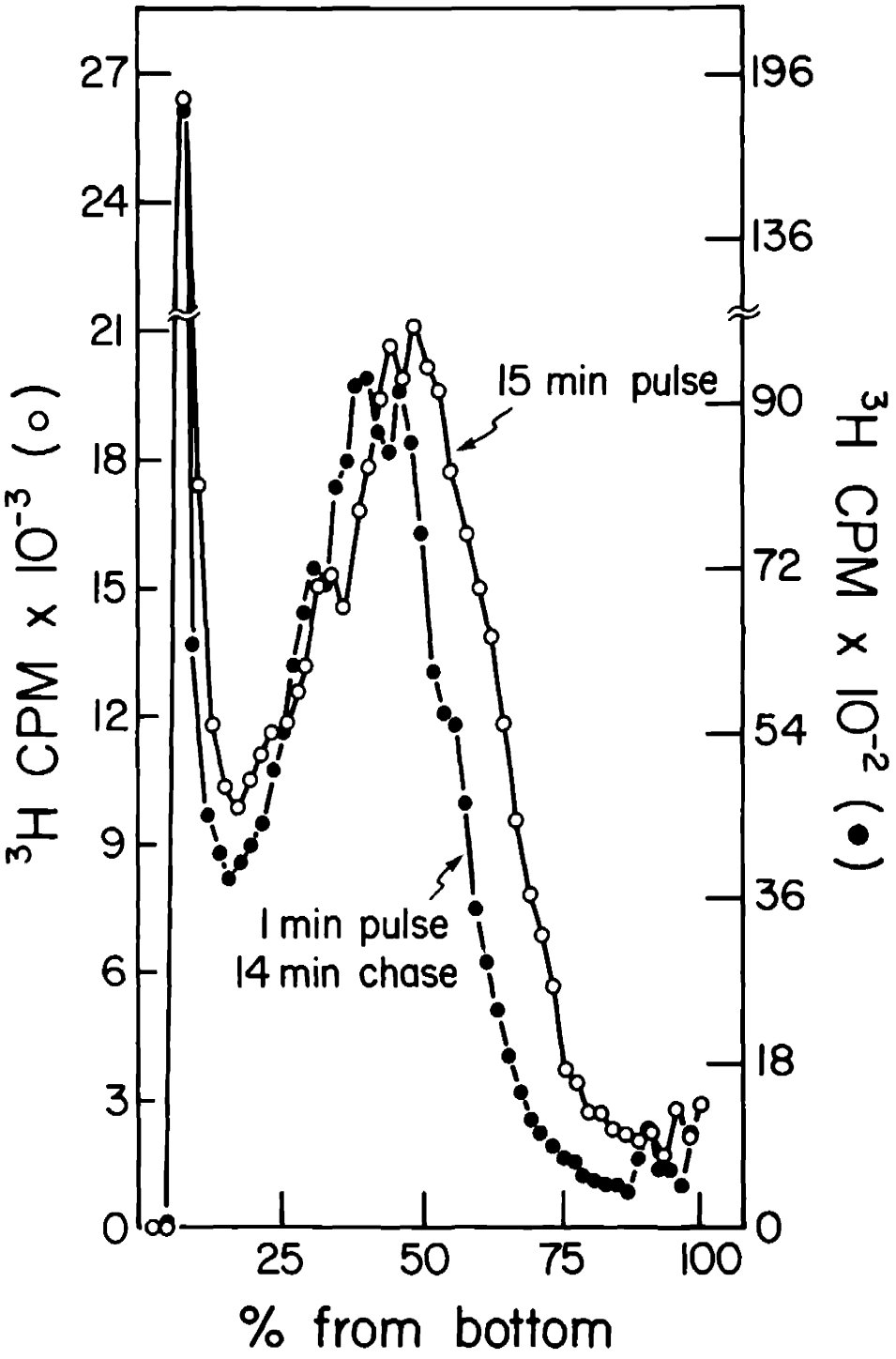
Figure 30. Sucrose gradient profiles of pulse chased DNA. Cells, some of which were prelabeled with (^{14}C) thymidine, were pulse labeled for one minute at 37 °C with (^{3}H) thymidine and chased for various lengths of time with unlabeled thymidine and deoxycytidine. Cells were lysed, treated, and centrifuged as in Figure 28.


A 1 min pulse
B 1 min pulse
1 min chase
C 1 min pulse
4 min chase
D 1 min pulse
14 min chase
E 1 min pulse
24 min chase
F 1 min pulse
59 min chase
P22
DNA
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-3} (o)
% from bottom of gradient

	1 min pulse	1 min pulse-1 min chase	1 min pulse-4 min chase	1 min pulse-14 min chase	1 min pulse-59 min chase
Total precipitable counts	50,996	95,804	125,557	219,604	223,962
Increase over previous sample	-	44,808	29,753	94,047	4,358
% Increase over previous sample	-	87%	31%	74%	1%
Increase over 1 min pulse	-	44,808	74,561	168,608	172,966
% Increase over 1 min pulse	-	87%	146%	330%	339%

Table I. Increase in total amount of acid precipitable radioactivity during chases of a 1 minute pulse. Experimental procedure is described in Figure 30.

Figure 31. Sucrose gradient profiles of pulse labeled and pulse chased DNA. Cells were pulse labeled for 15 minutes at 37°C with (^{3}H) thymidine or pulse labeled for 1 minute and chased for 14 minutes with cold thymidine and deoxycytidine. Cells were lysed, treated, and centrifuged as in Figure 28.


27
24
21
18
15
12
9
6
3
0
196
136
90
72
54
36
18
0
25
50
75
100
% from bottom
^{3}H CPM x 10^{-3} (○)
^{3}H CPM x 10^{-2} (●)
15 min pulse
1 min pulse
14 min chase

minute and a chase of 14 minutes the intermediate peak traveled farther through the gradient than did the intermediate peak of a 15 minute pulse.

C. Pulse labeling at 25°C

At 25°C, the rate of thymidine incorporation into DNA of CHO cells is only about one-fifth to one-fourteenth of the rate at 37°C (Painter & Schaefer, 1969; Huberman, unpublished). By pulse-labeling at temperatures of about 25°C it is therefore possible to examine in greater detail the sequence of events taking place during pulse labelings equivalent to very short pulse times and to observe more exactly the transition from the Okazaki peak to the intermediate peak.

Cells were pulse labeled at room temperature (22-26°C) (Fig. 32) for various time periods. Notice that it is easy to follow the course of events during what are essentially very short pulse labelings. At these temperatures, a clear Okazaki peak is still visible after 10 minutes (Fig. 32E), while after 15 minutes,only a shoulder remains (Fig. 32F). During very short (15 seconds) pulses, most (75%) of the acid precipitable radioactivity is found in the Okazaki peak region (Fig. 33).

As noted for pulses at 37°C, the sedimentation patterns of DNA pulse labeled at room temperature are similar whether the DNA is prepared by isolating nuclei or by purifying with proteinase K, chloroform and ethanol (Fig. 34).

Figure 32. Sucrose gradient profiles of DNA pulse labeled at room temperature and purified by nuclear isolation. Cells, some of which were prelabeled overnight with (^{14}C) thymidine, were pulse labeled for various times at room temperature (22-26°C) with (^{3}H) thymidine. Cells were lysed, treated, and centrifuged as in Figure 28.

(A) 1 minute pulse.

(B) 2 minute pulse.

(C) 4 minute pulse.

(D) 6 minute pulse.

(E) 10 minute pulse.

(F) 15 minute pulse.

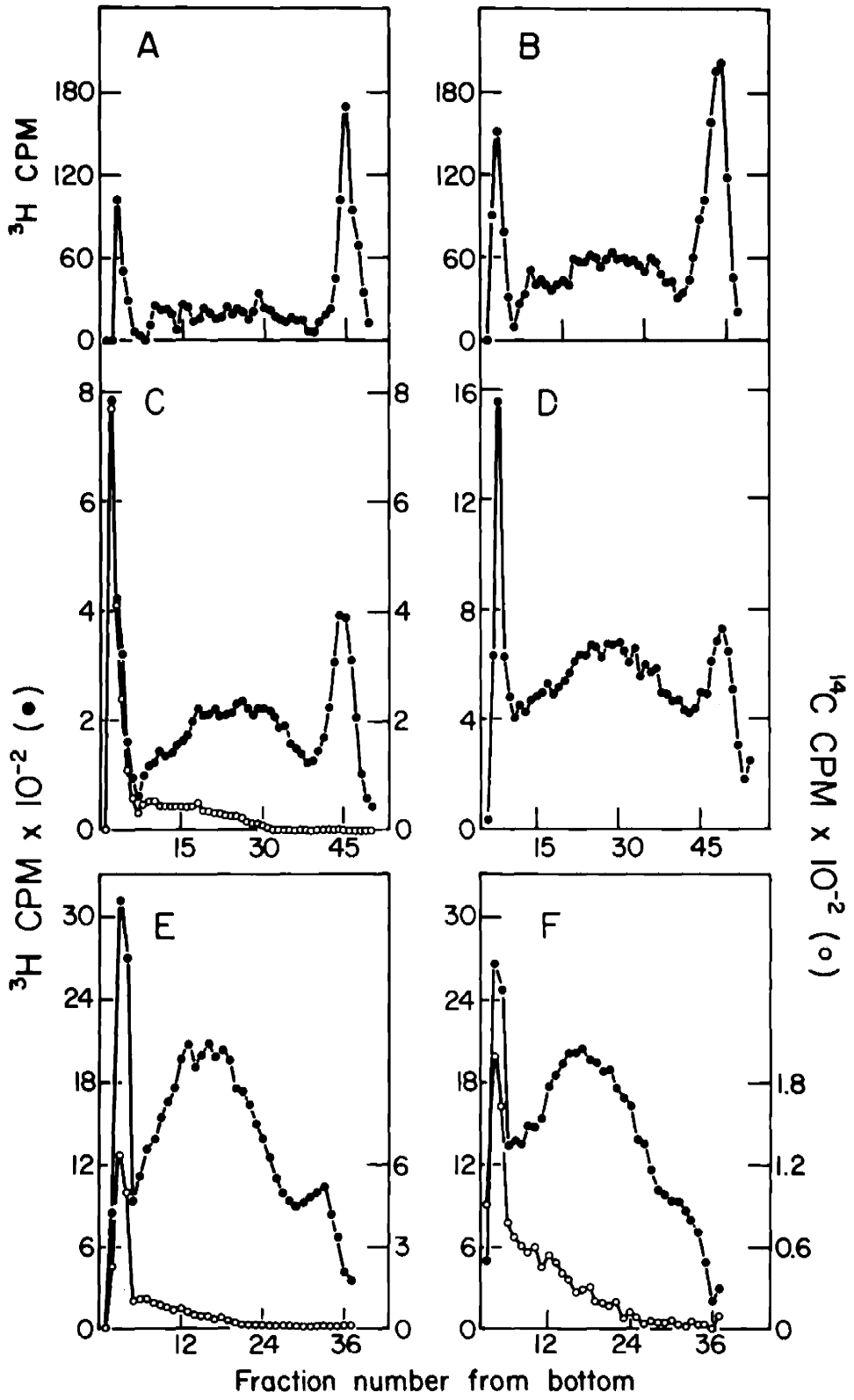

A
B
C
D
E
F
^{3}H CPM
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (o)
Fraction number from bottom

Figure 33. Sucrose gradient profile of DNA pulse labeled for 15 seconds at room temperature. Cells were pulse labeled for 15 seconds at room temperature with (^{3}H) thymidine, then lysed, treated, and centrifuged as in Figure 28.

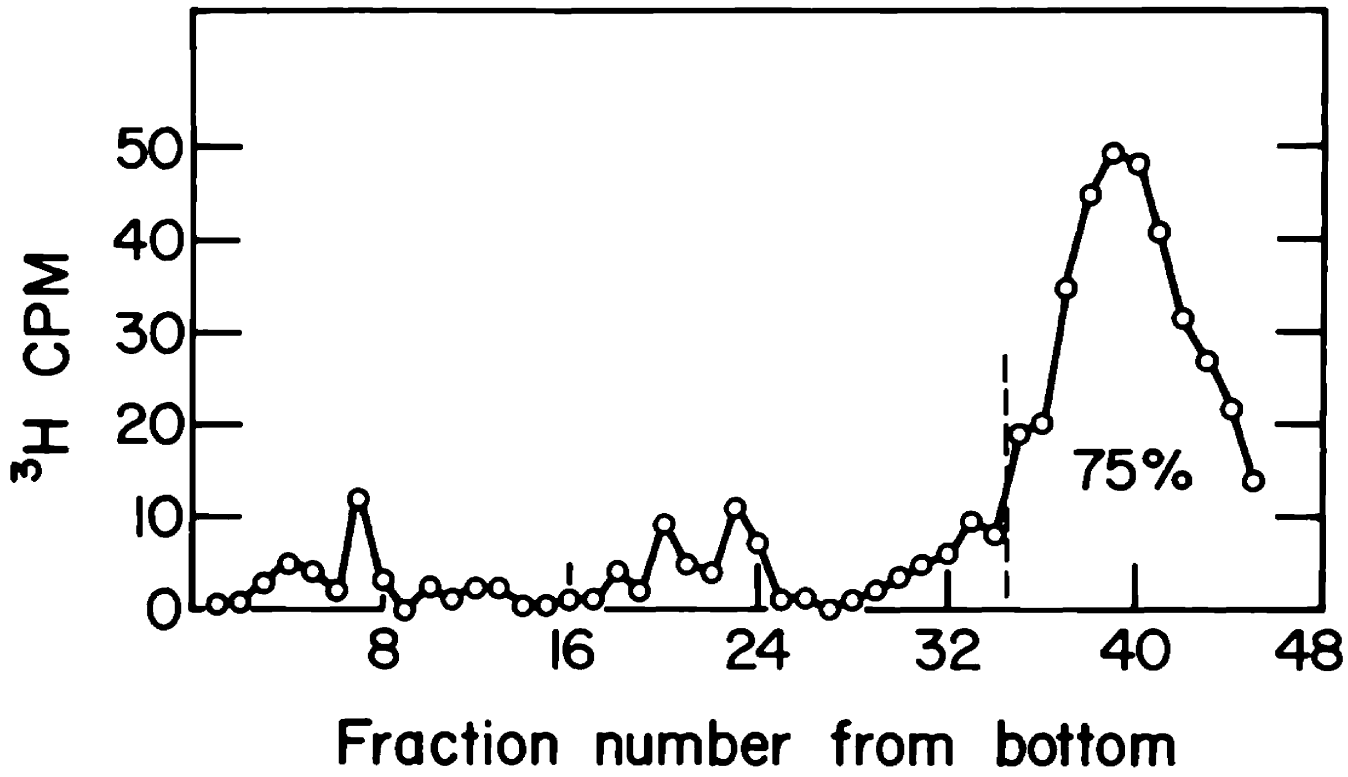
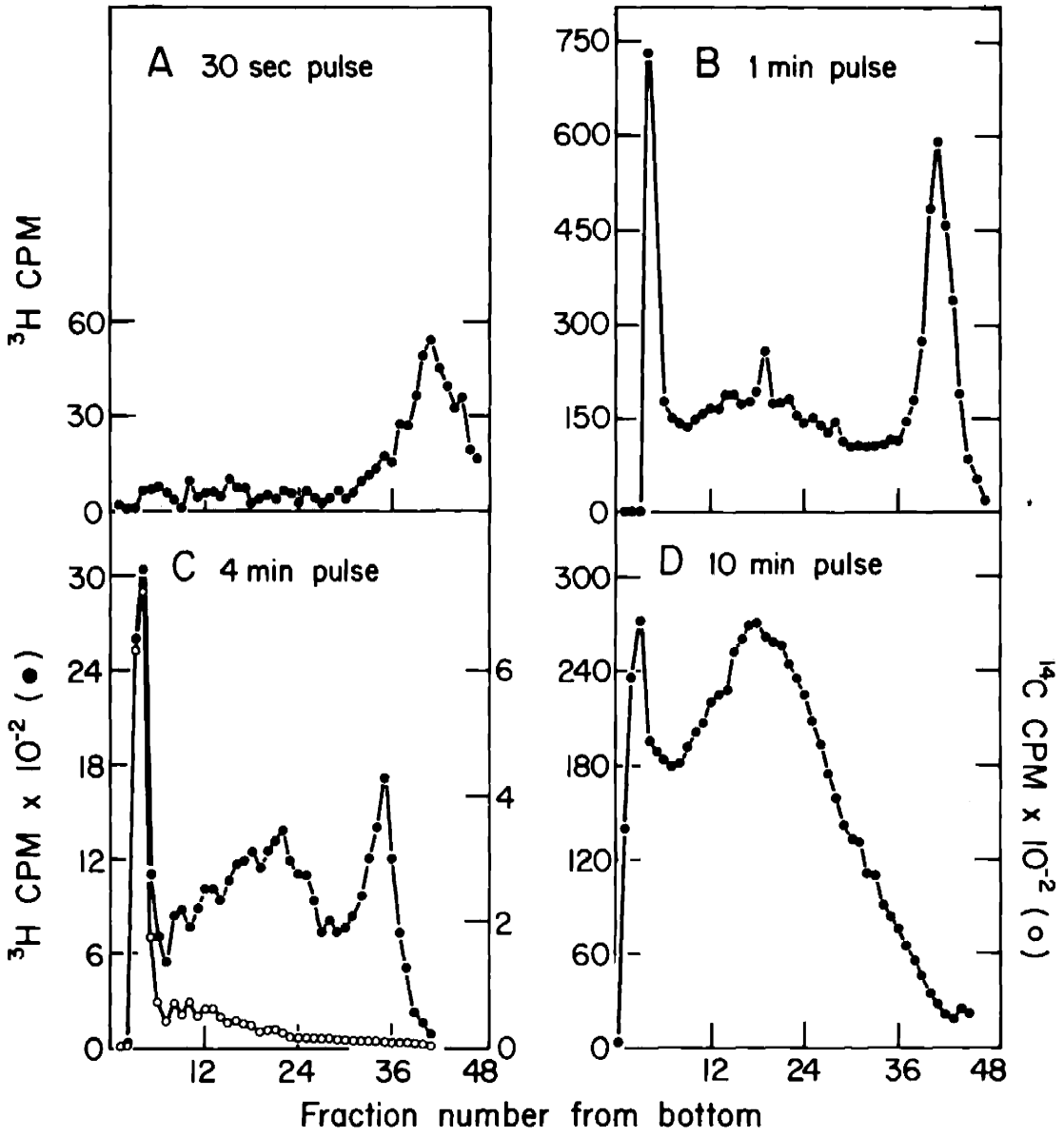

^{3}H CPM
50
40
30
20
10
0
8
16
24
32
40
48
75%
Fraction number from bottom

Figure 34. Sucrose gradient profiles of DNA pulse labeled at room temperature and purified by proteinase K, chloroform extraction and ethanol precipitation. Cells, some of which were pre-labeled overnight with (^{14}C) thymidine, were pulse labeled for varying times at room temperature with (^{3}H) thymidine. Cells were lysed, treated, and centrifuged as in Figure 33.


A 30 sec pulse
B 1 min pulse
C 4 min pulse
D 10 min pulse
^{3}H CPM
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (o)
Fraction number from bottom

D. Size of Okazaki fragments

Under the sedimentation conditions used above, the Okazaki peak barely moves into the gradient, making it impossible to determine how large the strands are. The exact size of the Okazaki peak was therefore measured by sedimenting it much farther into the sucrose gradient and using marker DNA fragments of known length to calculate the strand size. As shown in Figure 35, the average size of the strands in the Okazaki peak from a 40 second pulse at 37°C is about 100 nucleotides. Similar profiles were observed when Okazaki peaks from a 20 second pulse at 37°C and from 4 and 7 minute pulses at room temperature were measured (results not shown).

This size estimate was confirmed by gel electrophoresis (Fig. 36). The Okazaki peak fractions (#23-24) from a 2 minute pulse-label at room temperature (Fig. 36A) were run on a 5% polyacrylamide formamide gel. Also run were markers of known length and the distribution of these markers could be graphed as a straight line on a plot of log molecular weight versus mobility (Fig. 36B). The Okazaki peak fractions migrated as shown in Figure 36C, indicating that the Okazaki fragments are heterogeneous in size, ranging from less than 50 nucleotides to about 150 nucleotides, with a peak at about 100 nucleotides.

Although the Okazaki peak represents a heterogeneous distribution of strand sizes, the average size of the Okazaki strands does not appear

Figure 35. Sucrose gradient profile of Okazaki peak. Cells were pulse labeled for 40 seconds at 37 °C with (^{3}H) thymidine. They were then lysed and treated as in Figure 14 and centrifuged on an alkaline sucrose gradient in an SW50. 1 rotor at 45, 000 rpm for 18 hours at 23 °C. The numbers indicate where markers of different sizes (in nucleotides) sedimented when added to the sample before heating. The 47-nucleotide marker was added to the pulse labeled DNA solution before heating, while the other markers were run in parallel tubes.

