Synthesis of Deoxyribonucleic Acid in BHK-21/C13 Cells

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Newly synthesized ^3H -labelled DNA was extracted from baby hamster kidney cells (BHK-21/C13 cells) and was shown to possess single-stranded properties when examined by column chromatography on benzoylated naphthoylated DEAE-cellulose, hydroxyapatite and methylated albumin on kieselguhr, and by its affinity for nitrocellulose filters. Some of the newly synthesized DNA was shown to be of lower molecular weight than the bulk of the DNA when examined by alkaline sucrose-density-gradient centrifugation. The properties observed were not affected by treatment of the DNA with ribonuclease, Pronase or amylase. The effect of the size of the DNA on its observed properties was examined and is discussed. It is concluded that DNA synthesis in BHK-21/C13 cells proceeds according to the discontinuous-mechanism model in at least one of the strands.

It has been suggested (Okazaki *et al.*, 1968) that synthesis of DNA in *Escherichia coli* occurs in a discontinuous manner, that is, short fragments are synthesized first and are subsequently joined, presumably by the action of a polynucleotide ligase, to yield long stretches of DNA. Other work indicated that this idea was substantially correct for the synthesis of DNA in both bacterial and mammalian systems (Yudelevich *et al.*, 1968; Kidwell & Mueller, 1969; Schandl & Taylor, 1969; Sato *et al.*, 1970).

Much of the earlier work was based on the separation of newly synthesized (nascent) DNA from the bulk DNA by centrifugation on alkaline sucrose density gradients. This separation was generally assumed to be attributable to the fact that nascent DNA was of lower molecular weight than the bulk DNA (Habener *et al.*, 1969; Berger & Irvin, 1970). However, Lehmann & Ormerod (1970) have criticized the interpretation of some workers (Berger & Irvin, 1970) who obtained large discontinuous pieces of nascent DNA, about 30S. They have shown that this is an artifact of the different isotopic labelling pattern of pulse-labelled DNA. This criticism does not apply, however, to smaller pieces of nascent DNA (about 10S), which are obtained by alkaline sucrose-density-gradient centrifugation. These are the true 'Okazaki fragments' postulated by the model of discontinuous synthesis.

Many of the techniques used to study nascent DNA synthesis utilize the fact that some materials, such as

hydroxyapatite and nitrocellulose, exhibit differences in their affinity for native and denatured DNA. Studies on DNA replication in *Bacillus subtilis* have revealed the presence of intermediates that display properties consistent with their being single-stranded (Oishi, 1968*a,b,c*), and similar observations have been demonstrated in mammalian systems (Painter & Schaefer, 1969; Sato *et al.*, 1970).

Techniques that can be used to study the secondary structure of newly synthesized DNA are continually being developed, and this paper presents results obtained for baby hamster kidney cells (BHK-21/C13 cells) by using many of the techniques currently available. We have established that DNA synthesis in this mammalian cell line proceeds according to the discontinuous-mechanism model originally postulated for *E. coli* by Okazaki *et al.* (1968).

Experimental

Chemicals

Chemicals used were A. R. grade or the most pure available. [^3H]Thymidine (26Ci/mmol) and [^{14}C]thymidine (66mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Tissue culture. Baby hamster kidney cells (BHK-21/C13 cells; Macpherson & Stoker, 1962) were used throughout this work and were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, U.K., and occasionally from Bio-Cult Laboratories Ltd., Glasgow,

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U.K. Cells were grown in monolayer culture at 37°C in the Glasgow modification of Eagle's medium containing 10% (v/v) tryptose-phosphate broth (Difco Laboratories, East Molesey, Surrey, U.K.) and 10% (v/v) calf serum (ETC₁₀ medium). The calf serum was purchased from Flow Laboratories Ltd.

Labelling of DNA. Cells were grown in ETC₁₀ medium in the presence of [¹⁴C]thymidine to label the bulk DNA with ¹⁴C. When growth was on a burrler scale (80-oz glass bottles; House & Wildy, 1965), each burrler was seeded with 5×10^7 cells, which were then grown for 3 days in the presence of 1.6 nCi of [¹⁴C]thymidine/ml. For growth on glass Petri dishes seeding was at 3×10^6 cells/Petri dish and growth was for 24 h in the presence of 0.033 μ Ci of [¹⁴C]thymidine/ml. Cells were subsequently pulsed for 0.5, 1.0 or 3.0 min by exchanging the medium for minimal Eagle's medium containing [³H]thymidine at 20 μ Ci/ml.

In pulse-'chase' experiments the ³H-labelled medium was removed, the cells were washed twice in ETC₁₀ medium containing 10 μ M-thymidine and were then incubated at 37°C in media similar to the washing medium for appropriate times before extraction of the DNA from the cells.