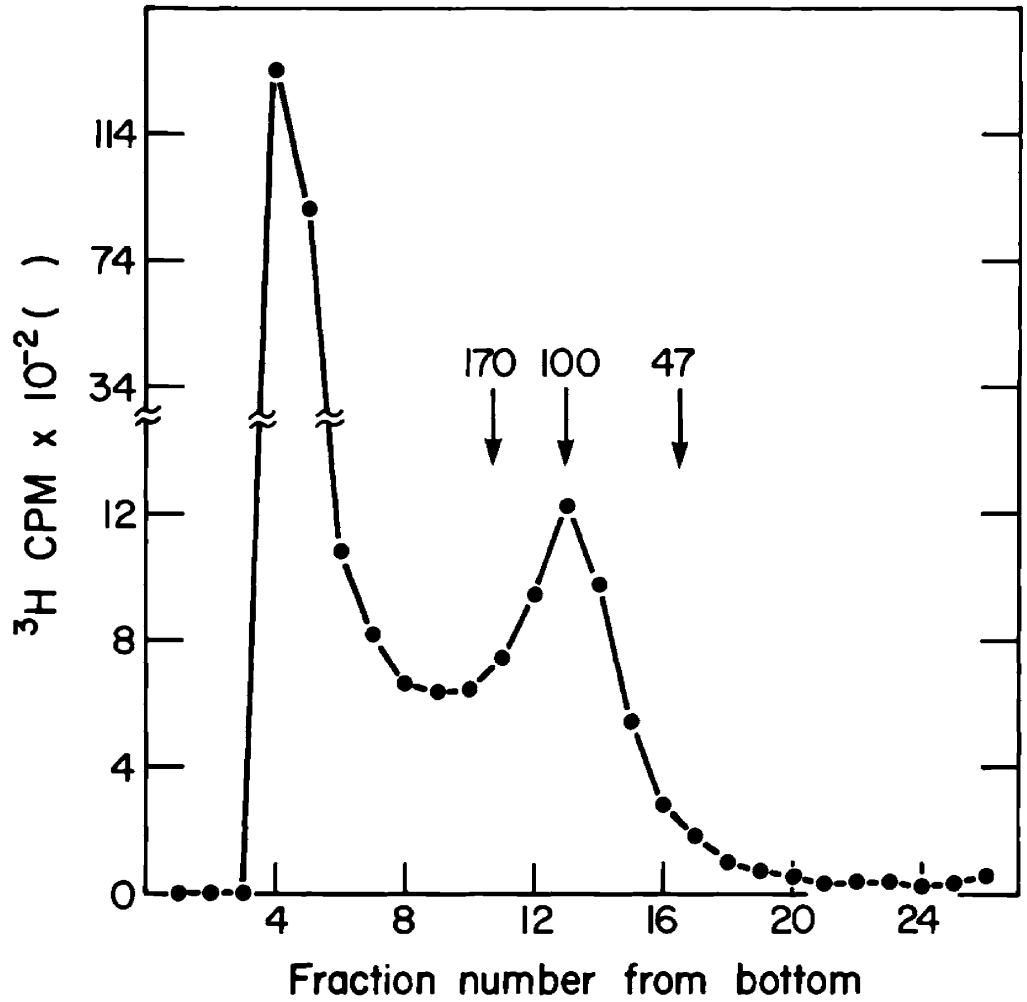
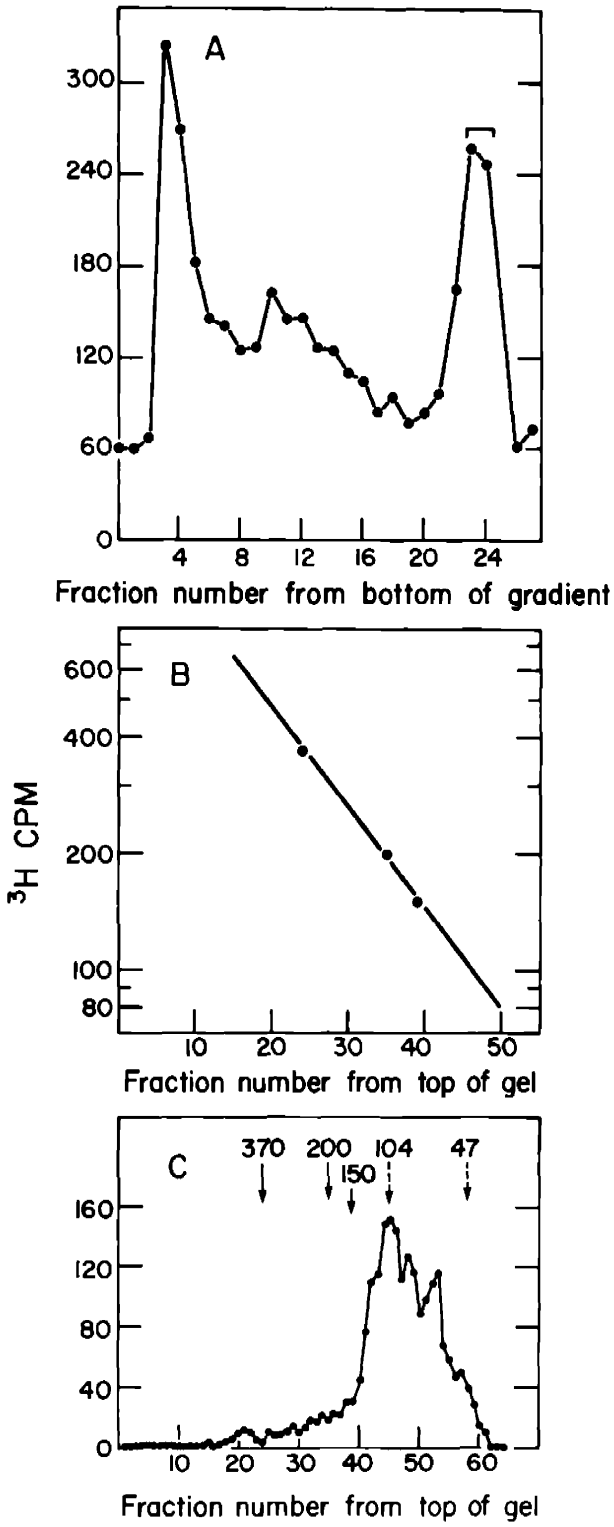

114
74
34
12
8
4
0
^{3}H CPM x 10^{-2}( )
170
100
47
4
8
12
16
20
24
Fraction number from bottom

Figure 36. Sucrose gradient and gel profiles of pulse labeled DNA. Cells were pulse labeled for 2 minutes at room temperature with (^{3}H) thymidine, lysed and treated as in Figure 14, and centrifuged on an alkaline sucrose gradient in an SW27 rotor at 25,000 rpm for 16 hours at 0°C. The peak fractions (#23-24) were pooled, the DNA was precipitated with ethanol and washed several times with 70% ethanol, and the precipitated DNA was dissolved in formamide and run on a 5% acrylamide formamide gel. Markers of known size (in nucleotides) were run on the same gel, and their final distribution forms a straight line on a plot of log molecular weight versus mobility.

(A) Sedimentation profile of 2 minute pulse centrifuged on alkaline sucrose gradient.

(B) Plot of log molecular weight of marker DNA pieces versus their mobility.

(C) DNA from peak of (A) run on a 5% polyacrylamide formamide gel. The numbers above the solid arrows indicate the positions of markers; the numbers above the broken arrows give the calculated size of the (^{3}H)-labeled DNA at the indicated positions.


A
300
240
180
120
60
0
4
8
12
16
20
24
Fraction number from bottom of gradient
B
600
400
200
100
80
^{3}H CPM
10
20
30
40
50
Fraction number from top of gel
C
370
200
150
104
47
160
120
80
40
0
10
20
30
40
50
60
Fraction number from top of gel

to change with longer pulse times. Figure 37 shows the sedimentation profiles of DNA pulse labeled at room temperature from 1 to 8 minutes. Although there is some variation in the position of the peaks, it is obviously a random variation rather than a monotonicly changing one.

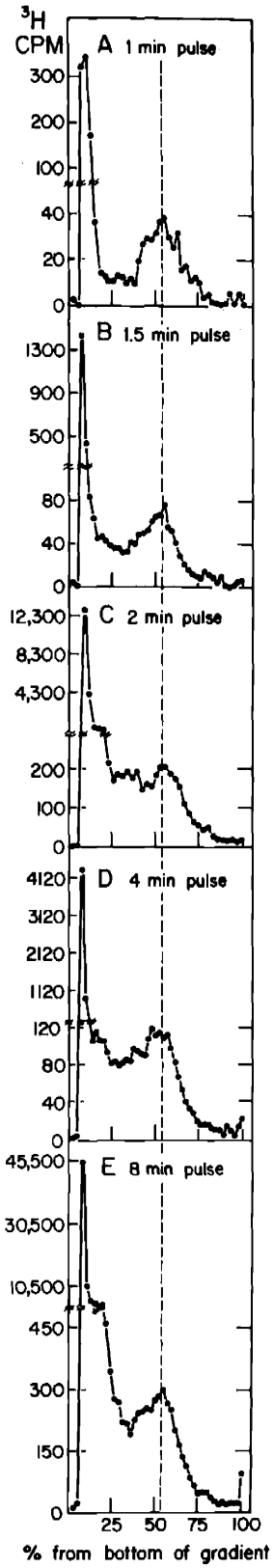
III. Inhibitors

Once the pattern of discontinuous DNA replication is known, it can be used to study the effects of inhibitors of protein and DNA synthesis. Details of the type of inhibition taking place can be observed by comparing the sedimentation pattern of DNA made by cells grown in the presence of an inhibitor with the normal patterns. Since it is important to observe the transition from Okazaki fragments to larger DNA as carefully as possible, all pulse labeling was done at room temperature, after the cells had been grown in the presence of the inhibitor for 1.5 hours at 37°C and then 30 minutes at room temperature. Table II shows the percentage of normal thymidine uptake remaining after the cells were incubated under these conditions with various inhibitors.

A. Inhibitors of protein synthesis

Figure 38 shows how sedimentation profiles are affected when CHO cells are grown in 0.5 μg/ml emetine before and during pulse labeling. Although the amount of label incorporated into acid precipitable counts is greatly reduced (less than 10% of control) the

Figure 37. Sucrose gradient profiles of the DNA from cells pulse labeled for various times. Cells were pulse labeled for varying times at room temperature with (^{3}H) thymidine, and lysed and treated as in Figure 17. The DNA was centrifuged through the alkaline sucrose gradients in an SW50. 1 rotor at 40, 000 rpm for 19 hours at 23°C.


^{3}H
CPM
A 1 min pulse
300
200
100
40
20
0
B 1.5 min pulse
1300
900
500
80
40
0
C 2 min pulse
12,300
8,300
4,300
200
100
0
D 4 min pulse
4120
3120
2120
1120
120
80
40
0
E 8 min pulse
45,500
30,500
10,500
450
300
150
0
25
50
75
100
% from bottom of gradient

Inhibitor	Concentration	% of Normal Uptake
Cycloheximide	10 μg/ml	7%
Emetine	0.5 μg/ml	7%
	2.0 μg/ml	7%
	5.0 μg/ml	5%
Puromycin	100 μg/ml	8%
Ara C	1×10^{-5} M	8%
	5×10^{-5} M	3%
FUdR	2×10^{-7} M	6-10%
	1×10^{-6} M	6-10%
	5×10^{-6} M	6-10%
Hydroxyurea	100 μg/ml	2%
	500 μg/ml	2%

Table II. Percentage of normal uptake remaining after incubating cells with inhibitors. Uptake was assumed to be the total acid-precipitable radioactivity recovered from alkaline sucrose gradients, and comparisons were made between pulses of cells preincubated with inhibitors for 2 hours and those grown under standard conditions. Each percentage is an average of comparisons made at different pulse times and should be taken as an approximation. Pulse labeling was done with (^{3}H) thymidine in all cases except FUdR, when (^{3}H) deoxycytidine was used.

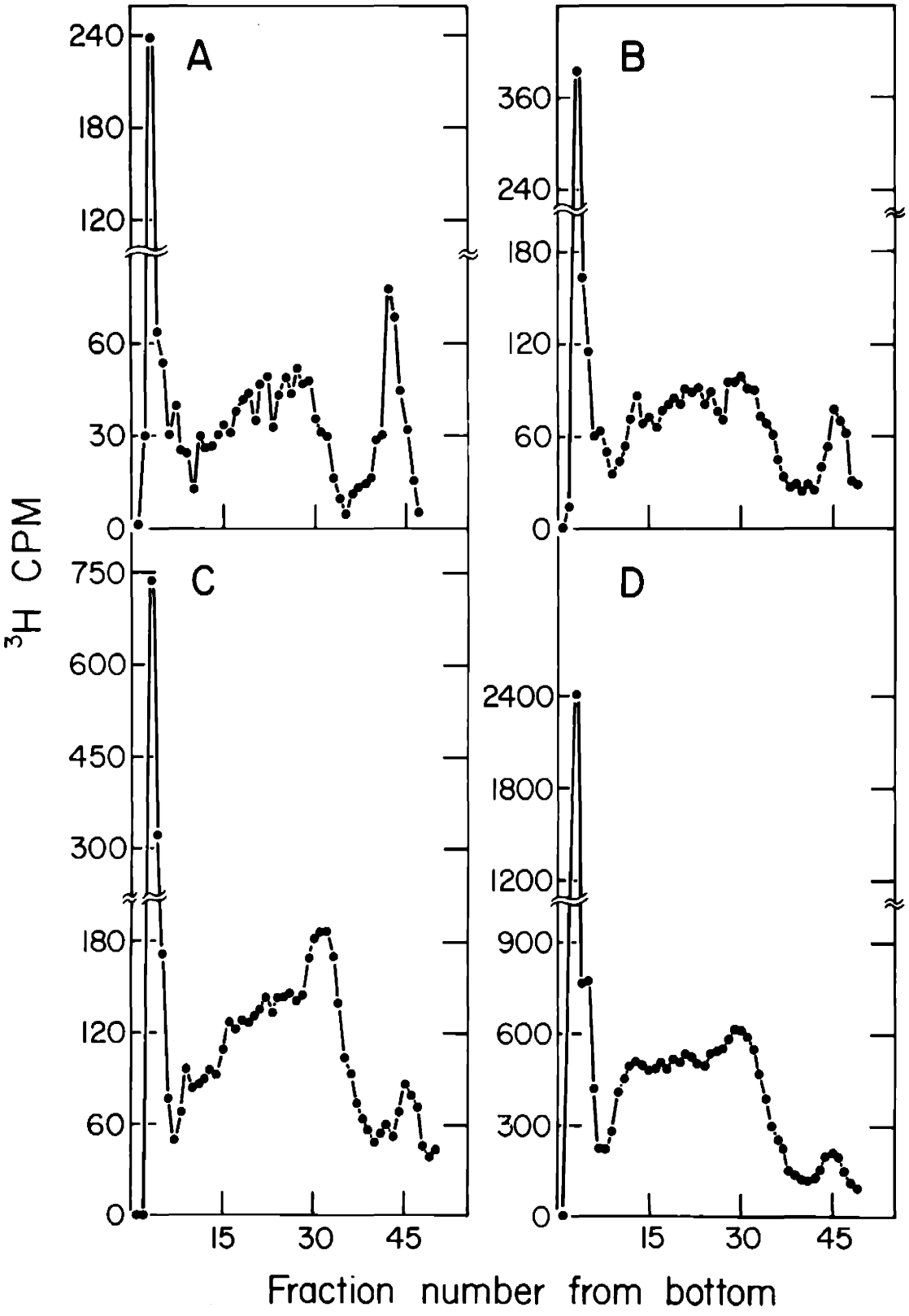
Figure 38. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with emetine. Cells were preincubated for 2 hours in the presence of 0.5 μg/ml emetine and then pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figue 28.

(A) 3 minute pulse.

(B) 5 minute pulse.

(C) 10 minute pulse.

(D) 15 minute pulse.


A
B
C
D
240
180
120
60
30
0
360
240
180
120
60
0
750
600
450
300
180
120
60
0
2400
1800
1200
900
600
300
0
15
30
45
^{3}H CPM
Fraction number from bottom

sedimentation patterns do not seem to be grossly altered from those of uninhibited cells (Fig. 32). The transition from Okazaki to intermediate peaks seems to happen at the same rate that it does under normal conditions, with the only noticeable difference being a sharper division between these two peaks, causing the Okazaki peak to remain a noticeably separate peak at longer pulse-times.

The same general pattern is seen when cells are grown in 100 μg/ml puromycin (Fig. 39) or 10 μg/ml cycloheximide (Fig. 40).

The pattern is not altered as the concentration of emetine is raised to 2 μg/ml (Fig. 41A) or 5μg/ml (Fig. 41B) even though incorporation of radioactive label drops slightly from about 7% to 5%.

When examined under more severe sedimentation conditions, the size of the Okazaki peaks does not seem to be affected either by the concentration of protein inhibitor (Fig. 42) or the length of the pulse label (Fig. 43). (Skewing of the peak in 42A makes it seem slightly larger than the others, but this is probably just a random variation. The peak seems to be further down the gradient than the 100 nucleotide marker and it has been shown that,even when no emetine is present, the Okazaki peak cosediments with this marker.) Note that although the position of the peak does not change with time, the 3 minute sedimentation pattern (Fig. 43A) seems to show a trailing edge that disappears with longer pulses (Figs. 43B,C). Experiments done with emetine and cycloheximide (results not shown) give the

Figure 39. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with puromycin. Cells, some of which were prelabeled overnight with (^{14}C) thymidine, were preincubated for 2 hours in the presence of 100 μg/ml puromycin and then pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 28.

(A) 4 minute pulse.

(B) 10 minute pulse.

(C) 15 minute pulse.

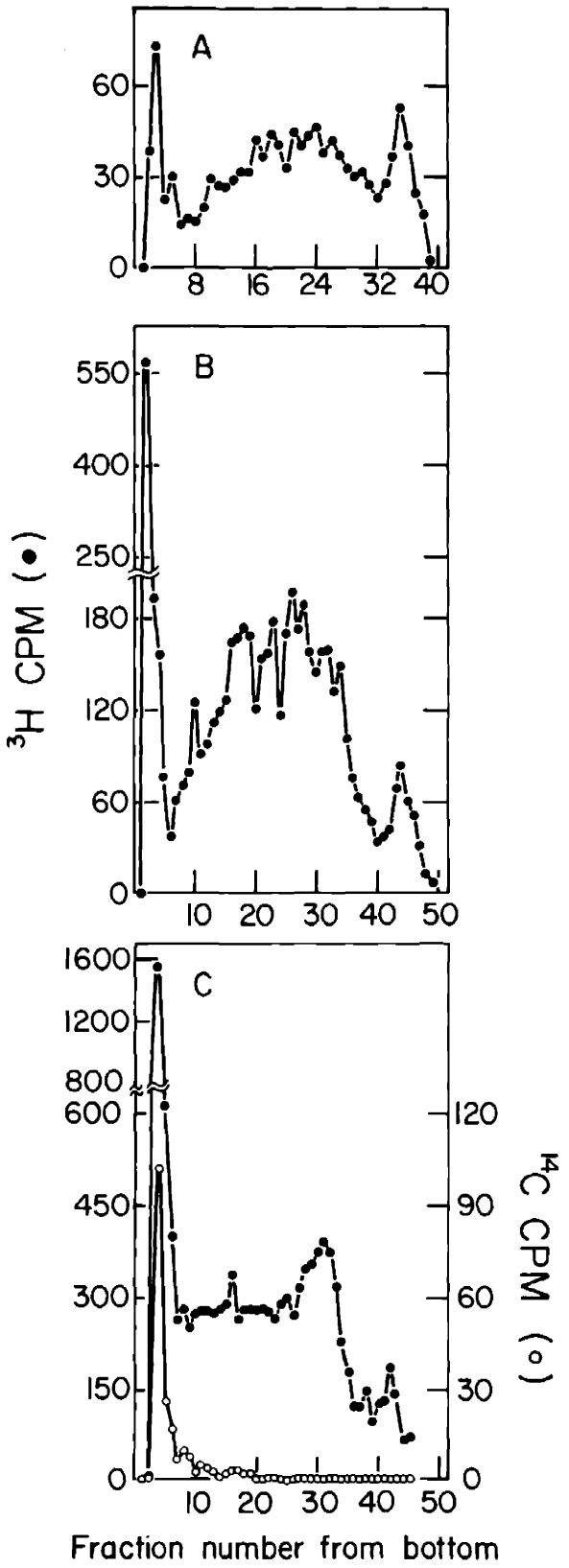

A
B
C
60
30
0
8
16
24
32
40
550
400
250
180
120
60
0
10
20
30
40
50
1600
1200
800
600
450
300
150
0
120
90
60
30
0
^{3}H CPM (•)
^{14}C CPM (○)
Fraction number from bottom

Figure 40. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with cycloheximide. Cells, some of which were prelabeled overnight with (^{14}C) thymidine, were preincubated for 2 hours in the presence of 10 μg/ml cycloheximide and then pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 28.

(A) 4 minute pulse.

(B) 10 minute pulse.

(C) 15 minute pulse.

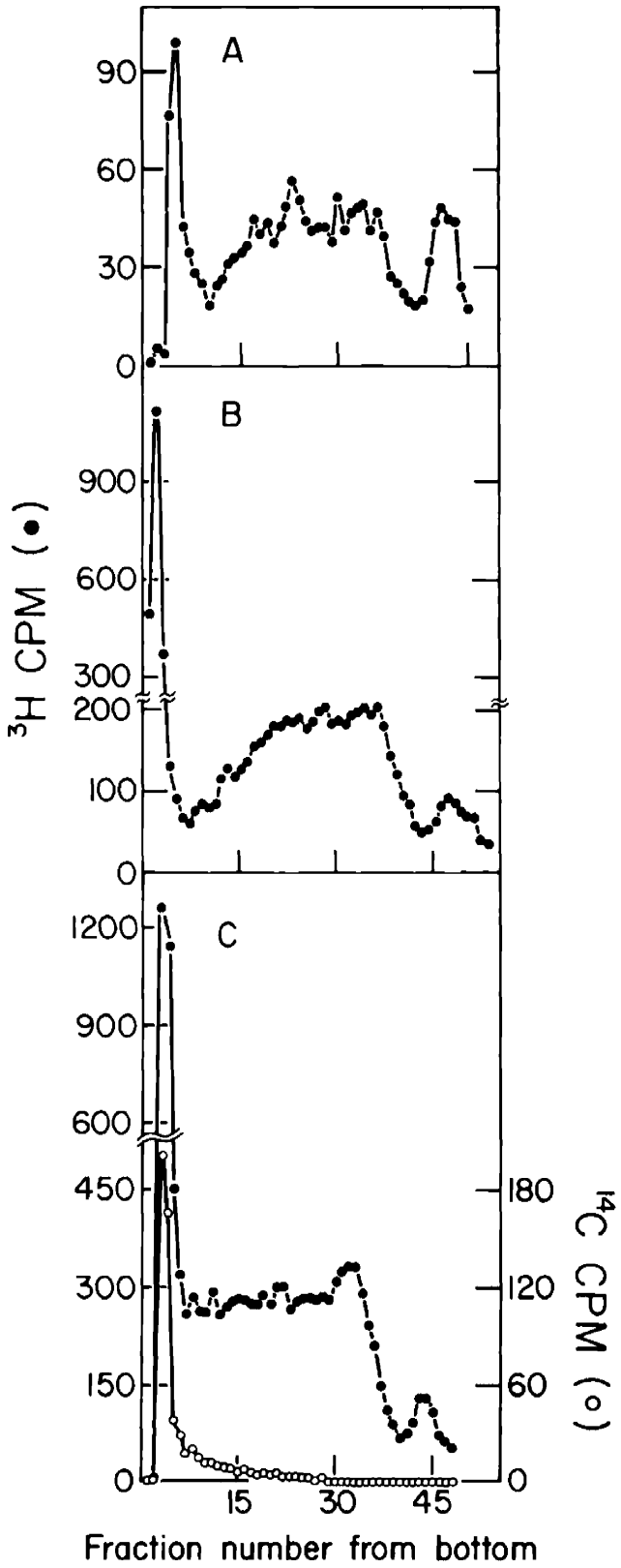

A
B
C
90
60
30
0
900
600
300
200
100
0
1200
900
600
450
300
150
0
180
120
60
0
15
30
45
^{3}H CPM (•)
^{14}C CPM (o)
Fraction number from bottom

Figure 41. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with various concentrations of emetine. Cells were preincubated with emetine, labeled, treated, and centrifuged as in Figure 38, except that the concentration of emetine was different. Cells were pulse labeled for 6 minutes at room temperature.