After the pulse had been given, incorporation of radioisotope was stopped by the addition of an ice-cold aqueous solution containing SSC medium (0.15 M-NaCl-0.015 M-sodium citrate) and KCN (1 mM).

Extraction of DNA. Cells were lysed *in situ* by incubation with SSC medium containing 2% (w/v) sodium dodecyl sulphate at 37°C for 2 h. The lysate was deproteinized by shaking it gently with an equal volume of phenol saturated with SSC medium and containing 8-hydroxyquinoline at a concentration of 0.1% (w/w). The liquid phases were separated by centrifugation for 10 min at 4°C and 10000g. The supernatant fluid was removed, re-extracted with phenol, and the DNA was precipitated by addition of 2.5 vol. of ethanol or 1.0 vol. of ethoxyethanol. It was spooled on to a glass rod (Marmur, 1961), washed in three separate 10 ml portions of ethanol-water (9:1, v/v), to remove free thymidine, and redissolved in 1/10 SSC medium to a concentration of 100 μ g/ml. Samples were stored at 4°C over chloroform and were always used within 2 weeks of preparation.

Liquid-scintillation spectrometry. For counting radioactivity of samples of DNA that had been precipitated on glass-fibre filter papers of diameter 2.5 cm (Whatman GF/C), the scintillation fluid contained 4.0 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1.0 litre of toluene, and for aqueous samples it contained 4.0 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1.0 litre of toluene-Triton X-100 (2:1, v/v).

All samples were counted for radioactivity in an Intertechnique liquid-scintillation spectrometer (model SL30) by using appropriate channel settings for ³H and ¹⁴C. Results were corrected for quenching and overlap.

Density-gradient centrifugation. (a) Sucrose. Sucrose gradients [5 ml; 5–20% (w/w)] were prepared in either 0.9 M-NaCl containing EDTA (1 mM) or in 0.1 M-NaOH-0.9 M-NaCl containing EDTA (1 mM) (Okazaki *et al.*, 1968). Approx. 1 μ g of DNA was sedimented by centrifugation in a 3×5 ml swing-out rotor for 2 h at 4°C and 130000g (r_{av} , 7.265 cm) in an MSE Superspeed 65 ultracentrifuge. Fractions were collected by piercing the base of the tube and dripping the fluid on to glass-fibre filters (Whatman GF/C). Filters were rinsed twice in 5% (w/v) trichloroacetic acid, once in ethanol, and once in diethyl ether. They were then dried before radioactivity counting. *s* values were calculated by using the formula of Abelson & Thomas (1966) and the accuracy was checked by using the DNA of bacteriophage λ as a reference.

(b) Caesium chloride. Solutions prepared for centrifugation were composed of DNA (20 μ g/ml), 0.5 M-potassium phosphate buffer, pH 11.0, 1 mM-EDTA, sodium dodecyl sulphate (25 μ g/ml) and CsCl to give a density of 1.696 g/ml. Samples (5 ml) were centrifuged isopycnicly for 66 h at 2°C and 70000g (r_{av} , 5.9 cm) in a type 40 rotor on a Spinco L-2 ultracentrifuge.

Column chromatography. (a) Benzoylated naphthoylated DEAE-cellulose (BND-cellulose). BND-cellulose was prepared by the method of Gillam *et al.* (1967). Preparation of columns for chromatography was based on the procedure of Iyer & Rupp (1971). A slurry of BND-cellulose was made in 0.3 M-NaCl-Tris-EDTA (Tris-EDTA: 1 mM-Tris-HCl buffer, pH 8.0, and 1 mM-EDTA) and columns (1.3 cm $\times$ 4 cm) were packed. DNA (100 μ g) in 5 ml of 0.3 M-NaCl-Tris-EDTA was adsorbed by slow passage through the column. The column was then washed consecutively with 10 ml of 0.3 M-NaCl-Tris-EDTA, 10 ml of 1 M-NaCl-Tris-EDTA, 20 ml of a 0–2% (w/v) gradient of caffeine in 1 M-NaCl-Tris-EDTA and finally with 10 ml of 6 M-guanidine thiocyanate, which was used to remove denatured DNA (Ellem & Rhode, 1969). The flow rate was 0.5 ml/min. Fractions (1 ml) were collected and assayed for radioactivity by using the toluene-Triton X-100 scintillation fluid.

(b) Methylated albumin on kieselguhr. Methylated albumin on kieselguhr (MAK) was prepared as described by Mandell & Hershey (1960) and the methodology was based on that of Harrison (1968). A column (10 cm $\times$ 1 cm) was packed with a slurry of MAK in 0.1 M-NaCl buffered with 0.5 M-potassium phosphate (pH 6.7). DNA (100 μ g) was loaded on to the column in a similar solution. A gradient of buf-

ferred saline was obtained by running 1.6M-NaCl–0.5M-potassium phosphate into a 100ml reservoir of 0.1M-NaCl–0.5M-potassium phosphate and 80 1 ml fractions were collected. The gradient was followed by 5ml of 0.75M-guanidine thiocyanate and then 5ml of 1% (v/v) NH_3 in 1.6M-NaCl–0.5M-potassium phosphate, 1 ml fractions being collected. The flow rate was 6ml/h and recoveries of radioactivity were usually about 90%.

(c) Hydroxyapatite. Hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was loaded into a column (1.3 cm $\times$ 4 cm) in a slurry in 0.01M-potassium phosphate buffer, pH 6.75. The column was equilibrated with 0.05M-potassium phosphate buffer, pH 6.75. The DNA (100 μg) was loaded in 5 ml of the same buffer and then washed with 5 ml of the buffer. The DNA was eluted with a 20 ml gradient of 0.1M–0.4M-potassium phosphate buffer, pH 6.75, at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected and were counted for radioactivity directly as described above. Recovery of radioactivity was usually about 85–90%.

(d) Affinity of DNA for nitrocellulose filters. Solutions containing DNA were diluted to give a concentration of 1 $\mu\text{g}/\text{ml}$ in 0.9M-NaCl–0.09M-trisodium citrate. Triplicate 5 ml portions were filtered at 1 ml/min through pre-washed nitrocellulose filters (Sartorius SM11306; 25 mm), which were then washed with 3 $\times$ 10 ml of 0.9M-NaCl–0.09M-trisodium citrate at a flow rate of 2 ml/min. (We later found that the flow rate was not critical.) The filters were dried and counted for radioactivity in the toluene-based scintillation fluid.

Sonication. Solutions containing DNA were sonicated for the required times by using a Dawe Soni-probe (Model 1130A) operating on setting number 4.

Results

Sucrose-density-gradient centrifugation

Following the work of Sakabe & Okazaki (1966) the size of nascent DNA from BHK cells was examined on both alkaline and neutral sucrose gradients.

Cells were grown in monolayer culture in media containing [^{14}C]thymidine and were subsequently pulsed for 0.5, 1.0 or 3.0 min with media containing [^3H]thymidine as described above. The DNA was extracted with phenol and its sedimentation properties on alkaline sucrose-density-gradient centrifugation were determined (Fig. 1). Although the size of the DNA obtained after the extraction procedure varied, there was a clear difference in the size of the ^3H -labelled DNA compared with the ^{14}C -labelled bulk DNA. In all cases there was a small peak of ^3H at about 10S, which represents the so-called 'Okazaki fragments', short stretches of polydeoxyribonucleo-

Fig. 1. Alkaline sucrose-density-gradient centrifugation of pulse-labelled DNA extracted with phenol

Cells, which had been growing in medium containing [^{14}C]thymidine, were pulsed for (a) 0.5, (b) 1.0 or (c) 3.0 min with media containing [^3H]thymidine. DNA was extracted and centrifuged for 2 h at 20°C and 130000g. $\blacktriangle$, ^3H ; $\bullet$, ^{14}C .

tide that, it is presumed, are synthesized during a short pulse with thymidine. The proportion of this 10S material decreased with increasing pulse-time. The larger peak of ^3H -labelled material, the 30S peak, is not an indication of discontinuous replication, as suggested by Berger & Irvin (1970) for a rat liver system, but is probably an artifact (Lehmann & Ormerod, 1970). These results indicated that if 'Okazaki fragments' did occur, then the proportion in the population of ^3H -labelled DNA molecules should increase with decreasing pulse time. The shortest pulse we were able to give was 15 s and a temperature of 20°C was chosen to slow down the rate of DNA synthesis. Alkaline sucrose-density-gradient centrifugation of a cell lysate containing 1 μg

of DNA showed that the 10S peak was now large (Fig. 2), suggesting that this material is a precursor of DNA of higher molecular weight.

Fig. 2. Alkaline sucrose-density-gradient centrifugation of a cell lysate containing pulse-labelled DNA

Pulse-labelled cells were lysed *in situ* with SSC medium+2% (w/v) sodium dodecyl sulphate at 50°C for 30 min. Amounts containing about 1 µg of DNA were centrifuged as described for Fig. 1. ▲, ³H; ●, ¹⁴C.