(A) 2 μg/ml emetine.

(B) 5 μg/ml emetine.

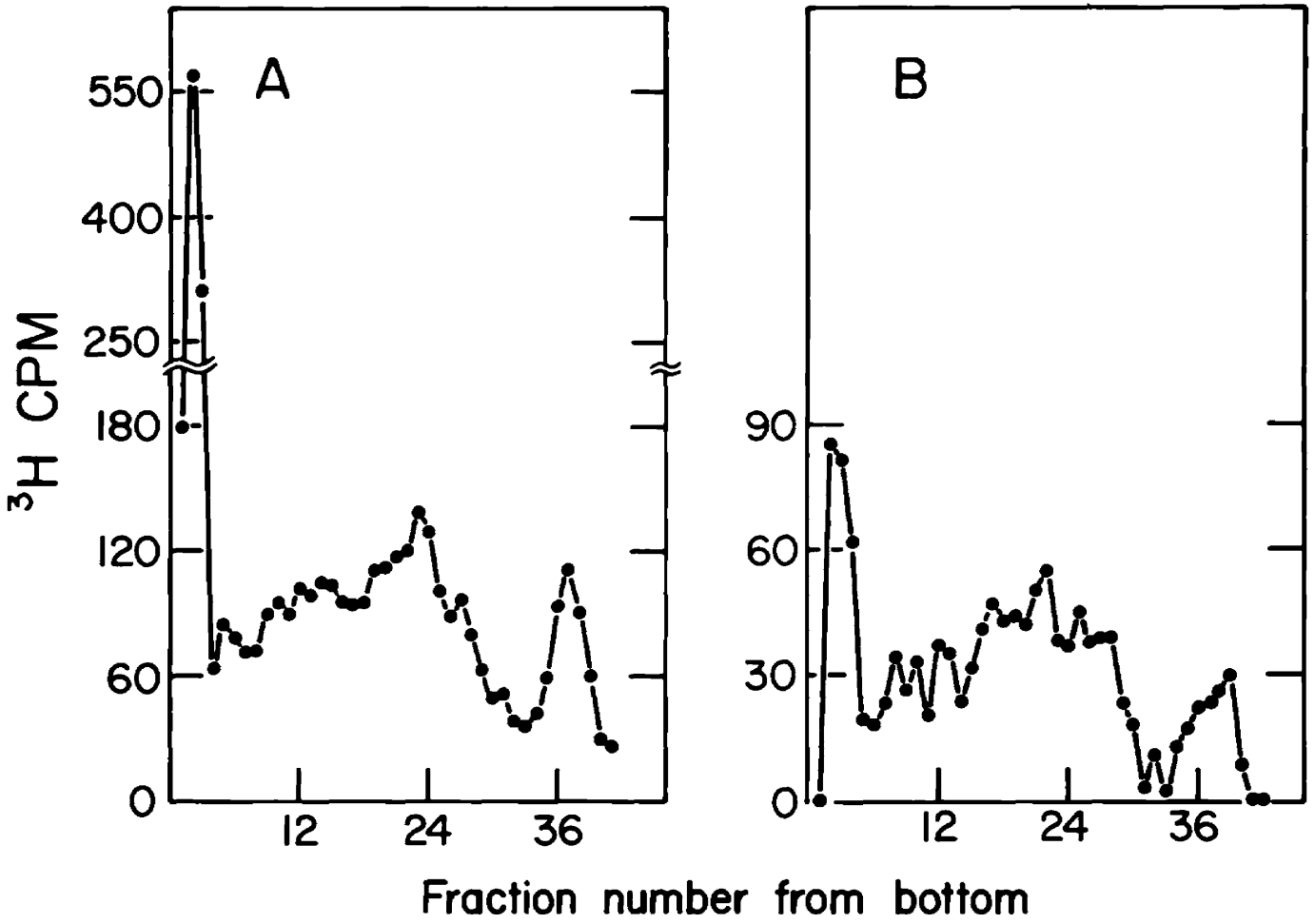

A
B
550
400
250
180
120
60
0
90
60
30
0
12
24
36
12
24
36
^{3}H CPM
Fraction number from bottom

Figure 42. Sucrose gradient profiles of the pulse labeled DNA from cells preincubated with various concentrations of emetine. Cells were preincubated with different concentrations of emetine and pulse labeled in its presence for 4 minutes at room temperature with (^{3}H) thymidine. DNA was prepared as in Figure 17 and centrifuged on alkaline sucrose gradients in an SW50. 1 rotor at 45, 000 rpm for 18 hours at 23°C. (^{32}P) labeled markers of known size were added to the samples before heating.

(A) 0. 01 μg/ml emetine; 100 nucleotide marker.

(B) 0. 1 μg/ml emetine; 47 nucleotide marker.

(C) 1. 0 μg/ml emetine; 170 nucleotide marker.

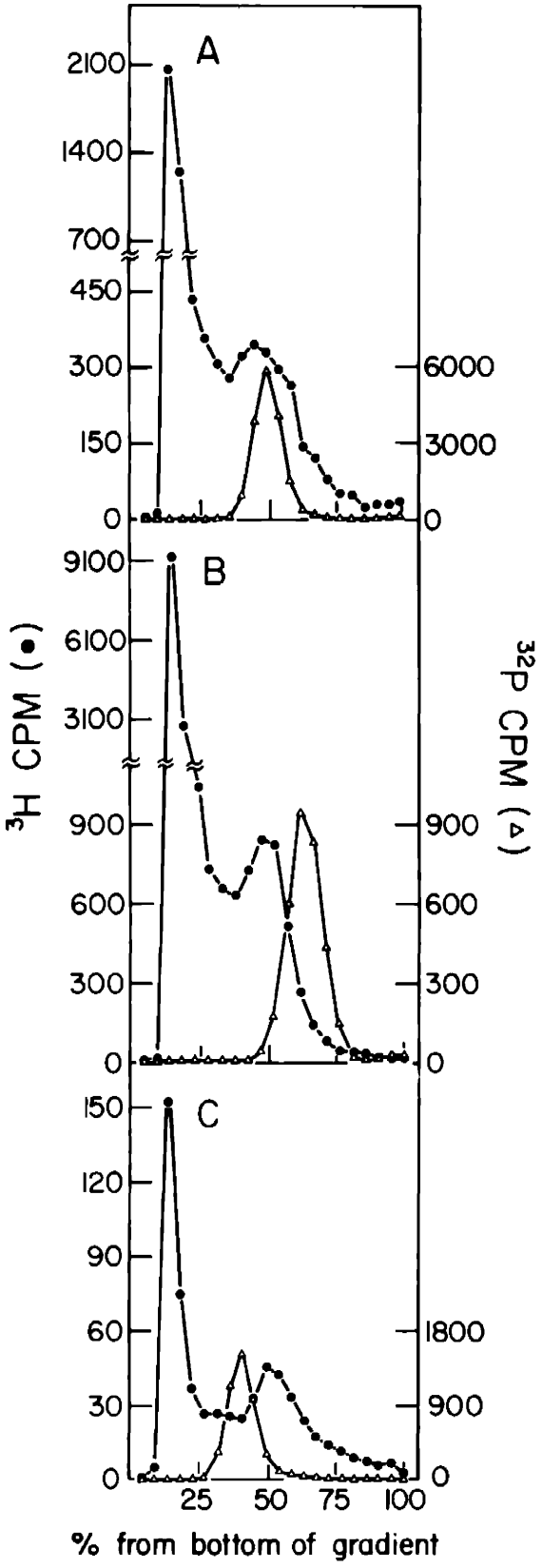
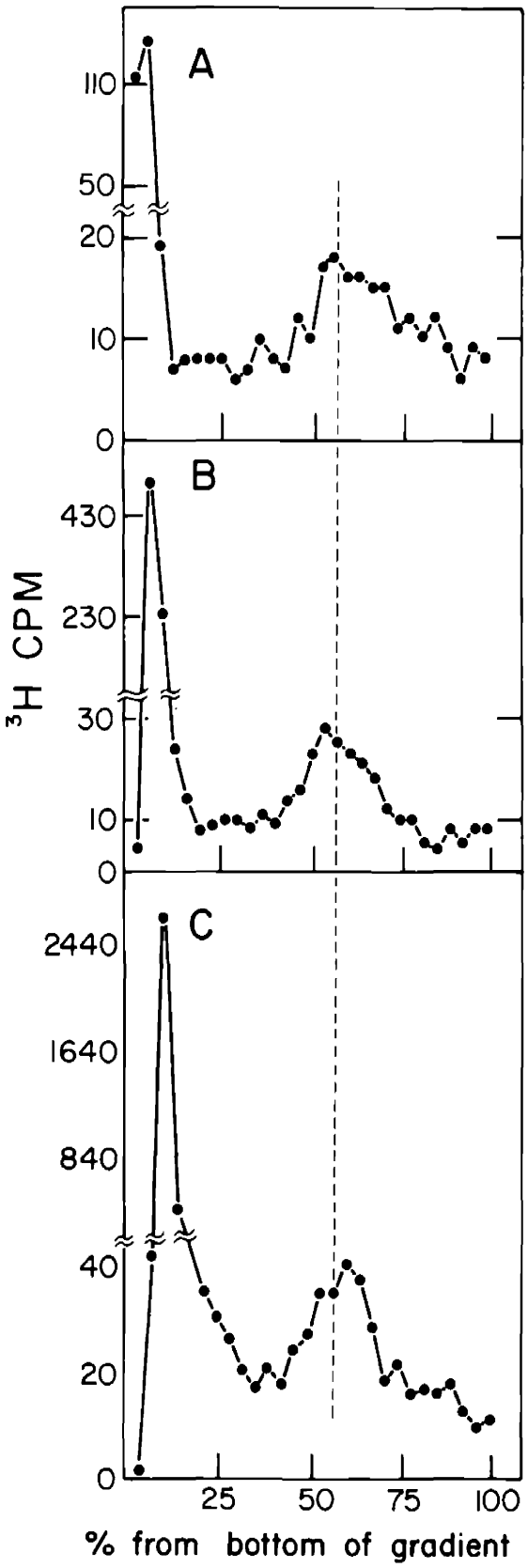

A
B
C
2100
1400
700
450
300
150
0
6000
3000
0
9100
6100
3100
900
600
300
0
900
600
300
0
150
120
90
60
30
0
1800
900
0
25
50
75
100
^{3}H CPM (•)
^{32}P CPM (△)
% from bottom of gradient

Figure 43. Sucrose gradient profiles of DNA from cells preincubated with emetine and pulse labeled for various times. Cells were preincubated with 100 μg/ml puromycin, pulse labeled and treated as in Figure 38. The DNA was centrifuged on alkaline sucrose gradients in an SW50.1 rotor at 40,000 rpm for 19 hours at 23°C.

(A) 3 minute pulse.

(B) 5 minute pulse.

(C) 10 minute pulse.


A
B
C
110
50
20
10
0
430
230
30
10
0
2440
1640
840
40
20
0
^{3}H CPM
25
50
75
100
% from bottom of gradient

same results--under all conditions the Okazaki peak is at a position of about 100 nucleotides, just as it is when no inhibitors are used (Fig. 37), but there is a trailing edge that gradually disappears.

B. Inhibitors of DNA synthesis

1. Hydroxyurea

While all the inhibitors of protein synthesis seem to have the same effect on discontinuous DNA synthesis, inhibitors of DNA synthesis present a more complicated picture. When cells are grown and pulse labeled in the presence of 100 μg/ml hydroxyurea, a large proportion of acid precipitable radioactivity remains in the Okazaki peak region (Fig. 44). There does not seem to be any build up of either intermediate- or bulk-sized DNA and the sedimentation pattern shows no real change from 5 to 30 minutes. In all cases slightly more than 50% of the acid-precipitable counts appear in the Okazaki peak.

Chasing with medium not containing (^{3}H)thymidine (Fig. 45) does not seem to change the pattern much (the counts on the bottom may or may not be an indication of labeled bulk DNA--see above, Section I, C1) until the hydroxyurea is removed (Fig. 45C). Then the counts quickly move into the "intermediate" area. Note the tremendous increase in acid precipitable radioactivity when the hydroxyurea is removed. If a 15 minute pulse is followed by a 30 minute chase with hydroxyurea, the number of acid-precipitable counts increases by a factor of 3, while if the hydroxyurea is removed for the last 15 minutes of the chase, the number increases by a factor of 15-16.

Figure 44. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with hydroxyurea. Cells were preincubated with 100 μg/ml hydroxyurea and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared as in Figure 17 and centrifuged on alkaline sucrose gradients in an SW41 rotor at 40,000 rpm for 3.5 hours at 23°C. Numbers indicate the percent of total acid precipitable radioactivity appearing in the Okazaki peak.

(A) 5 minute pulse.

(B) 15 minute pulse.

(C) 30 minute pulse.

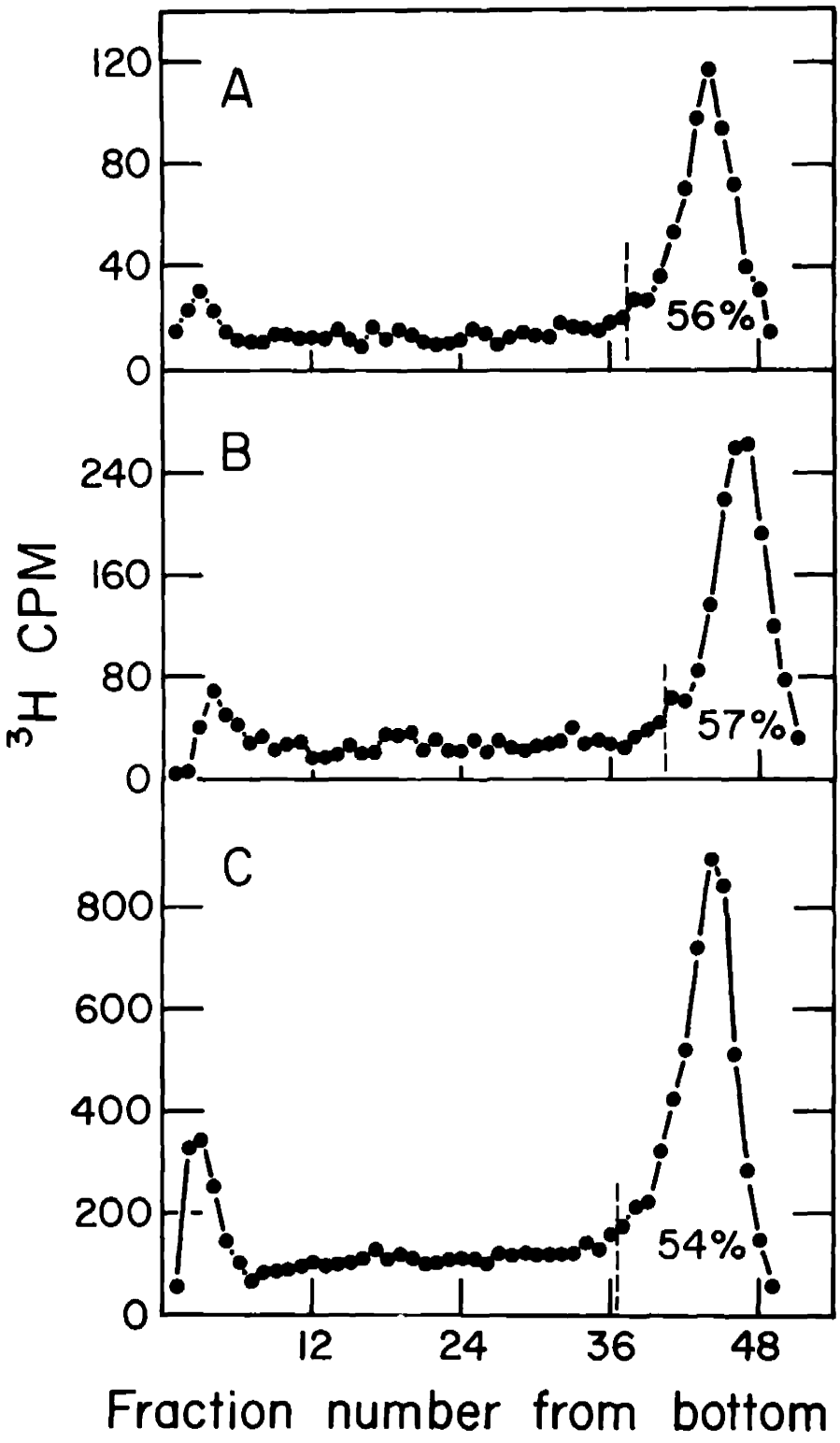
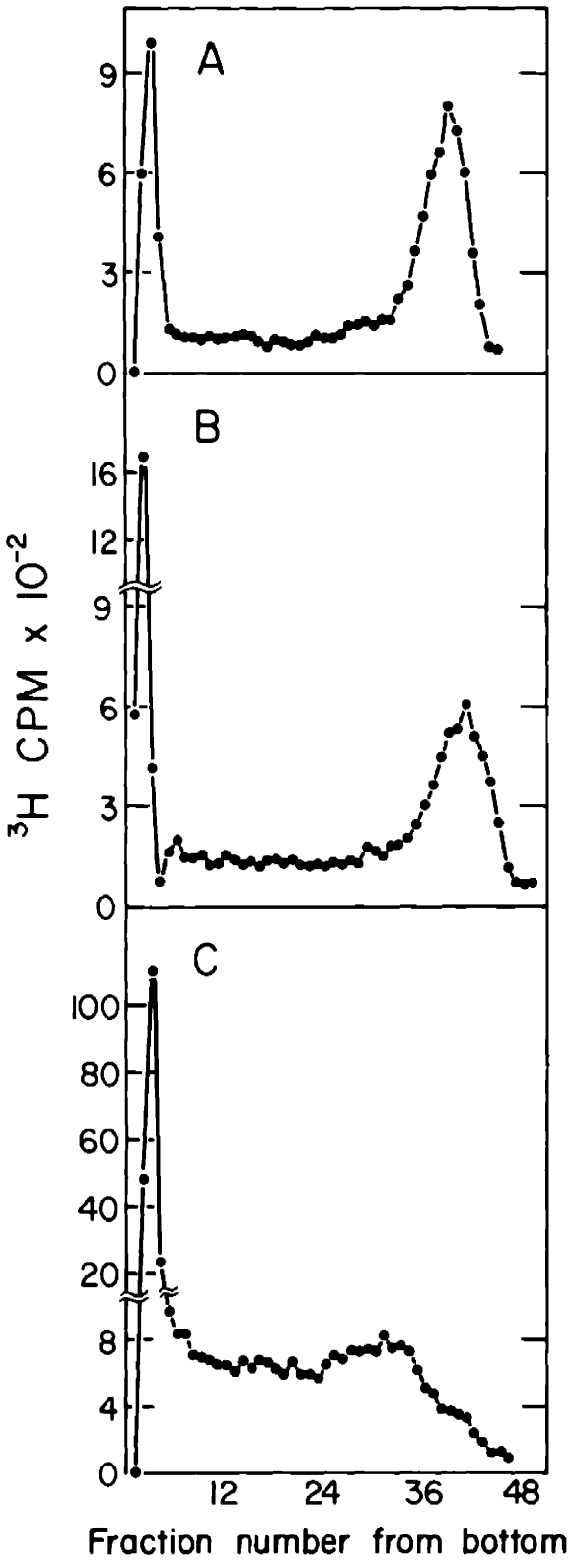

A
B
C
120
80
40
0
240
160
80
0
800
600
400
200
0
56%
57%
54%
^{3}H CPM
12
24
36
48
Fraction number from bottom

Figure 45. Sucrose gradient profiles of pulse chased DNA from cells preincubated with hydroxyurea. Cells were preincubated with 100 μg/ml hydroxyurea, pulse labeled in its presence at room temperature for 15 minutes with (^{3}H) thymidine, and chased with medium lacking (^{3}H)thymidine. DNA was prepared and centrifuged as in Figure 44.

(A) 15 minute chase with hydroxyurea.

(B) 30 minute chase with hydroxyurea.

(C) 15 minute chase with hydroxyurea followed by 15 minute chase without hydroxyurea.


A
B
C
^{3}H CPM x 10^{-2}
Fraction number from bottom
0
3
6
9
12
16
20
40
60
80
100
4
8
12
24
36
48

At low concentrations (5 μg/ml) hydroxyurea, like inhibitors of protein synthesis, reduces the amount of incorporated radioactivity without grossly affecting the sedimentation pattern (Figs. 46A, B). At higher concentrations (50 μg/ml), it does change the pattern (Fig. 46C) toward the accumulation of Okazaki fragments described above (Fig. 44). In fact, as the concentration of hydroxyurea is raised to 500 μg/ml (Fig. 47), more than 80% of the radioactivity appears in the Okazaki peak, and the pattern does not change for at least one hour.

Another effect of hydroxyurea is that the fragments in the Okazaki peak seem to get larger with increasing pulse times (Fig. 48). To examine this phenomenon more precisely in the absence of so much trailing material from the bottom of the gradient, Okazaki peak fractions were isolated from preparative sucrose gradients, then sedimented under identical conditions. The resulting patterns clearly show that the DNA in the Okazaki peak increases in average size as the pulse-time increases (Fig. 49).

2. Ara C

When Ara C, another inhibitor of DNA synthesis, is used, the results are more complicated. When cells are grown and pulse labeled in the presence of 1×10^{-5} M Ara C (Fig. 50), the shortest pulse labelings give sedimentation patterns (Figs. 50A,B) with a pronounced Okazaki peak and with considerable radioactivity in the form of a

Figure 46. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with various concentrations of hydroxyurea. Cells, some of which were prelabeled overnight with (^{14}C) thymidine, were preincubated with varying concentrations of hydroxyurea and pulse labeled in its presence for 4 minutes at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 44.

(A) 0 μg/ml hydroxyurea.

(B) 5 μg/ml hydroxyurea.

(C) 50 μg/ml hydroxyurea.

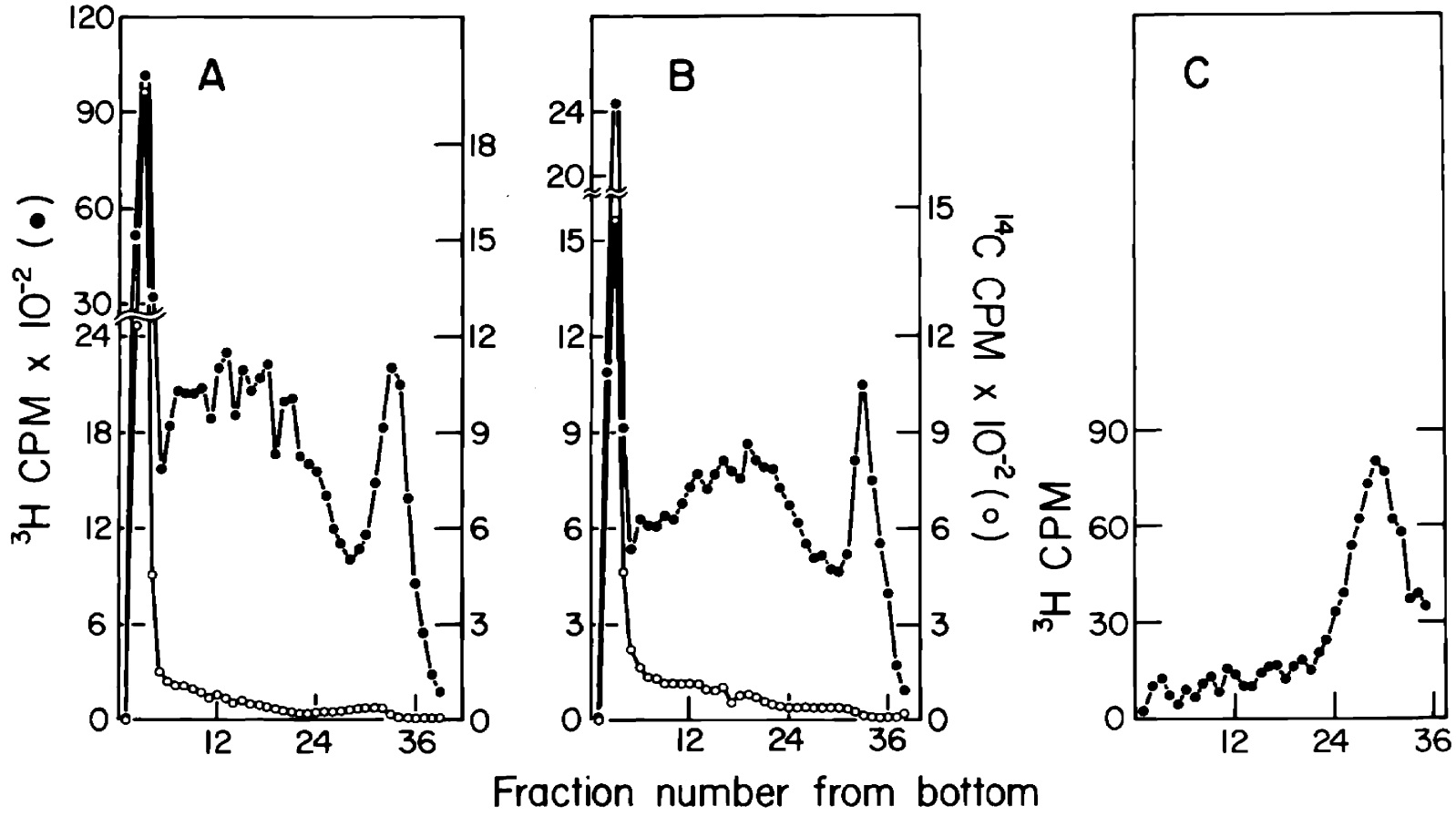

A
B
C
^{3}H CPM x 10^{-2} (•)
^{14}C CPM x 10^{-2} (○)
^{3}H CPM
Fraction number from bottom

Figure 47. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with 500 μg/ml hydroxyurea. Cells were preincubated with 500 μg/ml hydroxyurea and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 28. The numbers indicate the percentage of the total acid precipitable radioactivity that appears in the Okazaki peak.

(A) 15 minute pulse.

(B) 30 minute pulse.

(C) 1 hour pulse.

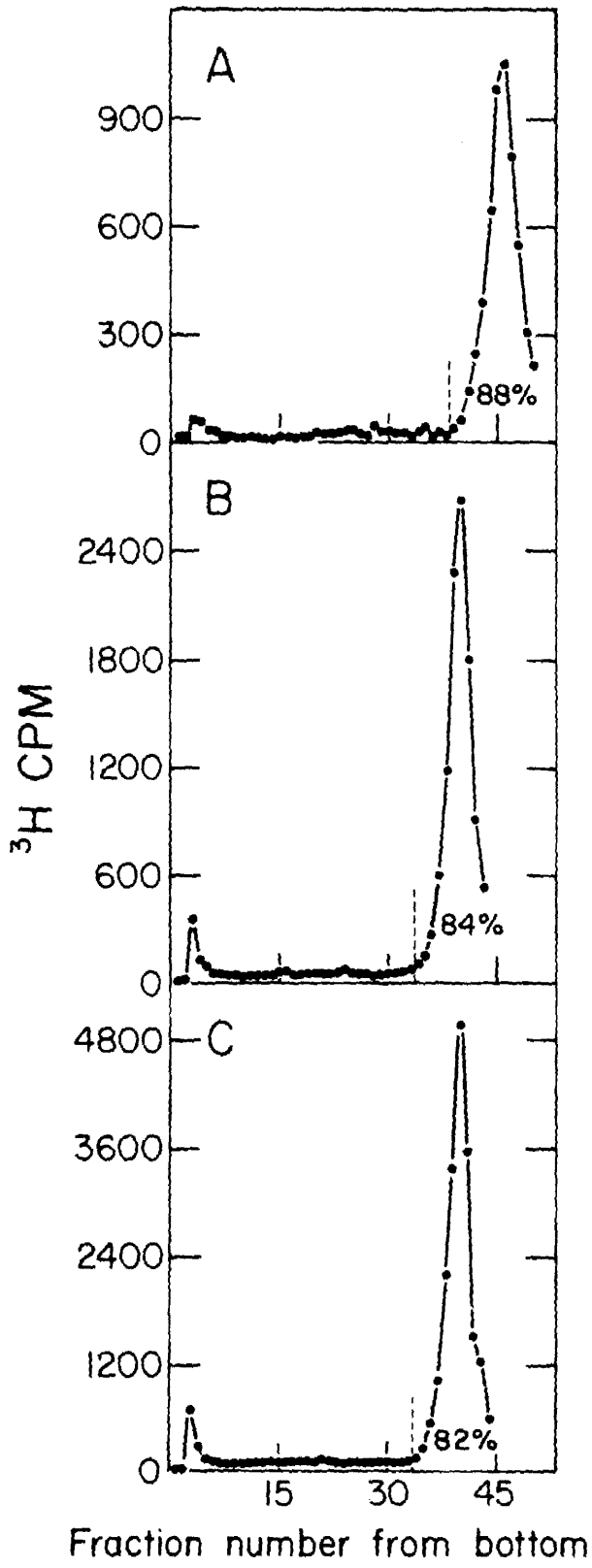

A
900
600
300
0
88%
B
2400
1800
1200
600
0
84%
C
4800
3600
2400
1200
0
82%
15
30
45
^{3}H CPM
Fraction number from bottom

Figure 48. Sucrose gradient profiles of the DNA from cells preincubated with hydroxyurea and pulse labeled for various times. Cells were preincubated in 100 μg/ml hydroxyurea and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 43.

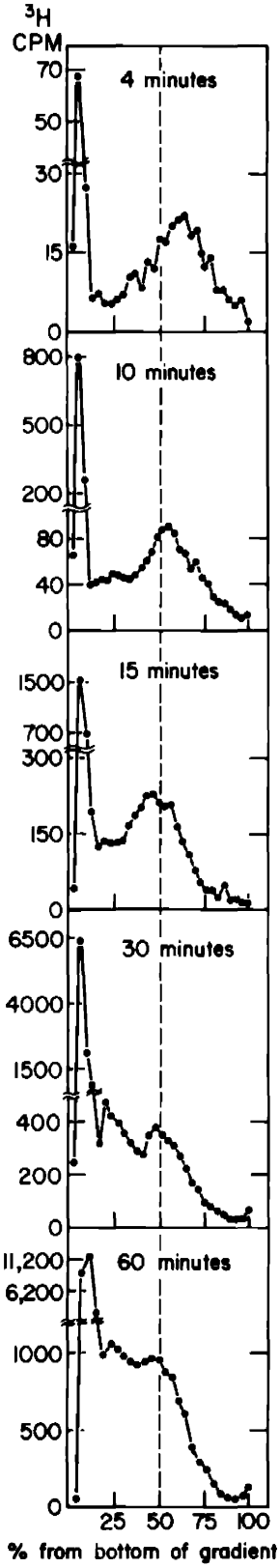

^{3}H
CPM
4 minutes
70
50
30
15
0
10 minutes
800
500
200
80
40
0
15 minutes
1500
700
300
150
0
30 minutes
6500
4000
1500
400
200
0
60 minutes
11,200
6,200
1000
500
0
25 50 75 100
% from bottom of gradient

Figure 49. Sucrose gradient profiles of the Okazaki peaks from cells preincubated with hydroxyurea and pulse labeled for various times. Cells were preincubated in 100 μg/ml hydroxyurea and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 17 (not shown). The Okazaki peak fractions were pooled, diluted 1:1 with water and centrifuged on alkaline sucrose gradients in an SW50. 1 rotor at 40,000 rpm for 19 hours at 23°C. The numbers indicate the fraction of total length traveled down the gradient at that point.

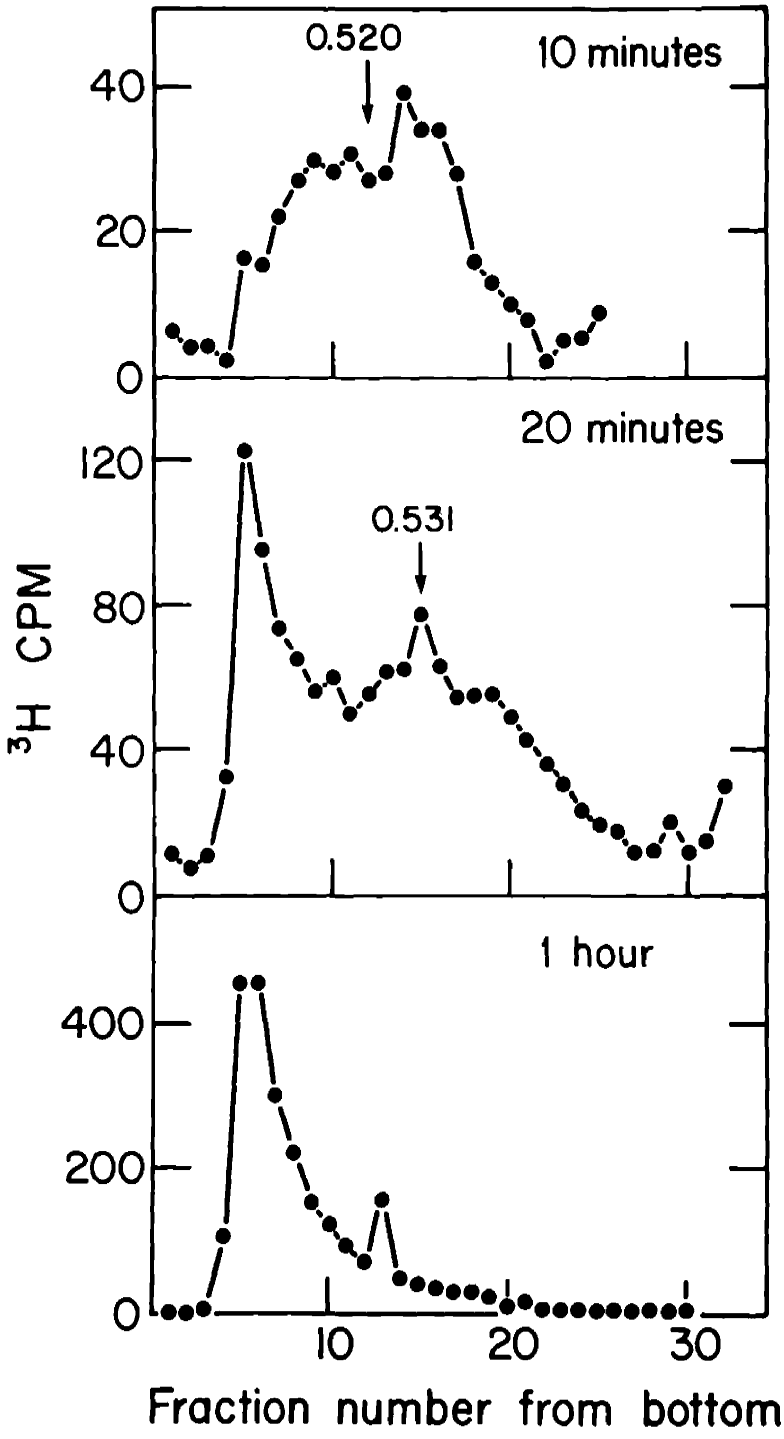

0.520
10 minutes
40
20
0
20 minutes
120
0.531
80
40
0
^{3}H CPM
1 hour
400
200
0
10
20
30
Fraction number from bottom

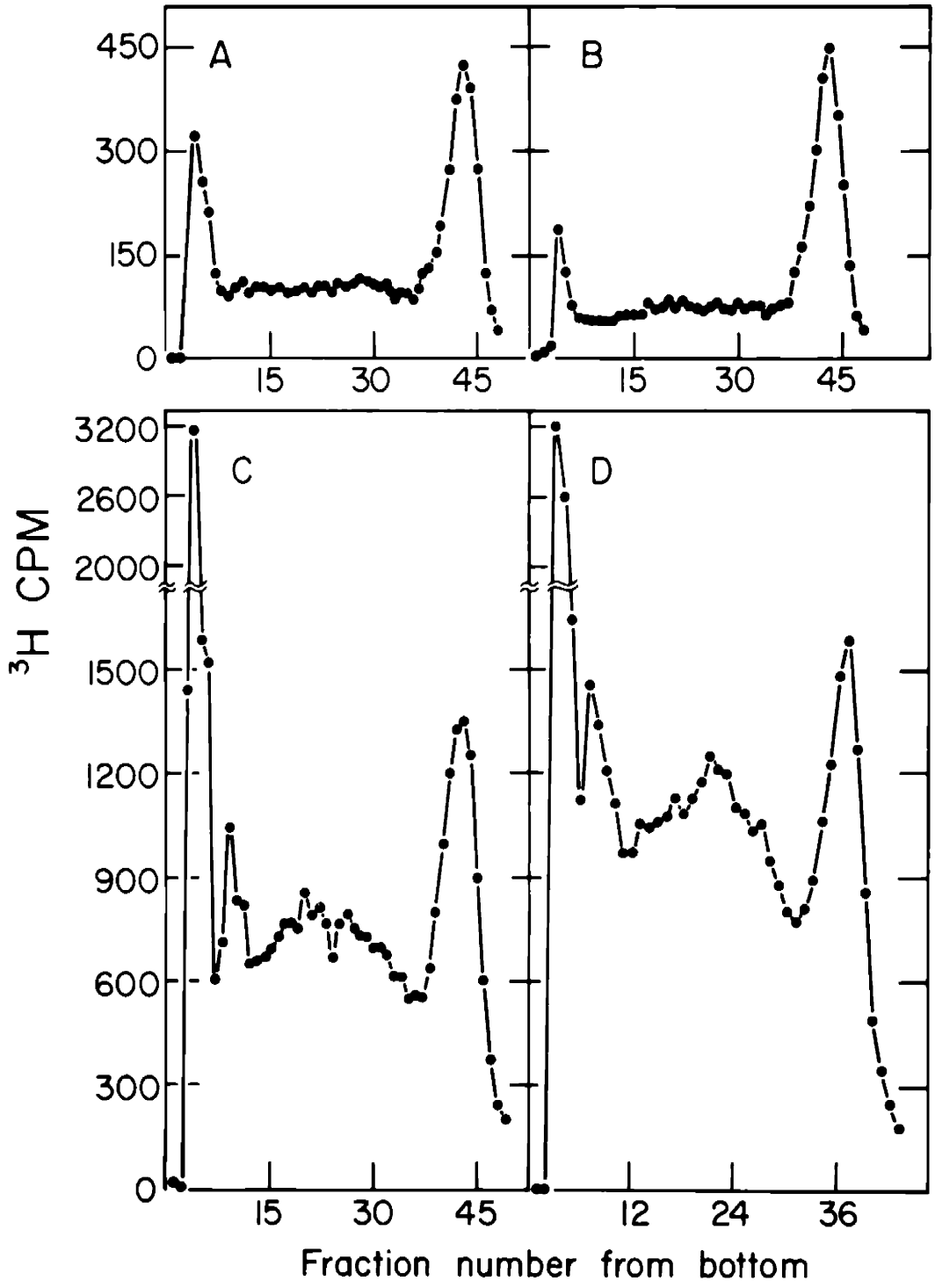
Figure 50. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with 1 x 10^{-5}M Ara C. Cells were preincubated in 1 x 10^{-5} M Ara C and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 17.

(A) 3 minute pulse.

(B) 7 minute pulse.

(C) 10 minute pulse.

(D) 15 minute pulse.


A
B
C
D
450
300
150
0
3200
2600
2000
1500
1200
900
600
300
0
15
30
45
12
24
36
^{3}H CPM
Fraction number from bottom

plateau in the intermediate region. However, a 10 minute pulse (Fig. 50C) shows indication of a distinct peak in the intermediate region, and by 15 minutes this peak is quite evident (Fig. 50D).

At a higher concentration (5×10^{-5}M) of Ara C, when the amount of DNA synthesis is reduced still further (from 8% of control to 3%), the sedimentation pattern starts out with an even more pronounced Okazaki peak and less radioactivity in the intermediate region (Fig. 51A). Gradually, however, radioactivity builds up near the bottom of the gradient (Fig. 51B) until after an 80 minute pulse there is a definite peak (Fig. 51D). This sedimentation pattern is unlike those obtained either with hydroxyurea (when only a plateau is observed in the "intermediate" region) or under uninhibited conditions (when the intermediate peak appears in the middle of the gradient rather than being skewed toward the bottom).

The DNA fragments in the Okazaki peak seem to be larger (Fig. 52) than those made under uninhibited conditions (Fig. 37) or under the influence of hydroxyurea (Fig. 48, 4 min and 10 min). In addition, they seem to get still larger with increasing pulse times, as they do when inhibited by hydroxyurea. Again, resedimentation of the Okazaki peaks taken from preparative sucrose gradients confirms this increase in size (Fig. 53).

3. FUdR

a. Pulse labeling with deoxycytidine

Figure 51. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with 5×10^{-5} M Ara C. Same procedure as in Figure 50, except concentration of Ara C was 5×10^{-5} M.

(A) 5 minute pulse.

(B) 20 minute pulse.

(C) 40 minute pulse.

(D) 80 minute pulse.

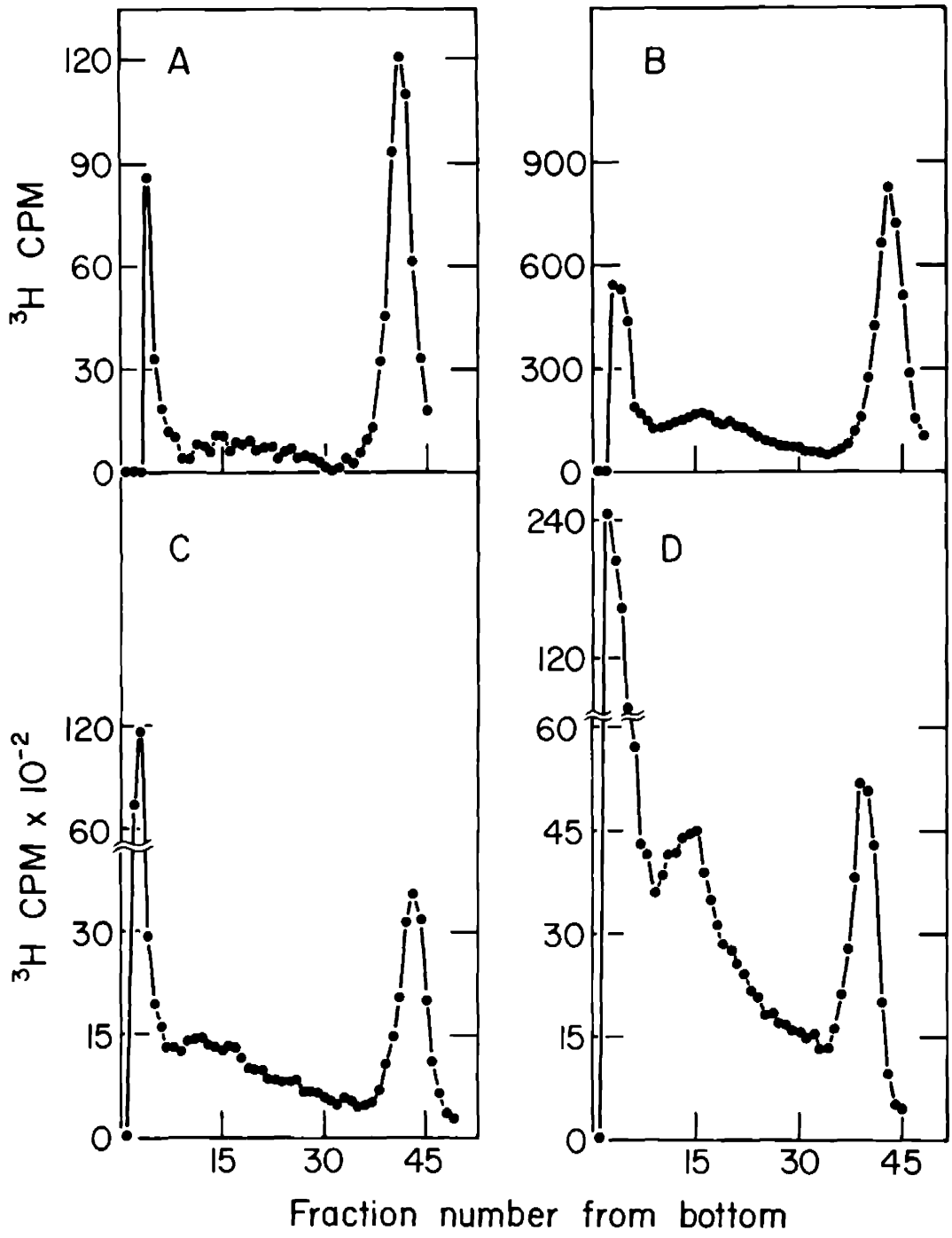

A
B
C
D
120
90
60
30
0
900
600
300
240
45
15
^{3}H CPM
^{3}H CPM x 10^{-2}
15
30
45
Fraction number from bottom

Figure 52. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with Ara C. Cells were preincubated for 2 hours with 5×10^{-5} M Ara C and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 48.

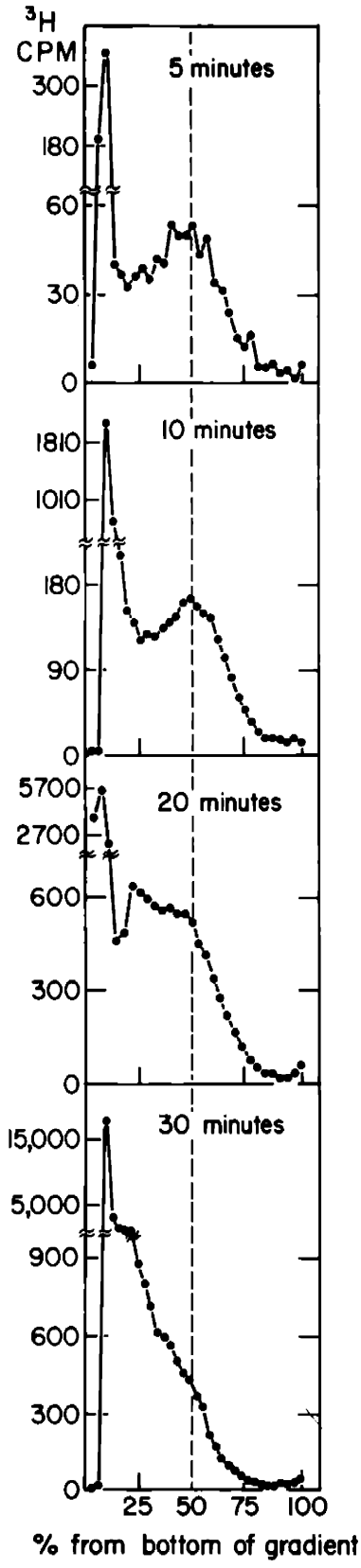
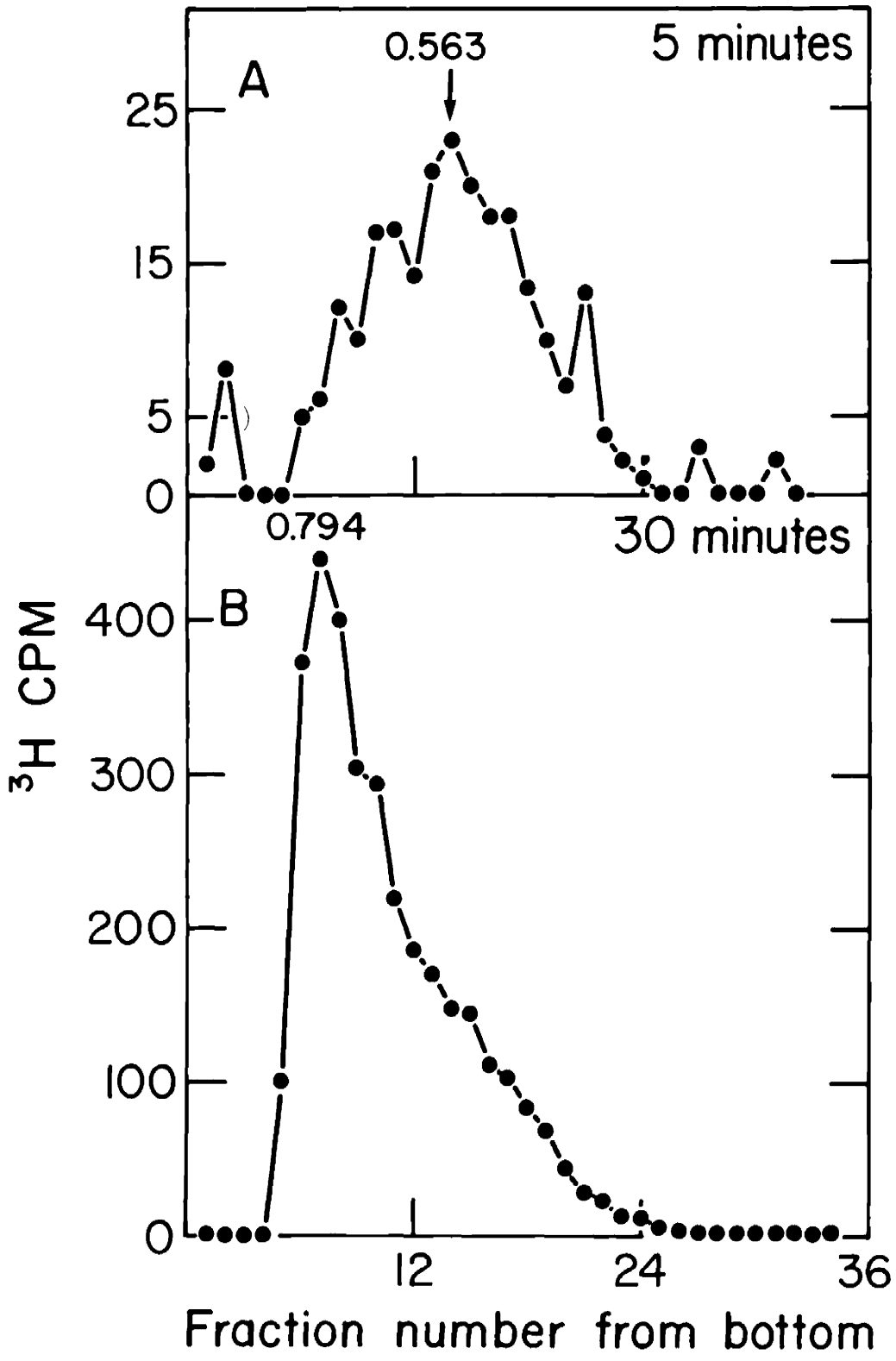

^{3}H
CPM
5 minutes
300
180
60
30
0
10 minutes
1810
1010
180
90
0
20 minutes
5700
2700
600
300
0
30 minutes
15,000
5,000
900
600
300
0
25
50
75
100
% from bottom of gradient

Figure 53. Sucrose gradient profiles of Okazaki peaks from cells preincubated with Ara C. Cells were preincubated for 2 hours with 5×10^{-5} M Ara C and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared, centrifuged and re-centrifuged as in Figure 49.


A
0.563
5 minutes
25
15
5
0
0.794
30 minutes
B
400
300
200
100
0
^{3}H CPM
12
24
36
Fraction number from bottom

When FUdR is used as the inhibitor of DNA synthesis, other unusual aspects of the inhibition are noted. At a concentration of 2×10^{-7}M, the level of incorporation of (^{3}H)deoxycytidine is reduced to about 6-10% of control, but no change in the gross sedimentation pattern is observed (Fig. 54). At a concentration of 5×10^{-6}M, without any noticeable further decrease in incorporation, the sedimentation patterns become similar to those obtained with hydroxyurea. Here the increase in size of the Okazaki peak is quite noticeable. The leading bulge of the Okazaki peak seen during short pulse-labelings (Figs. 55A,B) is eliminated when the FUdR concentration is raised to 2×10^{-5}M (Fig. 56).

b. Pulse labeling with thymidine

It has been assumed that when thymidine is added to cells growing in medium containing FUdR, inhibition is removed and normal DNA synthesis commences. However, the sedimentation pattern of DNA from cells pulse labeled with radioactive thymidine after having been grown for two hours in the presence of FUdR is not at all normal. At an FUdR concentration of 1×10^{-6}M the pattern shows a peak slightly larger than the Okazaki peak with considerable skewing into the intermediate region (Fig. 57). With increasing pulse-times, the peak seems to move down the gradient and an increasing percentage of radioactivity appears in the intermediate area. At 5×10^{-6}M FUdR (Fig. 58) the sedimentation patterns appear remarkably similar

Figure 54. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with 2×10^{-7} M FUdR. Cells were preincubated for 2 hours with 2×10^{-7} M FUdR and pulse labeled in its presence for 10 minutes at room temperature with (^{3}H) deoxycytidine. DNA was prepared and centrifuged as in Figure 17.

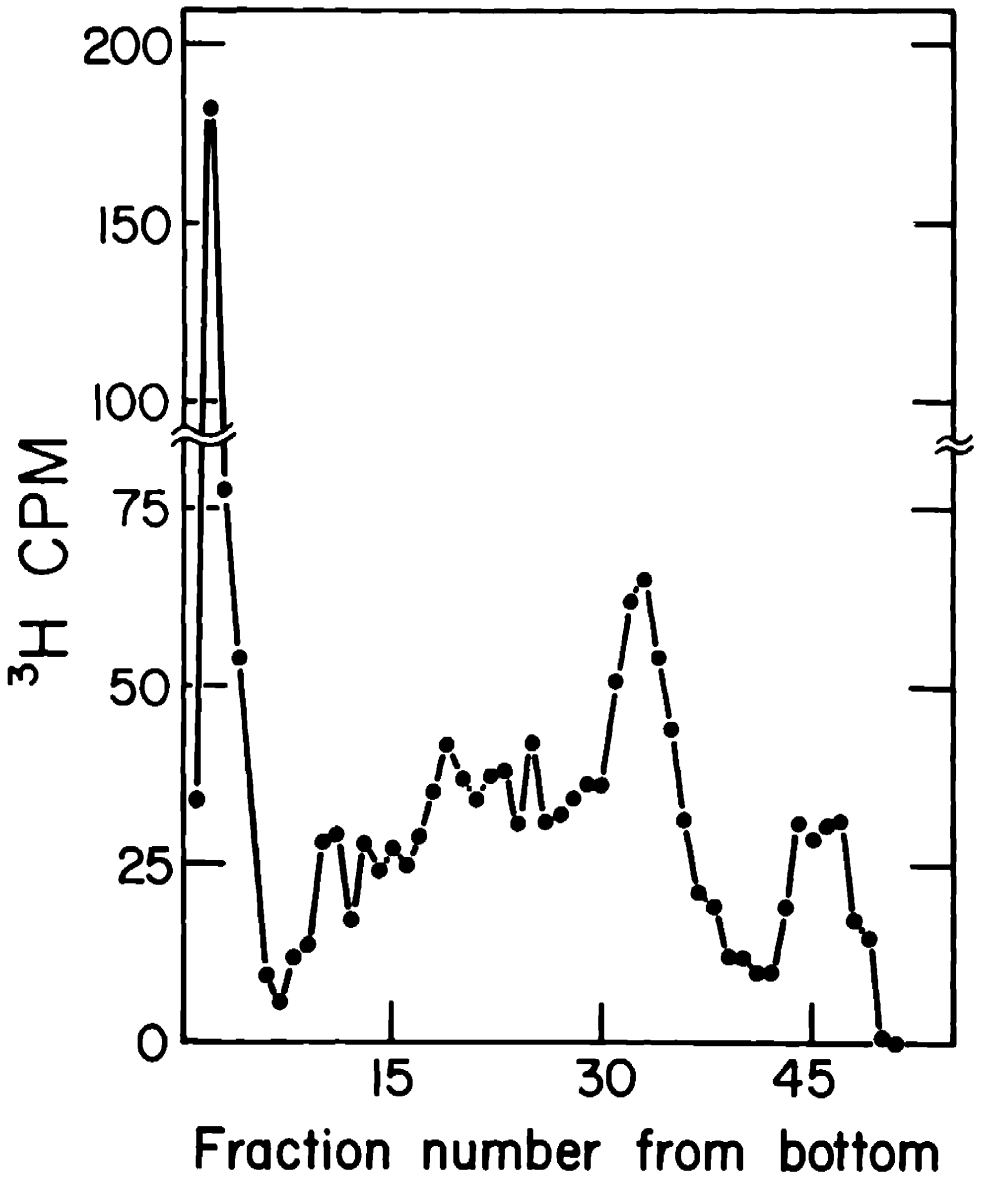

200
150
100
75
50
25
0
^{3}H CPM
15
30
45
Fraction number from bottom

Figure 55. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with 5×10^{-6} M FUdR. Cells were preincubated for 2 hours with 5×10^{-6} M FUdR and pulse labeled in its presence at room temperature with (^{3}H) deoxycytidine. DNA was prepared and centrifuged as in Figure 17.

(A) 5 minute pulse.

(B) 10 minute pulse.

(C) 15 minute pulse.

(D) 30 minute pulse.

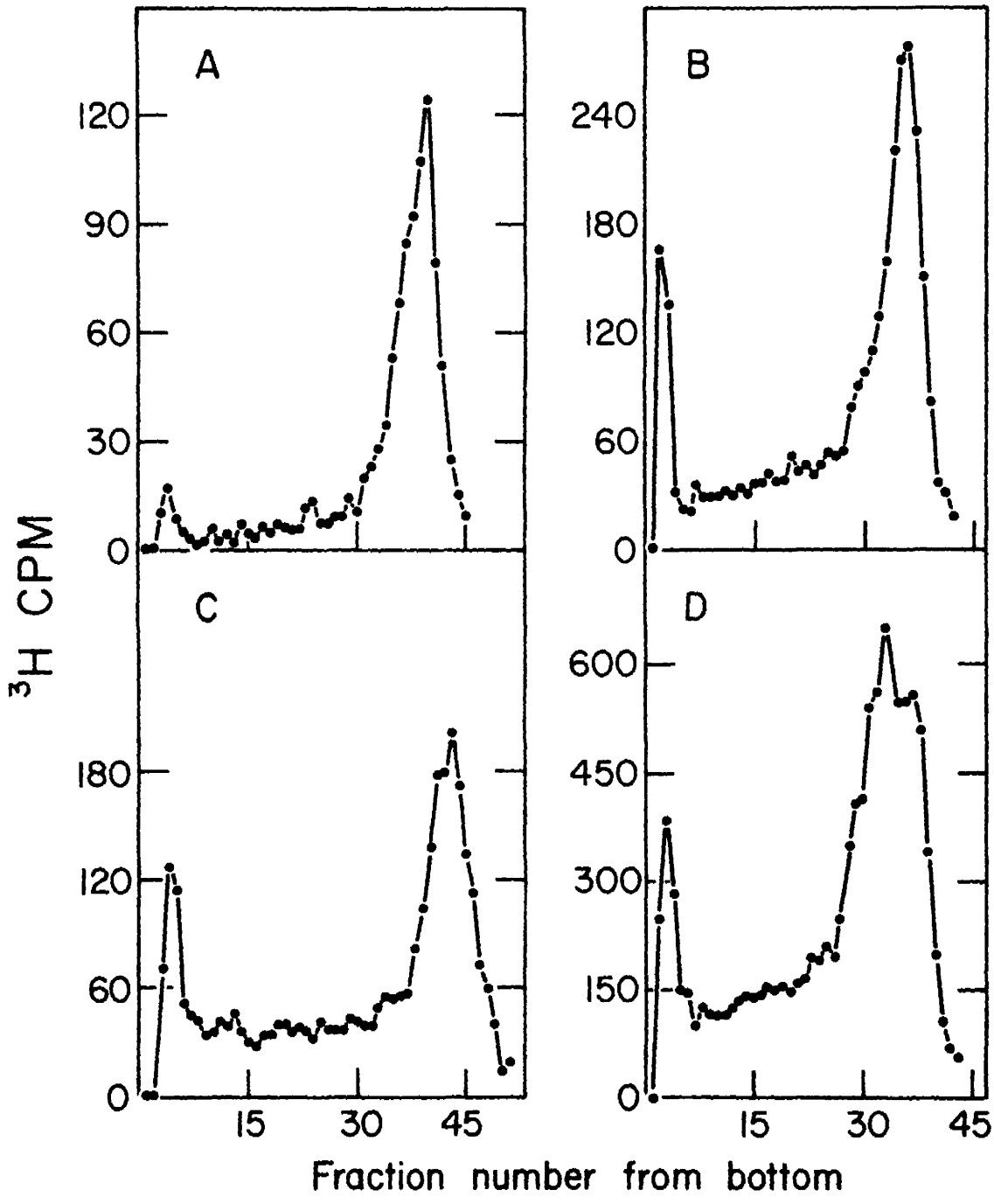

A
B
C
D
120
90
60
30
0
240
180
120
60
0
180
120
60
0
600
450
300
150
0
15
30
45
^{3}H CPM
Fraction number from bottom

Figure 56. Sucrose gradient profile of pulse labeled DNA from cells preincubated with 1×10^{-5} FUdR. Cells were preincubated for 2 hours in 1×10^{-5} FUdR and pulse labeled in its presence for 10 minutes at room temperature with (^{3}H) deoxycytidine. DNA was prepared and centrifuged as in Figure 17.

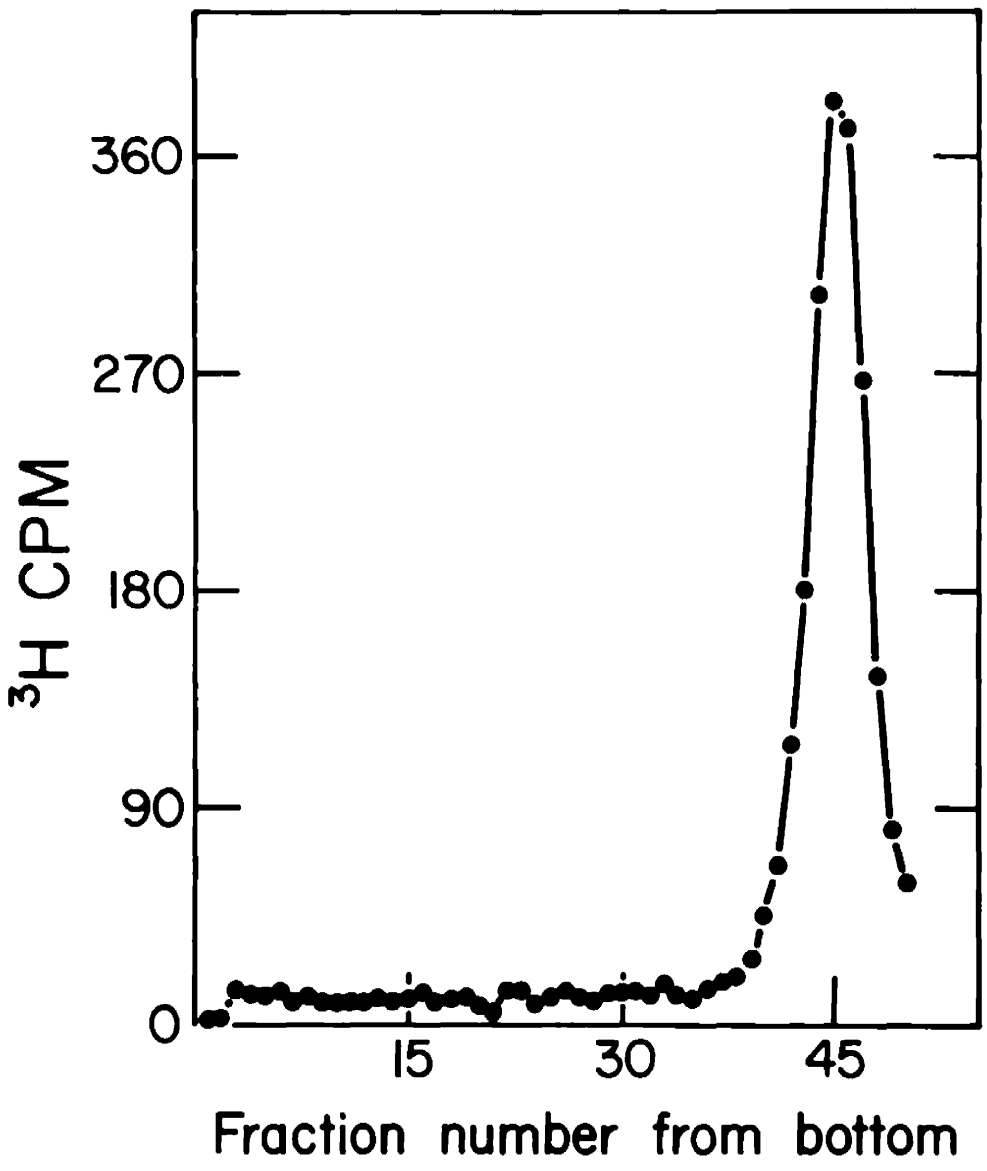

360
270
180
90
0
^{3}H CPM
15
30
45
Fraction number from bottom

Figure 57. Sucrose gradient profiles of DNA from cells preincubated with 1×10^{-6} M FUdR and pulse labeled with thymidine. Cells were preincubated for 2 hours in 1×10^{-6} M FUdR and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 17.

(A) 4 minute pulse.

(B) 15 minute pulse.

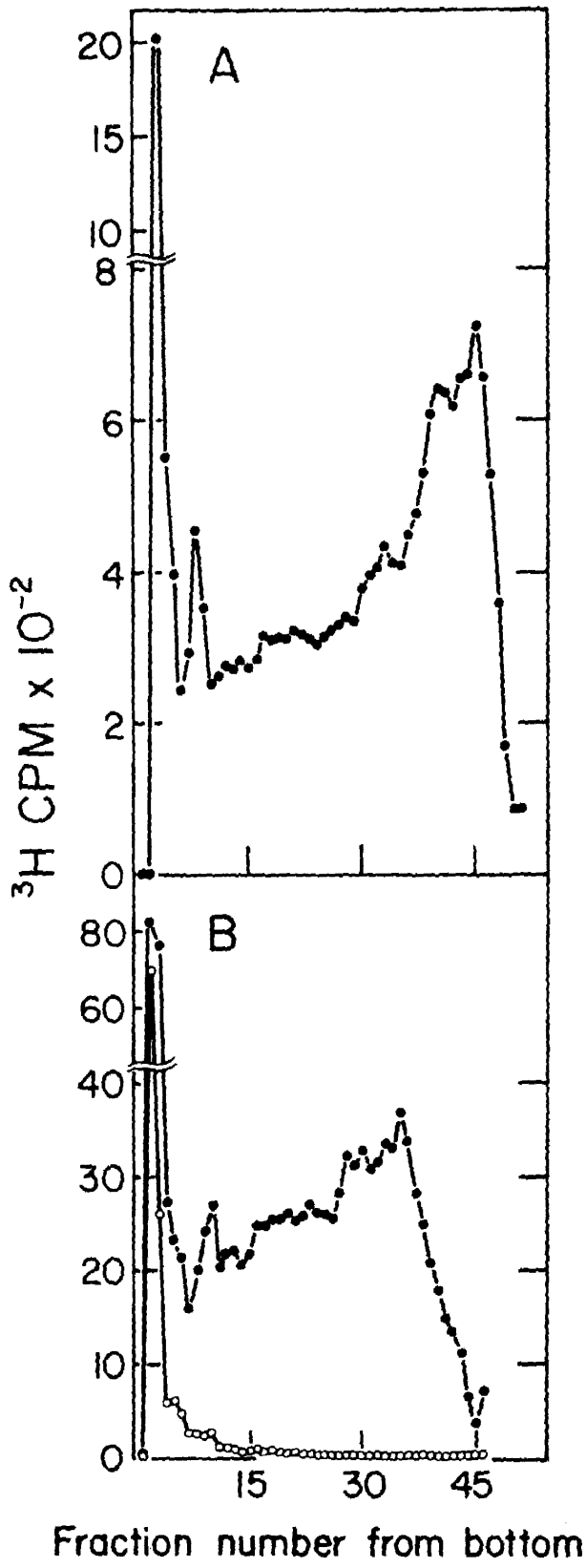
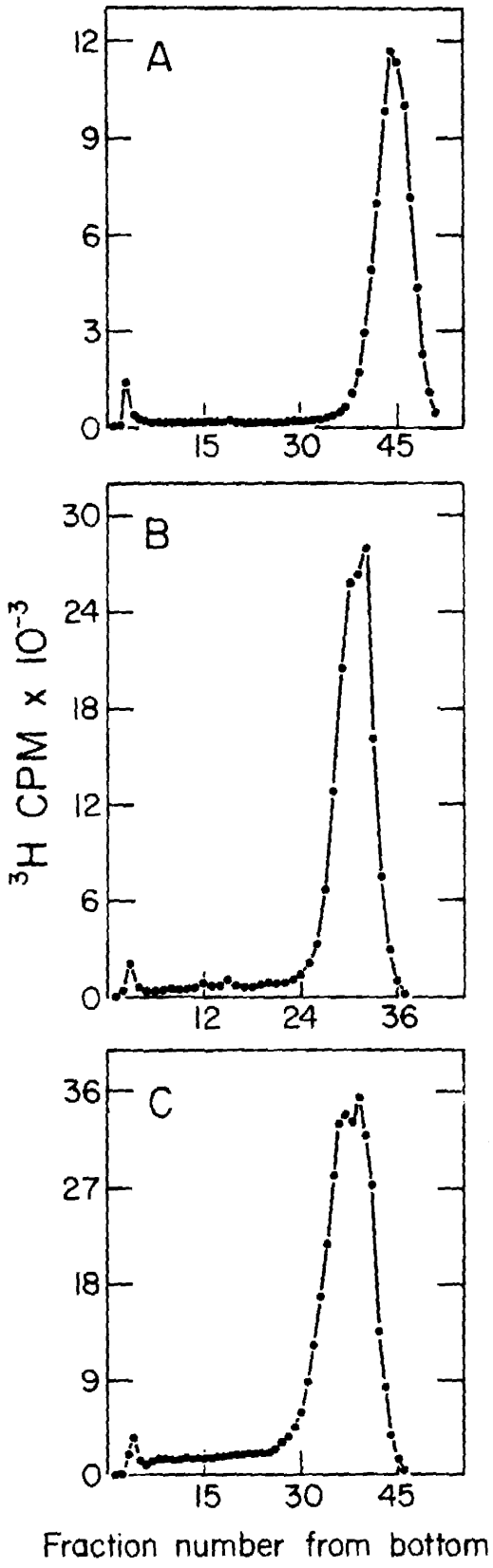

A
B
20
15
10
8
6
4
2
0
80
60
40
30
20
10
0
15
30
45
^{3}H CPM x 10^{-2}
Fraction number from bottom

Figure 58. Sucrose gradient profiles of DNA from cells preincubated with 5×10^{-6} M FUdR and pulse labeled with thymidine. Cells were preincubated for 2 hours in 5×10^{-6} M FUdR and pulse labeled in its presence at room temperature with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 17.

(A) 5 minute pulse.

(B) 10 minute pulse.

(C) 30 minute pulse.


A
B
C
12
9
6
3
0
30
24
18
12
6
0
36
27
18
9
0
15
30
45
12
24
36
^{3}H CPM x 10^{-3}
Fraction number from bottom

to those obtained with 500 μg/ml hydroxyurea (Fig. 47) and show an even larger proportion of acid precipitable counts in the Okazaki peak than when cells grown in the same concentration of FUdR are pulse-labeled with deoxycytidine (Fig. 55). Increasing the concentration of FUdR to 1 x 10^{-5} M does not seem to cause any further changes in the pattern (Fig. 59).

Since DNA synthesis at room temperature proceeds at only 7-20% of the rate at 37°C, experiments were done at 37°C to see how long the abnormal pattern of synthesis continues after the addition of thymidine. Cells were grown in the presence of 5 x 10^{-6}M FUdR for 2 hours and then unlabeled thymidine was added. After varying lengths of time the cells were pulse labeled with an equal amount of radioactive thymidine for 5 minutes at 37°C. As shown in Figure 60, if the (^{3}H) thymidine is added at the same time as the cold thymidine, the sedimentation pattern is similar to that obtained with hydroxyurea. As the time of prelabeling with cold thymidine increases, the peak moves down the gradient, and after a prelabeling of between 15 minutes (Fig. 60B) and one hour (Fig. 60C) the pattern becomes similar to that obtained under normal conditions of synthesis (Fig. 23A).

Figure 59. Sucrose gradient profile of DNA from cells preincubated with 1×10^{-5} M FUdR and pulse labeled with thymidine. Cells incubated, pulsed, treated and centrifuged as in Figure 58C except FUdR concentration was 1×10^{-5} M.

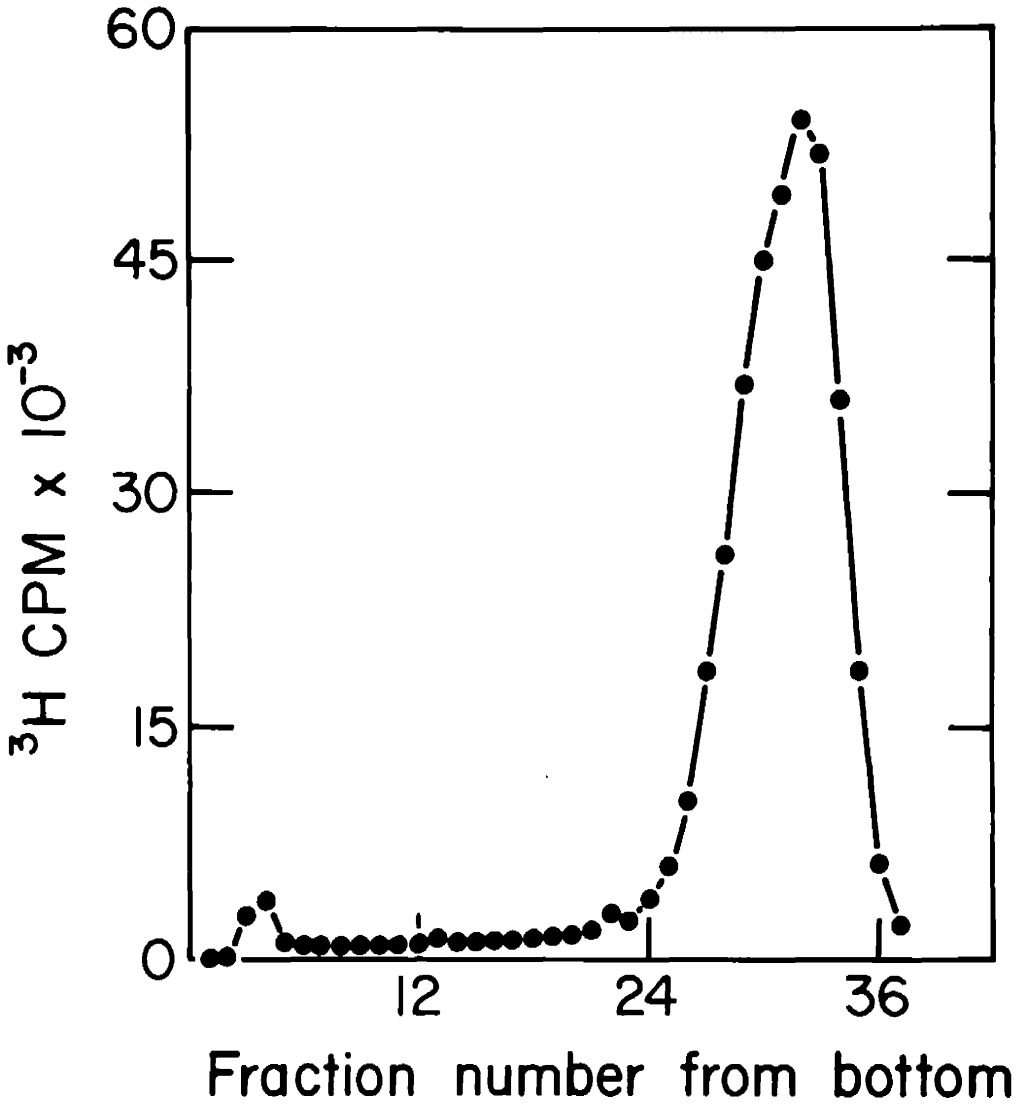

60
45
30
15
0
^{3}H CPM x 10^{-3}
12
24
36
Fraction number from bottom

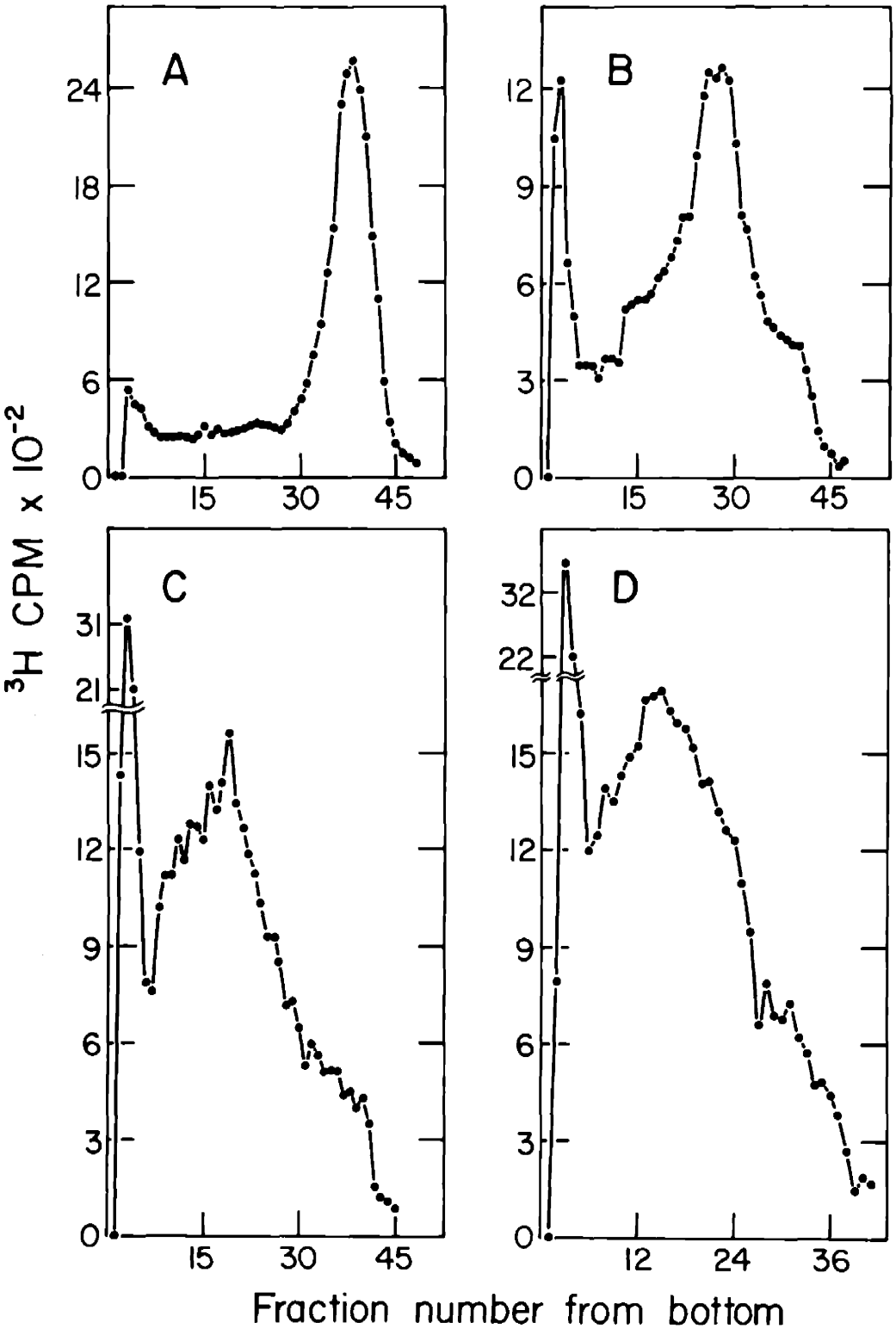
Figure 60. Sucrose gradient profiles of pulse labeled DNA from cells preincubated with FUdR and then thymidine. Cells were preincubated for 2 hours in 5×10^{-6} M FUdR and pulse labeled for varying times at 37°C with 3.3×10^{-6} M unlabeled thymidine followed by a 5 minute pulse at 37°C with (^{3}H) thymidine. DNA was prepared and centrifuged as in Figure 17.

(A) No prepulse with cold thymidine.

(B) 15 minute prepulse with cold thymidine.

(C) 1 hour prepulse with cold thymidine.

(D) 2 hour prepulse with cold thymidine.


A
B
C
D
^{3}H CPM x 10^{-2}
Fraction number from bottom
0
3
6
9
12
15
18
21
22
24
31
32
36
45

DISCUSSION

I. Artifacts

A. Adsorption

1. The "Okazaki peak"

My results clearly show that when mammalian cells are exposed to (^{3}H) thymidine and then lysed, if the total lysate is centrifuged through a sucrose gradient, a peak appears in the "Okazaki fragment" region whether or not the cells had a chance to incorporate radioactivity into their DNA. Although I did not do any experiments with bacteria, it is quite possible that they too can produce this artifact.

It appears that this artifact has two aspects, both of which must be dealt with in order to eliminate it. There seems to be a large intracellular pool of labeled nucleotides, and these adsorb to some cellular component that sediments in a sucrose gradient but does not band at the density of DNA in a cesium chloride gradient. Dialyzing or precipitating and washing with ethanol seems to remove most of the excess nucleotides, but the adsorbed molecules remain. Therefore, an additional step is needed to get rid of material that the nucleotides adsorb to. Thus the proteinase K incubation and chloroform-isoamyl extraction or the preparation of nuclei is also required.

The fact that preparation of nuclei is sufficient to remove the artifact suggests that the cellular component to which nucleotides adsorb

is a cytoplasmic one. Preparation of nuclei simultaneously removes this cytoplasmic component and the intracellular pool.

Many of the groups that have reported finding Okazaki pieces in organisms ranging from bacteria and phage to mammalian cells have in fact used the total lysate method (Newman & Hanawalt, 1968b; Okazaki et al., 1968a; Pauling & Hamm, 1969; Schandl & Taylor, 1969; Bird & Lark, 1970; Ensminger & Tamm, 1970; Graham & Whitmore, 1970b; Hyodo et al., 1970, 1971; Nuzzo et al., 1970; Cheevers et al., 1972; Fujiwara, 1972; Gautschi & Kern, 1973; Gautschi et al., 1973; Goldstein & Rutman, 1973; Friedman et al., 1975; Gautschi & Clarkson, 1975; Lark & Wechsler, 1975; Mendelsohn et al., 1975; Rajalakshmi & Sarma, 1975). Others have done some purifying steps, such as chloroform-isoamyl extraction (Schandl & Taylor, 1971; Schandl, 1972) or ethanol precipitating without further washings with ethanol (Tseng & Goulian, 1975a), but have not gone through enough of a purification to be sure that the artifact has been completely removed. In light of the fact that the artifact accentuates any real Okazaki peak in a sucrose gradient but does not band in cesium chloride, it is interesting to note that in at least one report (Okazaki et al., 1968a) Okazaki's group used the total lysate method for all their sucrose gradients and a phenol extraction (Thomas procedure) for all the cesium sulfate gradients.

2. Nascent DNA complex

Several groups have reported that nascent DNA seems to be complexed in some way, because it goes to the interface in a chloroform or phenol extraction and/or does not band in cesium chloride (Ben-Porat et al., 1962; Levis et al., 1967; Friedman & Mueller, 1968, 1969; Pearson & Hanawalt, 1971; Fakan et al., 1972; Weintraub, 1972). While some groups feel that this phenomena just represents the single-stranded nature of the nascent DNA (Fakan et al., 1972), others argue the DNA must be bound to lipo-polysaccharides (Friedman & Mueller, 1969) or protein molecules such as polymerases (Levis et al., 1967). However, it is quite possible that this "complex" is nothing more than the artifact I have demonstrated. Rather than nascent DNA adhering to some cellular component, the phenomenon may simply be free radioactive nucleotides adhering to this component. The complex would then appear to chase (into the aqueous layer or the cesium chloride gradient) as the radioactive nucleotides were incorporated into DNA.

3. Further problems in analysis

It is important to remember that this artifact presents problems in analysis even when labeled pieces of DNA of Okazaki-fragment size are present. Since the artifact will increase the amount of radioactivity appearing in the Okazaki peak beyond that incorporated into DNA, estimates of the percentage of total radioactivity appearing in the

Okazaki peak will be misleading (see below, II). Also, when sucrose gradients are used to measure the size of Okazaki fragments, the artifact will cause inaccurate determinations if it sediments to a slightly different position than that of the Okazaki fragments.

Unfortunately, many of the procedures used to extract or purify DNA seem to degrade it as well. I have found that whenever the DNA is left in solution overnight there is breakage of long strands. This may be due to nucleases that are not eliminated by prior purification steps. One group (Berger & Huang, 1974), after dialyzing three times, finds that the bulk DNA is very small. Another group (Habener et al., 1969b) suggests that pulse-labeled DNA breaks down when it is left in solution because tritium radiolysis causes degradation. However, this would not account for the overnight breakdown of (^{14}C)-labeled DNA.

B. Sedimentation

1. Entangling of different-sized strands

Besides artifactual results arising during cell lysis and preparation of the DNA, the major area of concern is artifacts caused by sedimentation conditions. Several groups have reported that large DNA gets entangled and sediments anomalously (Lett et al., 1967; Lehmann & Ormerod, 1971; Ormerod & Lehmann, 1971a; Ormerod & Stevens, 1971). I have shown that the sedimentation profile of bulk DNA is changed when large amounts of DNA are put on the gradient

and there is sometimes a variable amount of pulse-labeled DNA at the bottom of the gradient. (Gautschi & Clarkson, 1975, also report this phenomenon and show by use of agarose gels that it is an artifact of centrifugation.) However, under conditions which sediment this large DNA to the bottom, the shape of the curve of pulse-labeled DNA appearing in the rest of the gradient is not changed. Other groups (Lehmann & Ormerod, 1971; Gautschi & Kern, 1973) also report that intermediate-sized pieces of DNA are not entangled by bulk DNA.

2. Speed of centrifugation

There are also reports that a high speed of centrifugation (23,000-37,000 rpm) causes anomalous sedimentation patterns, especially for large DNA (Elkind, 1971; Lehmann & Ormerod, 1971; McBurney et al., 1971; Ormerod & Lehmann, 1971b); although one group (Lehmann & Ormerod, 1971) does point out that the intermediate peak looks the same at high and low speeds. I have shown that, at least for the size and amount of DNA that I usually use, there is no difference in the shape of the curves of either bulk or intermediate DNA when sedimented at 13,500 or 38,000 rpm.

C. Denaturation

I have also shown that extending any one of the conditions of denaturation--i.e., raising the temperature or the pH or lengthening the time of incubation--causes some breakdown of large pieces of DNA.

The effect of alkali on DNA has been reported by others (Taylor et al., 1970; Elkind, 1971; Hozier & Taylor, 1975) and my results seem to indicate that prolonged exposure to high pH causes a continuous breakdown of DNA. The decrease in size of DNA strands does not seem to stop at a specific point, and thus gives no support to the idea that DNA strands are joined by "linkers" of non-DNA material that might be more susceptible to alkali attack.

The EM study of the changes in the DNA caused by raising the denaturing temperature also shows that just about all of the DNA is in single-stranded form. This answers a possible objection of one group (Simpson et al., 1973) that the "complex" of bulk DNA that is sometimes observed (Elkind, 1971; Ormerod & Lehmann, 1971a; Elkind & Chang-Liu, 1972; Friedman et al., 1975) is really double-stranded DNA and that even the "main peak" contains some double-stranded DNA.

II. Discontinuous Synthesis of DNA

A. Okazaki fragments

Results presented in this thesis clearly indicate that DNA is synthesized in a discontinuous fashion and is originally made in at least three different size classes. Some, if not all, of the radioactive label goes first into small (~100 nucleotides) pieces of DNA, thus reinforcing the opinion of almost all groups that, in at least one strand of the helix, nucleotides are first incorporated into Okazaki fragments.

1. Why they weren't seen by other groups

There are several possible explanations for the fact that some investigators did not find Okazaki pieces. Since the amount of radioactivity in the intermediate peak soon overshadows and masks that in the Okazaki region, a pulse must be quite short if a clear Okazaki peak is to be seen. I have demonstrated that if experiments are carried out at 37°C, a pulse-time of 2 minutes (Fig. 28D) or in some cases even 1 minute (Fig. 29C) is too long for observation of the peak. Many of the unsuccessful attempts to see this peak suffered from this problem (Habener et al., 1969b; Lehmann & Ormerod, 1970; Chiu & Rauth, 1972; Lynch et al., 1972; Winnacher et al., 1972).

Certainly the pulse labeling of intact organs (Tsukada et al., 1968; Berger & Irvin, 1970) does not allow the accurate stopping of a pulse within this time limit. Okazaki's group has pointed out (Kurosawa & Okazaki, 1975) that even if a sufficiently short pulse-time is chosen, incorporation very often continues after it has been supposedly stopped. Adding to this problem is the fact that very short pulses at 37°C or pulses at room temperature do not allow the incorporation of very much labeled nucleotide per cell. This means that unless a lot of cell lysate or DNA is put on the gradient there may be too little radioactivity in the Okazaki pieces for the peak to be clearly seen.

Another difficulty lies in the fact that Okazaki fragments can be selectively lost during purification or centrifugation. It has been noted

that these fragments often appear in a single-stranded form after cell lysis,when the bulk DNA is still double-stranded (Okazaki et al., 1968a; Painter & Schaefer, 1969, Sato et al., 1970; Cheevers et al., 1972; Mendelsohn et al., 1975); and single-stranded DNA can be selectively lost during a phenol extraction or by selective adsorption to cellulose nitrate centrifuge tubes (Fakan et al., 1972).

Even when the Okazaki fragments are not lost they may not appear as a separate entity on a sucrose gradient. Sometimes the sample is not sedimented far enough into the gradient to separate the peaks of intermediate and Okazaki strands, especially when the bulk DNA has been severely broken down (Lehmann & Ormerod, 1969, 1970; Ensminger & Tamm, 1970; Hyodo et al., 1970).

Finally, some groups have worked with in vitro systems (Lynch et al., 1972; Winnacher et al., 1972), and it is never possible to be sure that observations made with such systems indicate what is happening in intact cells.

Okazaki pieces have been pretty well established to be the first step in DNA synthesis. Only Werner (1975) has claimed that, at least in E. coli, these pieces are made only in repair synthesis and are seen because thymidine is only utilized for that type of synthesis. Several groups have shown that this is not the case (Wang & Sternglanz, 1972, 1974; Okazaki et al., 1973). These Okazaki fragments seem to all be made in the 5' $\rightarrow$ 3' direction (Okazaki & Okazaki, 1969; Sugino

& Okazaki, 1969), although there is one group (Diaz & Werner), 1975; Diaz et al., 1975) that feels they are made by random "crystallization" of nucleotides on the parental template.

2. Size of Okazaki fragments

By careful measurements on both sucrose gradients and gels, I have shown that the Okazaki pieces are about 100 nucleotides long. Although some of the wide discrepancy in reported sizes for Okazaki fragments (see Introduction I. C.) might be explained on the basis of differing cell types making pieces of different lengths, most of the problem is probably due to faulty techniques. Many of the measurements in the literature are arrived at on the basis of sedimentation done so that the Okazaki peak is so close to the top of the gradient as to make accurate measuring impossible (Kidwell & Mueller, 1969; Hyodo et al., 1970, 1971; Nuzzo et al., 1970; Fujiwara, 1972; Friedman, 1974; Hershey & Taylor, 1974). Also, as I have demonstrated, putting a total lysate on the gradient produces artifactual radioactivity in the region of the Okazaki peak. Even if there are Okazaki pieces present, the artifact may cause a shift in the position of the peak's midpoint, and therefore in the apparent sizes of the Okazaki fragments. This problem may have affected the size estimates of Schandl & Taylor (1969), Nuzzo et al. (1970), Cheevers et al. (1972) and Goldstein & Rutman (1973).

3. Does the size change with time?

If the Okazaki pieces are not joined to the growing strand soon after they are completed, several of them may lie end to end before joining

takes place. In that event the fragments may be joined randomly rather than sequentially, thus forming pools of dimers, trimers, tetramers and even polymers of Okazaki fragments that may be substantial relative to the pool of single fragments. The fact that I observe no change in the size of the Okazaki peak for as long as it is visible (Fig. 37) indicates that if there are multimers of Okazaki fragments, their relative frequencies remain constant from 1 to 8 minutes at room temperature. (Although material from the higher molecular weight region at the bottom of the gradient might have trailed into the Okazaki peak, such trailing could only cause the Okazaki pieces to appear to have sedimented further down the gradient than they actually did. Thus a false impression would be created only if the DNA strands in the Okazaki peak region are actually getting smaller with time. This is such an unlikely possibility that it need not be considered.)

Several groups report that they observe the Okazaki peak increasing in size with time. Some notice this as a gradual shift of the position of the peak from one size to another (Gautschi & Clarkson, 1975; Tseng & Goulian, 1975a) while others report two distinct peaks (Taylor et al., 1973; Friedman, 1974). It has been suggested that the peak might become larger either because of nucleotide addition (in which case the distinct peak of small pieces must result from a slowing or pause in growth, Tseng & Goulian, 1975a)

or because several small pieces are linked together (Taylor et al., 1973a; Friedman, 1974; Tseng & Goulian, 1975a).

However, much of the evidence presented is not convincing. In some cases it is not at all clear that the position of the peak is changing (Gautschi & Clarkson, 1975) while in others the two peaks seen are more probably just one peak with a slight reduction in the radioactivity seen in one fraction (Friedman, 1974). Also, both Friedman (1974) and Tseng & Goulian (1975a) observe this increase for in vitro synthesis only, so it may not be comparable to my results.

4. Are both sides made discontinuously?

I found that during very brief pulse labelings at room temperature, 75% of the acid precipitable radioactivity appeared in the Okazaki peak (Fig. 33). The simplest interpretation of this finding is that both strands of the DNA helix are replicated discontinuously. (If one strand was being synthesized as a continuous piece, at least 50% of the acid precipitable radioactivity would appear in large (intermediate or bulk) DNA, no matter how short the pulse.) The fact that when the cells were pre-incubated with hydroxyurea at a concentration of 500 μg/ml, more than 80% of the acid precipitable radioactivity appeared in the Okazaki peak (Fig. 47) reinforces this observation.

However, this interpretation is certainly not conclusive. It may be that there are different deoxyribonucleoside triphosphate pools used for synthesis of the two strands, and the one used for the 3' $\longrightarrow$ 5'

strand equilibrates with exogenous nucleosides much more quickly (a possibility noted by Edenberg and Huberman, 1975). Also, excess fragments may be being synthesized on the discontinuous side only to be continually degraded (as suggested by Huberman & Horwitz, 1973).

Several groups have reported that they never observe more than 50% of the acid precipitable radioactivity in the Okazaki peak (Painter & Schaefer, 1969; Hyodo et al., 1970, 1971; Iyer & Lark, 1970; Eisenberg & Dernhardt, 1974; Francke & Hunter, 1974a; Friedman, 1974; Hershey & Taylor, 1974; Louarn & Bird, 1974). Some of the reports are not convincing because pulse-times were so long that the percentage of the total acid precipitable radioactivity found in the Okazaki peak is probably misleadingly small (Hyodo et al., 1970; Hershey & Taylor, 1974). Also, some of the observations were made with in vitro systems (Francke & Hunter, 1974a; Friedman, 1974; Hershey & Taylor, 1974), so they may not accurately reflect the in vivo situation.

Even if at least 50% of the acid precipitable radioactivity were found in DNA strands considerably longer than Okazaki fragments, it would not prove that only one side is replicated discontinuously. Perhaps both sides are made discontinuously, but on one side the pieces are joined much more rapidly (suggested by Laipis & Levine, 1973 and Kurosawa & Okazaki, 1975). Also, the radioactive label may be used for gap filling as well as making new fragments, so some label appears immediately in bulk DNA (suggested by Qasba, 1974b).

Bonhoeffer's group (Hermann et al., 1972; Olivera & Bonhoeffer, 1972) has shown that both sides of the E. coli helix are synthesized discontinuously, but the size of the small pieces made on the possibly continuous side varies considerably as the in vitro conditions are changed. I looked carefully for such a phenomenon with CHO cells, but was unable to find any indication of two different size-classes of small pieces.

B. Joining the fragments

The fact that Okazaki pieces are seen as a peak on sucrose gradients implies that there is a pool of such fragments and they are not joined to larger strands of DNA immediately upon being completed (Edenberg & Huberman, 1975). Several steps are involved in the transition, and it is not clear which, if any, of these steps is rate limiting. Although my experiments did not examine any of these steps directly, I would like to briefly mention them as an aid in the elucidation of the overall scheme.

It seems that mammalian Okazaki fragments are primed by short stretches of RNA (Sato et al., 1972; Fox et al., 1973; Taylor et al., 1973b; Neubort & Bases, 1974; Tseng & Goulian, 1975b; Waqar & Huberman, 1975b). Although Taylor's group has proposed a short piece of DNA as the primer (Taylor et al., 1970; Schandl & Taylor, 1971; Schandl, 1972), there is strong evidence that these pieces are really just an artifact caused by the cells' triphosphate pools (Huberman, 1974).

Before the Okazaki pieces can be joined, the stretch of RNA primer must be removed and the gap caused by this removal must be filled in. Several different enzymes are needed for these processes, and the DNA polymerase that fills in the gaps may not be the same one that is used to synthesize the fragments. In E. coli these steps are probably performed by DNA polymerase I, its exonuclease activity, and the DNA ligase (see Introduction, I. A. 2), but in the case of mammalian cells things are not so clear. For a slight amplification of this question, see below (Discussion, III).

Depending on whether removal of the RNA and filling in of the gap or joining of the completed pieces is the rate limiting step, the pool of Okazaki fragments will be made up of primarily fragments the length of the original DNA segment or of fragments the length of the DNA plus RNA segments. Even if both types of fragments are present there would probably be only one peak, since the RNA primer seems to be small in proportion to the total piece (Sugino et al., 1972; Hirose et al., 1973; Hunter & Francke, 1974b; Neubort & Bases, 1974; Reichard et al., 1974; Waqar & Huberman, 1975).

C. Kinetics of synthesis and joining

My observations do not allow me to say very much about the time required to synthesize and join an Okazaki fragment. Goulian's group (Mendelsohn et al., 1973; Tseng & Goulian, 1975a) calculates the lifetime of an Okazaki fragment by multiplying the pulse-time by

the fraction of radioactivity appearing in the Okazaki peak. However, this equation seems to be incorrect, for it implies that (assuming both strands to be replicated discontinuously) during any pulse shorter than the lifetime of a fragment,no radioactivity will appear in the intermediate or bulk regions. This is certainly not true, for the Okazaki piece may have incorporated label just as it was nearing completion.

Nuzzo et al. (1970) say that the lifetime is equal to the longest pulse-time during which radioactivity appears only in the Okazaki peak. Although this does set a lower limit, the lifetime could actually be much longer, for the reason mentioned above.

Hunter & Francke (1974a) claim that the upper limit of the lifetime of a piece is equal to the pulse-time required to reach saturation of radioactivity in the Okazaki peak. (This is only an upper limit because of the time required for pool equilibration.) If this is so, then my data indicates a lifetime of 6-10 minutes at room temperature (Fig. 32). Since the rate of fork movement at this temperature is 300-600 nucleotides/min (5-10% of 3000 nucleotides/min or 1 μm/min, the rate at 37°C), a lifetime of 6-10 minutes would give 18-60 as the maximum number of unjoined (nascent or complete) Okazaki fragments per strand (at each replication fork). This figure seems quite high (Gautschi & Clarkson, 1975, feel that there are 2-3 fragments per strand). Also, the saturation point of radioactivity in the Okazaki

piece cannot be simply calculated from sedimentation profiles, since trailing of material from the increasingly large intermediate peak probably causes the Okazaki peak to appear as if it is still growing larger after it is actually no longer doing so.

However, by making a few assumptions we can say something about the kinetics of synthesis and ligation. After a 15 second pulse at room temperature, 25% of the acid precipitable radioactivity is in DNA larger than Okazaki strands (Fig. 33). This means that the time needed to join completed fragments (with filled-in gaps) is less than 15 seconds. In fact, since the RNA primer is probably less than 10% as long as the DNA portion of the fragment (see above, B), if 25% of the radioactivity is in large strands, most of it must have been incorporated into incomplete Okazaki fragments. Therefore, the total time required for removal of the RNA, gap-filling and joining is less than 15 seconds at room temperature. (Of course this estimate of 15 seconds depends on the assumption that the addition of lysis solution instantly stops incorporation.)

D. Nascent DNA passes through an intermediate size class

The results of my pulse labeling and pulse chase experiments prove and develop the idea that nascent DNA passes through an intermediate size class before it becomes full-length or "bulk." This size class is seen as an intermediate peak of pulse labeled DNA on a sucrose gradient, and the position of this peak moves down the gradient in a continuous fashion as the time of pulse labeling and/or chasing increases.

1. Discontinuous movement

Some groups, while reporting this phenomenon, have claimed to be observing discontinuous movement of the peak size from one S value to another. However, in most of the cases where several intermediate peaks are observed in a single gradient,the peaks are composed of only a single fraction (Hyodo et al., 1970; Friedman et al., 1975); this makes their interpretation as a distinct species of DNA highly suspect. Other groups only show one size for each pulse-time, but claim that the transition from one to the next is discontinuous (Berger & Irvin, 1970; Hyodo et al., 1970, 1971; Goldstein & Rutman, 1973; Berger & Huang, 1974). In any case, these groups do not eliminate the possibility that at pulse-times in between those they have chosen the peak occupies other positions in the gradients, thus indicating a continuously increasing average size.

2. Is the intermediate peak an artifact?

Lehmann and Ormerod (1969, 1970; Ormerod & Lehmann, 1974) demonstrate that if long DNA molecules are end-labeled and then randomly sheared, the pulse labeled DNA will appear to produce a peak with a lower average molecular weight than is obtained from fully labeled molecules. Also, when a short pulse label is followed by a chase, the peak size will increase with increasing chase time until it coincides with the peak formed by fully labeled molecules. They prove this by both mathematical analysis and experimental analysis and claim that the

intermediate peak observed by several groups can be totally accounted for by this artifact.

However, it is extremely unlikely that this phenomenon is the explanation for my observation of an intermediate peak. As other groups (Nuzzo et al., 1970; Goldstein & Rutman, 1974) point out, the objections of Lehmann and Ormerod don't apply to situations where two distinct size classes of labeled molecules are present (i.e., both the intermediate and the bulk peaks contain pulse-labeled DNA). Lehmann and Ormerod must also recognize this limitation to their objection, for they too report the existence of an intermediate-sized class of nascent DNA (Lehmann & Ormerod, 1971).

In many of the earlier reports, the bulk DNA was sheared quite severely, either on purpose to avoid sedimentation artifacts or inadvertently during purification (Habener et al., 1969b; Berger & Irvin, 1970; Fujiwara, 1972; Goldstein & Rutman, 1973; Berger & Huang, 1974; Friedman, 1974; Hershey & Taylor, 1974). Such shearing may not only cause the Lehmann and Ormerod phenomenon discussed above, but may also cause the intermediate DNA not to be observed as a separate class at all, if both bulk and intermediate DNA are sufficiently degraded. The fact that many extraction and purification schemes cause extensive aggregation also may tend to conceal the intermediate peak, since most or all of the pulse-labeled DNA may be pulled to the bottom by the bulk DNA.

E. The time course of discontinuous synthesis

The simplest interpretation of my data, and those of others, is that mammalian cells make DNA first in pieces of about 100 nucleotides, these pieces are joined to the growing daughter strands of replication units (replicons) and completed replicons are joined together to form bulk DNA (Fig. 61).

1. Okazaki fragments are precursors to intermediate DNA

Because of the fact that Okazaki fragments are made rapidly in relation to the time required for equilibration of the nucleotide pools (Edenberg & Huberman, 1975) and the fact that there is further incorporation of radioactive label during attempted chases (Cleaver & Holford, 1965; Painter & Schaefer, 1969b; Horwitz, 1971; Huberman, 1974; Tseng & Goulian, 1975a; my results - Table I), it is hard to prove that the Okazaki pieces are all precursors to intermediate-sized DNA. However, my results imply that this is indeed the case. Both the fact that the Okazaki peak seems to appear first (Figs. 28A, 32A, 33, 34) and the fact that it seems to disappear during a chase (Fig. 30) support this conclusion.

In vitro experiments carried out in our laboratory (Fraser, unpublished) also seem to confirm this sequence of events.

2. The intermediate peak is composed of growing replicons

All the observations I have made regarding the intermediate-sized strands are consistent with the hypothesis diagrammed in

Figure 61. An explanation of "intermediate strands." This is a diagrammatic representation of a bidirectional replication bubble. The heavy lines are newly synthesized DNA, and the light ones are template DNA. One or several Okazaki fragments are synthesized at each replication fork and subsequently joined to intermediate strands. The intermediate strands grow only by addition of Okazaki fragments at each end, not by incorporation of mononucleotides. The diagram as drawn represents totally discontinuous synthesis; semi-discontinuous synthesis would have Okazaki fragment(s) on only one side of each replication fork.

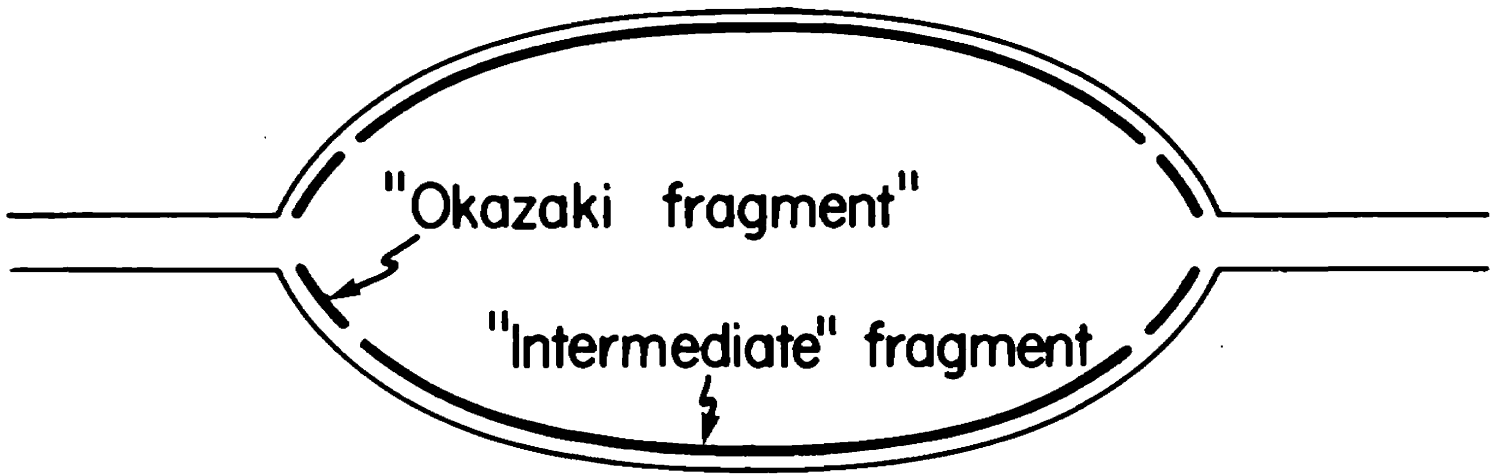
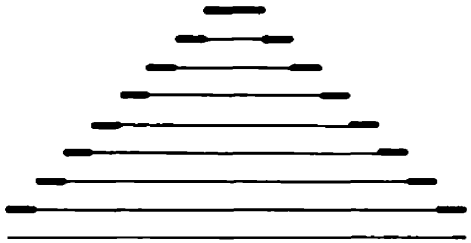

"Okazaki fragment"
"Intermediate" fragment

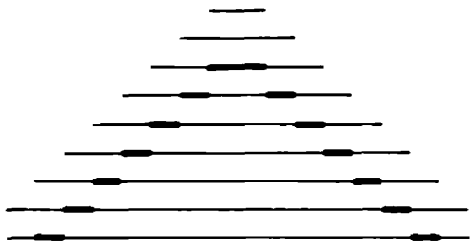
Figure 61 and mentioned above: that the intermediate strands are the growing replicons. The size of the intermediate strands is consistent with the size distributions reported for mammalian replicons (Edenberg & Huberman, 1975). The fact that it takes almost one hour (at 37°C) for the radioactive label in intermediate strands to move into bulk DNA (Fig. 30F), a fact confirmed by several other investigators (Berger & Irvin, 1970; Sato et al., 1970; Cheevers et al., 1972; Berger & Huang, 1974; Tseng & Goulian, 1975a), reinforces this idea, since this time span corresponds to the time needed to make complete replicons (Edenberg & Huberman, 1975). (However, the intermediate peak could also consist of a mixture of full-sized and broken replicons.)

The increase in average size of labeled intermediate strands with increasing chase times (Fig. 30), and the observation that the peak seems to move further down the gradient after a pulse-chase than after an equivalent pulse (Fig. 31), are also consistent with the idea that these strands are growing replicons. Figure 62 elucidates this point. As chase time is increased, the smaller strands become increasingly pieces that were made after the pulse was stopped, while the label moves into strands of longer and longer length, thus increasing the average length of labeled strands. Also, since a 15 minute pulse will produce labeled strands of all sizes while a one minute pulse-14 minute chase will put label only into relatively long strands, the peak of the former will not appear to sediment as far as the peak of the latter.

Figure 62. An explanation for the apparent increase in size of intermediate strands. Each group of lines represents the set of growing and completed replicons present with the cell at one time. The light lines indicate nonradioactive DNA and the heavy lines represent DNA made radioactive during a pulse. Immediately after a pulse all strands except some completed ones will contain a stretch of labeled DNA at each end. After a short chase the smallest strands will be completely unlabeled, and more of the completed strands will be labeled. After a longer chase, the average size of unlabeled chains will have increased, and the label will be present only in the larger growing strands and completed strands.



Pulse



Short chase

Long chase

One group finds that the intermediate peak never becomes larger than 50S and claims that the material near 50S in size is very quickly converted into bulk DNA and therefore doesn't pass through "well-defined stages of intermediate sedimentation" (Friedman et al., 1975). I did not find that the peak pauses at a size definitely smaller than bulk DNA. Rather, I found that it moves all the way down the gradient until the pattern of pulse-label coincides with that of bulk label.

III. Effect of inhibitors

A. Inhibitors of protein synthesis

The sedimentation patterns of DNA synthesized by cells inhibited in protein synthesis give us useful information about the effect of an inhibition of protein synthesis on DNA synthesis, although not enough to characterize the changed course of events in all its detail. On a gross level, we notice that the amount of incorporation of (^{3}H) thymidine into DNA is greatly reduced, but the sedimentation patterns are not greatly changed. All of the protein-synthesis inhibitors studied seem to produce the same patterns. This implies that the effect of inhibition does not depend on the inhibitor used, contrary to some reports in the literature (Hand & Tamm, 1973; Gautschi, 1974).

1. Reduction in the rate of replicon initiation

This observation might be explained in one of three ways. The sedimentation results are consistent with a reduction in initiation of new replicons without any change in the rate of fork movement. Since there is a preincubation time of two hours--one and a half of which

are at 37°C--all the replicons that had been started before the addition of inhibitor would have been completed. Therefore, a reduction in initiation would mean that only a fraction of the usual number of replicons were incorporating thymidine at any one time, but that that fraction was proceeding according to the usual scheme. Thus the sedimentation patterns would remain the same while incorporated radioactivity was reduced.

Although the observed sedimentation patterns are in accord with this explanation and it has been proposed by some other groups (Ensminger & Tamm, 1970; Fujiwara, 1972; Hori & Lark, 1973), most literature reports indicate that the rate of elongation is reduced when protein synthesis is inhibited (Weintraub, 1972; Weintraub & Holtzer, 1972b; Gautschi & Kern, 1973; Gautschi et al., 1973; Hand & Tamm, 1973; Gautschi, 1974). This has also been clearly demonstrated in recent autoradiographic studies by Eva Aufreiter (unpublished).

2. Reduction in the rate of chain elongation

a. Initiation of replicons must also be reduced

Assuming that the rate of elongation of DNA chains is reduced by inhibitors of protein synthesis, two different schemes can be proposed to explain my data, within certain limitations. There must be some reduction in the frequency of initiation of replicons as well, because if only the rate of fork movement were reduced there would be a gradual

build-up of small pieces of replicating DNA. This would occur because replicons would be initiating at the usual rate but growing longer at a much reduced rate, so a larger and larger percentage of replicons would be in a "just started" state as the time after addition of inhibitor increased. (Magnusson et al., 1974, describe a similar effect.) If this happended there would be a shift in the sedimentation pattern, with more labeled DNA appearing near the top of the gradient. Since this is not the case, the rate of replicon initiation must also be reduced. In fact, a similar scheme has been proposed by others (Gautschi & Kern, 1973; Hand, 1975).

b. All elongation steps are affected

The elongation of a strand of DNA really involves several processes. Besides nucleotide addition there is the initiation of each Okazaki fragment and the joining of completed fragments, which in itself involves removal of the RNA primer, filling the gap left by this removal and ligation of the completed piece. When all of these steps are slowed down equally, or when the rate limiting step is slowed down, the result should be similar to that which I (Figs. 32, 34) and others (Gautschi & Clarkson, 1975) have observed when the temperature is reduced from 37°C to room temperature. Under such circumstances I found that the transfer of radioactivity from the Okazaki peak to the intermediate peak was retarded, causing the sedimentation pattern observed after pulse labeling for two minutes at 37°C to be similar to that observed after pulse labeling for 15 minutes at room temperature. In essence,

this means that if the time scale is increased by a factor equal to the reduction in rate of elongation, the two sets of sedimentation profiles will be similar.

The above scheme is in fact proposed by Gautschi & Kern (1973) as a description of what happens when cycloheximide is used, and is consistent with their data. However, my results with three different inhibitors of protein synthesis indicate that this is not what is happening. Rather than requiring a change in the time scale to make the sedimentation profiles of DNA from inhibited cells correspond to those of DNA from control cells, a change in the Y-axis (that indicates the amount of radioactive label incorporated) is all that is needed. The conflict between my results and those of Gautschi and Kern may arise because of differences in the levels of inhibition. They preincubate for only 20-40 minutes and find that DNA synthesis is only reduced by 80%. I preincubate for 2 hours and find that synthesis is reduced by more than 90% (Table II). It is difficult to analyze their data in great detail because they never observe an intermediate peak, only a skewed smear of radioactivity in the intermediate region.

c. Only the initiation of Okazaki fragments is affected

My data indicate that the shapes of the sedimentation profiles of DNA from inhibited or uninhibited cells correspond fairly well for any short pulse. This would occur if the rate of initiation of Okazaki fragments were slowed down while nucleotide addition and ligation of

completed fragments proceeded at the normal rate. This combination of events would result in there being a smaller amount of labeled Okazaki fragments at any time, thus reducing the amount of radioactivity in the Okazaki peak. It would also cut down the number of Okazaki fragments moving into the intermediate peak, reducing the amount of radioactivity in that peak as well.

In fact, this scheme would explain one of the minor changes observed in the sedimentation patterns when the cells are inhibited. The valley between the Okazaki and intermediate peaks seems to be more pronounced under inhibitory conditions (Figs. 38, 39, 40) than normal ones (Fig. 32). This change also occurs in the sedimentation patterns used to measure the size of the Okazaki peaks (Figs. 43, 37). I have already noted that when Okazaki fragments are completed they might either be joined to a growing replicon or to other completed Okazaki pieces, thereby forming small polymers of Okazaki fragments. Assuming that both of these events take place, it is probable that the more completed but unjoined fragments there are at a single growing point, the more likely it is that they will join randomly together rather than sequentially to the growing strand. If we assume that the DNA in the valley between the two peaks is at least in part labeled polymers of Okazaki fragments, then it is understandable that we see less DNA in this size range when the cells are treated with inhibitors of protein synthesis. As I have indicated, if

only the rate of initiation of Okazaki fragments is slowed down, there will probably be a smaller than normal pool of Okazaki fragments at each growing point. The fragments would therefore be more likely to be joined sequentially, and fewer than normal polymers of Okazaki fragments would be formed. Thus there would be less labeled DNA in the area of the sucrose gradient corresponding to these polymers.

If the effect of inhibitors of protein synthesis is indeed to slow down the initiation of Okazaki fragments, they may be acting through an effect on the RNA polymerase needed to provide the presumptive RNA primer of Okazaki fragments.

d. Both the initiation and elongation of fragments are affected

It is also possible that inhibiting protein synthesis slows down the rate of both the initiation and elongation of Okazaki fragments while ligation is not affected. If that were the case, the number of nascent radioactive Okazaki fragments would remain the same while the pool of completed but unjoined ones would be greatly reduced. This would reduce the amount of radioactive DNA appearing in the Okazaki peak, and would also reduce the rate of conversion of Okazaki fragments to intermediate strands. Since the label would be entering the intermediate peak at a reduced rate, the intermediate peak would also have less radioactivity in it and the observed pattern would be obtained.

This sequence of events would explain another slight variation in the sedimentation pattern that is observed when protein synthesis is

inhibited. When the Okazaki peak is examined carefully (Fig. 43), the sedimentation profiles of the peaks created by short pulse labelings show a trailing edge of acid-precipitable counts. This would tend to indicate a larger proportion of incomplete to complete Okazaki fragments than is normally observed, and is exactly the condition predicted by this scheme.

e. Intermediate strands take longer to become bulk DNA

Many groups claim that under conditions of inhibition of protein synthesis there is an increase in the time required for intermediate DNA to become bulk sized (Hyodo et al., 1971; Gautschi & Kern, 1973; Gautschi, 1974; Seale & Simpson, 1975). My data do not deal directly with this question, but if there is such a slowdown it would explain another slight variation in the sedimentation profiles I obtained. All the 15 minute pulse-labelings (Figs. 38, 39, 40) seem to show an intermediate peak that does not decrease toward the bottom of the gradient as sharply as it does when no inhibitor is present (Fig. 32). This may indicate that there are more than the usual number of large replicons, which might be a result of completed replicons not being immediately joined into bulk DNA (see above for similar phenomena with polymers of Okazaki fragments). Further experiments would be needed to definitely establish this point.

3. Relation of my data to the work of other groups

I have examined the effect of inhibitors of protein synthesis on

DNA synthesis at a different level than have most other investigators. All the groups mentioned in this section (IIIA.Inhibitors of protein synthesis) looked at the rate of fork movement or the rate of movement of intermediate strands into bulk DNA. Only Gautschi & Kern (1973) considered the change from Okazaki strands to intermediate DNA. I have shown that focusing on this shift provides much useful information that has not been obtained by other methods. Because of this change in focus my data do not mainly serve to contradict or reinforce that of any other specific group, but rather to raise their results to a greater degree of precision.

My data do contradict the observations and conclusions of Fujiwara (1972). He claims that treatment with cycloheximide stops the initiation of replicons and eliminates the Okazaki peak. However neither his data nor his arguments are very convincing. His "Okazaki peak" appeared in the top fraction of the sucrose gradient and he used the total lysate method. Therefore, conclusions drawn about Okazaki fragments cannot be substantiated by the data. Also, he claims that elongation proceeds at a normal rate, but only demonstrated that it does not stop. Finally, he added FUdR at the same time as he did cycloheximide. As I have demonstrated (Figs. 57-60), incubation with FUdR affects DNA synthesis even when pulse labeling is done with thymidine. Therefore, no conclusions can be drawn about the effect of cycloheximide on DNA synthesis.

B. Inhibitors of DNA synthesis

1. Hydroxyurea

When cells were pulse labeled in the presence of inhibitors of DNA synthesis, several large scale changes in the sedimentation patterns were produced. When hydroxyurea was used as the inhibitor, I observed a proportional build-up of the Okazaki peak (Figs. 44, 46, 47). There seemed to be a constant percentage of acid precipitable radioactivity in the Okazaki peak (slightly above 50% at 100 μg/ml of hydroxyurea - Fig. 44).

a. All steps involved in DNA synthesis are affected equally

The simplest explanation of the data is that all steps involved in synthesis are reduced about 50-fold (to account for the new rate of incorporation being about 2% of the control rate), so that even after an hour long pulse labeling in the presence of hydroxyurea the sedimentation pattern observed is only equivalent to a one minute pulse under normal conditions (Fig. 32A). This would explain the observed results on a gross level, by implying that no change in the pattern was observed because it was examined over too narrow a time span and that an intermediate peak did not appear because too short a pulse label was used.

However, several aspects of the observed patterns tend to indicate that this explanation is not adequate. First of all, if the 50-fold reduction in incorporation simply means a 50-fold expansion of the time scale (as postulated for the change from 37°C to room temperature) then

a five minute pulse labeling in hydroxyurea would be equivalent to a six second pulse under normal conditions. But after such a short pulse label, much more than 56% of the acid precipitable radioactivity should have been in the Okazaki peak (Fig. 32 showed that even after a 15 second pulse, 75% of the radioactivity remained in the Okazaki region). Also, no matter how much the time scale is expanded, a 30 minute pulse is six times as long as a five minute one, so there should be some change in the sedimentation pattern. Finally, at higher (500 μg/ml) concentrations of hydroxyurea, the percentage of radioactivity in the Okazaki peak increased tremendously without a concurrent reduction in rate of incorporation (Fig. 47), a fact not accounted for by this explanation.

b. Initiation of fragments is affected

i. Rate of joining reduced more than that of initiation

The fact that there is a constant percentage of acid precipitable radioactivity in the Okazaki peak implies a change in the relation of the rate of initiation of Okazaki fragments to their rate of joining. Under normal steady-state conditions completed Okazaki fragments are being removed from the pool (by joining) as fast as they are introduced (by initiation). Therefore the amount of radioactive label in large DNA increases while the amount in Okazaki pieces remains constant. If hydroxyurea is added the situation changes, and joining is slowed in relation to initiation.

Observing 50% of the acid precipitable radioactivity in the Okazaki peak implies that initiation had been occurring twice as fast as joining, while finding 80% in the Okazaki peak means initiation had been occurring four times as fast as joining. This follows from the fact that maintaining equal amounts of radioactivity in both peaks demands that for every two radioactive molecules incorporated into Okazaki fragments, one molecule leaves the Okazaki fragment pool and enters the pool of intermediate DNA (by joining). This results in each pool being increased by one molecule. If 80% of the radioactivity is to remain in the Okazaki region, then one molecule must be removed from the pool of Okazaki fragments for every five that enter it.

ii. Which part of the joining step is affected?

A decrease in the rate of joining relative to the rate of initiation could be caused by a slowdown in the rate of removal of the RNA primer, the filling in of the gap, or the actual joining of completed fragments. Several groups (Laipis & Levine, 1973; Magnusson, 1973) suggest that it is the gap-filling step that is affected by hydroxyurea, and the finding that cells inhibited by hydroxyurea accumulate pieces having less than the usual amount of RNA and being a little shorter than usual (Magnusson et al., 1973) would tend to support this view. These groups suggest that gap-filling is done by a different polymerase than the one used to synthesize Okazaki fragments, and that this second polymerase is more sensitive to hydroxyurea.

iii. This imbalance increases the pool of Okazaki pieces

This proposed imbalance of initiation and ligation of Okazaki fragments would lead to longer and longer stretches of replicated DNA that are composed completely of unjoined Okazaki fragments. If replicon initiation were also reduced enough so that the number of replicating replicons began to decline, this pool of unjoined Okazaki fragments would gradually decline as active replicons reached their full length and stopped incorporating radioactive label. In fact, if the very slight decline with increasing pulse-time in percentage of radioactivity in the Okazaki peak is real (Figs. 44, 47), this decline might reflect a change in the ratio of initiation to elongation as the completion of replicons stopped the initiation of new fragments while the joining of previously synthesized fragments continued.

iv. An increase in polymers

Since many times the usual number of completed but unjoined Okazaki fragments are present, the joining of these fragments might be much more random than is normal (see discussion above-III. A. 2. c- of Okazaki fragment polymers). This might cause such a large number of polymers of Okazaki fragments, and polymers containing so many Okazaki fragments, that there would be no valley before a peak of intermediate length DNA. These polymers might sediment to a point in the sucrose gradients between the Okazaki peak and the peak of intermediate DNA, so that at the top half of the gradients the intermediate

peak is turned into a plateau. The effect might also account for the fact that the Okazaki peak, while starting out at the same size as under uninhibited conditions, seems to get larger with longer pulse times.

This effect might also be accentuated by a build-up of nascent replicons if elongation is reduced to a greater extent than initiation (see Discussion, III. A. 2. a and III. B. 3). The flattening out of the larger half of the intermediate peak can be interpreted in a similar fashion--there might be a delay in ligation of completed replicons so that more large replicons accumulate, thus filling in the area between the peaks of intermediate and bulk DNA. Alternatively, if initiation of replicons were inhibited to a greater extent than elongation, larger replicons would also accumulate.

v. What causes the reduction in incorporation?

Of course, the above discussion only rationalizes the shape of the sedimentation pattern. A relative reduction in speed of joining can explain the shift in the pattern, but not the tremendous decrease in the rate of incorporation of radioactive thymidine. There must also be a tremendous decrease either in the number of active replicons, or, more probably, in the total rate of elongation combined with some decrease in initiation. Others have noted a decrease in the rate of fork movement (Hand & Tamm, 1973). This reduced rate of elongation must be accompanied by an even greater reduction in the rate of joining. Since it seems that the inhibitory effect of hydroxyurea is

due to a reduction in some or all of the deoxynucleotide triphosphate pools (Neuhard, 1967; Adams et al., 1971; Skoog & Nordenskjold, 1971; Walters et al., 1973; Skoog & Bjursell, 1974), it is quite plausible to say that the polymerase that makes Okazaki fragments is affected by this reduction, but the putative gap-filling polymerase is affected to a greater extent.

c. Other effects of inhibition

Several other points relating to inhibition with hydroxyurea should be briefly noted. Chases do not work at all (Figs. 45A, B), because the pool of labeled thymidine is very large in proportion to the rate at which the hydroxyurea allows it to be used in DNA synthesis. When the hydroxyurea is removed (Fig. 45C) this relatively large pool causes a tremendous increase in the amount of radioactive label incorporated.

Parenthetically, this chase experiment (Fig. 45C) shows that inhibition by hydroxyurea is reversible, a fact noted by several other groups (Skoog & Nordenskjold, 1971; Amaldi et al., 1972; Wanka & Moors, 1972; Hershey et al., 1973; Magnusson, 1973; Walters et al., 1973; Manteuil & Girard, 1974; Vlak et al., 1975). Finally, at the concentration of hydroxyurea that I used (1.32 mM), the inhibitor does not seem to cause any breakdown of bulk DNA. Others have noted the same results at concentrations of 10 mM (Magnusson, 1973; Magnusson et al., 1973; Vlak et al., 1975), and the one group that reported a breakdown of DNA in solution (Jacobs & Rosenkranz, 1970) used a much higher concentration (0.2 M).

In conclusion, one further point must be emphasized. The fact that, at high concentrations of hydroxyurea, much more than 50% of the pulse label is found in Okazaki fragments (Fig. 47) seems to argue for discontinuous DNA synthesis on both sides of the fork. One group working with Ara C attempted to explain the proportional increase in radioactivity found in the Okazaki peak under inhibiting conditions by suggesting that the polymerase for the continuous side is inhibited more severely than the discontinuous polymerase (Hunter & Francke, 1975). However, this is probably not the case with inhibition by hydroxyurea, since it would create progressively longer stretches of unwound helix with one strand replicated and one strand not replicated. (Hunter & Francke add another hypothesis but even so this problem remains.)

2. Ara C

a. Intermediate peak eventually appears

Some groups have argued that the inhibition caused by Ara C is similar to that caused by hydroxyurea (Laipis & Levine, 1973; Hunter & Francke, 1975). My results certainly do not support this assumption. Rather, they indicate the same overall rate reduction that is noticed when the temperature is reduced, except, of course, that the rate is reduced by a much larger factor. While this explanation does not account for the skewing of the intermediate peak observed at the higher concentration of Ara C (Fig. 51), I am not sure that this skewing is a

significant variation. If it is,it may be due to an increase in the proportion of long replicons caused by a relative inhibition of replicon initiation (see above, III. B. 1. b. iv).

b. Okazaki peak gets bigger

The other aspect of Ara C inhibition that is qualitatively different than that caused by temperature reduction is the fact that the DNA contained in the Okazaki peak seems to increase in average size with increasing length of pulse labeling. This probably reflects an increase in the proportion of small polymers of Okazaki fragments due to an increase in random joining. This increase might be explained by assuming that there is a slight additional reduction in the rate of ligation. Although some groups postulate that, as in the case of hydroxyurea, this reduction is due to a reduced rate of gap filling (Gallo et al., 1973; Hunter & Francke, 1975), the fact that inhibition by Ara C prevents the RNA from being chased off Okazaki fragments (Tseng & Goulian, 1975b) tends to indicate that it is the removal of the RNA primer that becomes rate limiting.

Hunter & Francke (1975) find that Okazaki fragments synthesized during inhibition by Ara C are smaller than normal, and propose a very unusual scheme to explain their results. However, their experiments were done in vitro, so their observations may not be comparable to mine.

3. FUdR

a. Changes observed at different concentrations

My results with FUdR indicate a more complex series of effects than those caused by the other inhibitors. Since an intermediate level of the inhibitor causes a great reduction in uptake without changing the sedimentation pattern (Fig. 54), the initial effect of FUdR inhibition on DNA synthesis appears to be similar to that of the inhibition of protein synthesis. Probably initiation of Okazaki fragments is reduced. Certainly the overall rate of fork movement is reduced, as noted by Amaldi et al. (1972). At a higher concentration of inhibitor,inhibition of the joining of Okazaki fragments becomes the dominant factor. This latter effect has been postulated by others (Magnusson, 1973; Salzman & Thoren, 1973).

b. Elongation is inhibited more than initiation

Other aspects of the inhibition by FUdR can be explained by assuming that elongation is reduced to a much greater extent than is initiation of replicons,as suggested by Amaldi et al. (1972). Since this would result in replicons starting at the usual (or relatively close to the usual) rate, but being extended very slowly, there would be a build-up of very small active replicons. If so, when thymidine is added and replication returns to its normal rate, most of the replicons incorporating label would be very small and would first appear in the general area of the Okazaki peak. This large class of similarly-sized

nascent replicons would gradually grow to full length, and my results indicate that sometime between 15 minutes and one hour (at 37°C) this skewed distribution would be replaced by the normal range of active replicons.

c. The Okazaki peak grows bigger rapidly

This hypothesis explains why the Okazaki peak seems to grow so large so rapidly (Fig. 55). The peak created by this build-up of very small fragments of replicons gradually becomes predominant over the true Okazaki peak. Amaldi et al. (1972) first noted the increase in active replicons but did not mention the change this result would cause in the size distribution of active replicons. Magnusson et al. (1974) suggested a similar effect for inhibition with hydroxyurea, but did not examine the effect of FUdR. (Some of my hydroxyurea data might also be rationalized by this hypothesis.)

Another possible explanation for the unusual results obtained with thymidine labeling after FUdR inhibition is that, after such blockage a pulse of thymidine causes primarily a new round of synthesis, with no radioactivity being incorporated into previously started strands of DNA. Such a scheme has been suggested for SV40 synthesis (Salzman & Thoren, 1973) and although it seems quite unlikely that this happens in the case of mammalian DNA synthesis, the scheme is consistent with my data.

d. Thymidine does not immediately stop the FUdR inhibition

In any case, my data indicates that even 15 minutes (at 37°C) after the addition of thymidine, the pattern of DNA synthesis is quite unusual (Fig. 60B). This is a much more drastic effect than those noted by other groups, and may have wide implications. Many groups (including Huberman & Riggs, 1966; Schandl & Taylor, 1969; Hyodo et al., 1970; Fujiwara, 1972; Hand & Tamm, 1973; and Hori & Lark, 1973) use FUdR to synchronize cells or increase the cells' uptake of thymidine, and assume that they are observing normal DNA synthesis. My data prove that what they see is certainly not completely normal synthesis, and the effect of preincubation with FUdR may, in some cases, be sufficient to render their observations meaningless (Fujiwara, 1972).

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