Pulse-‘chase’ experiments showed that the time required for ‘chasing’ the ³H-labelled DNA into the bulk DNA was between 15 and 30 min (Fig. 3). By using the analysis of Lehmann & Ormerod (1970) an estimation of the approximate growth rate of the molecule is possible. They showed that if DNA, with an isotopically labelled terminus, was randomly sheared the molecular weight of the terminal fragments (labelled) was lower than the average molecular weight of all fragments produced by such shearing. Hence if the bulk DNA is labelled with ¹⁴C and the end with ³H, then the ³H-labelled material will sediment more slowly than that labelled with ¹⁴C. Only when the ³H label has moved, by ‘chasing’, away from the end of the molecule, by a distance greater than that of the average size of all fragments produced (50S), will it co-sediment with the ¹⁴C-labelled material. In this way it is possible to estimate the rate of replication of DNA in the system. The pulse-‘chase’ experiments show (Fig. 3) that co-sedimentation of the ³H- and ¹⁴C-labelled DNA occurred at some time between 15 and 30 min after the pulse. In this time the DNA chains must have grown by at least a further 30 µm, which represents synthesis of a piece of DNA of size 50S, about

Fig. 3. Alkaline sucrose-density-gradient centrifugation of pulse-‘chased’ DNA

Cells were pulsed with [³H]thymidine for 1 min and the label was then ‘chased’ with non-radioactive thymidine for (a) 0, (b) 5, (c) 10, (d) 15 or (e) 30 min. DNA was extracted and centrifuged as described for Fig. 1. ▲, ³H; ●, ¹⁴C.

27.5×10^6 daltons. Hence the rate of chain elongation is of the order of $1-2 \mu\text{m}/\text{min}$, which agrees with published values (Painter & Schaefer, 1969).

Binding of DNA to nitrocellulose

The greater affinity of denatured DNA, compared with native DNA, for nitrocellulose provides a useful technique for investigating the presence of single-stranded regions in the molecule, such as would occur if synthesis was discontinuous. Fig. 4 shows the results obtained after filtration of pulse-labelled DNA through nitrocellulose filters before and after various degrees of shear produced by sonication.

About 65–80% of the ^3H -labelled DNA bound to the filters compared with 18–25% of the bulk ^{14}C -labelled DNA. When the DNA sample was sonicated for up to 120s the degree of binding of both the ^3H - and ^{14}C -labelled material decreased. The decrease in binding was rapid in the first 30s of shear but was subsequently much slower. The degree of binding of the ^{14}C -labelled DNA was decreased almost to zero after 30s sonication, whereas 20–30% of the ^3H -labelled DNA was retained on the filter even after sonication for 120s. Sonication did not affect the binding properties of either heat- or alkali-denatured DNA, but that of native DNA was decreased from approx. 30% to zero.

In any pulse-labelled material some of the ^3H will be incorporated into native DNA, which is covalently linked to, and very close to, the presumed binding area. On shearing, this material will be lost along with much ^{14}C -labelled DNA, and because of the closer proximity of the ^3H -labelled native DNA to the binding site, compared with the bulk of the DNA, the binding of the bulk DNA will decrease more rapidly than that of the ^3H -labelled material. This is illustrated in the pulse-‘chase’ experiments shown in Fig. 5.

It was found that after a 2min ‘chase’ with non-radioactive thymidine extensive sonication (60s) was required to decrease the ^3H binding from 36% to background values, that is 8.1%. This value is low considering that the intracellular [^3H]thymidine pool is only partially depleted by the thymidine ‘chase’ (Cleaver & Holford, 1965). Thus the size of the binding site cannot be larger than the length of DNA synthesized in 2min. This also indicates that the size of DNA after 60s sonication is about the same as that synthesized in 2min, because one shear event must have occurred in the DNA synthesized during a 2min ‘chase’ to release all the ^3H -labelled DNA from the binding regions. With ‘chase’ times longer than 2min, sonication decreases binding much more rapidly. We found that the size of DNA produced after 1 min sonication was about 2×10^6 daltons when examined on neutral-sucrose gradients. Further sonication did not decrease the size. This explains the

Fig. 4. *Effect of sonication on the affinity of pulse-labelled DNA for nitrocellulose filters*

Pulse-labelled DNA ($5 \mu\text{g}$) in 5 ml of 0.09M -NaCl– 0.9M -trisodium citrate, after sonication as described in the text for various times, was passed through nitrocellulose filters which were then washed with 10 ml of the same medium. The filters were dried and assayed for radioactivity. Δ , ^3H ; $\bullet$, ^{14}C . Pulse times: (a) 0.5 min; (b) 1.0 min; (c) 3.0 min.

small differences in binding of the sonication-resistant ^3H -labelled DNA from the cells pulsed for 0.5, 1.0 and 3.0 min, that is 30, 28 and 22%. In the samples pulsed for 0.5 and 1.0 min the major proportion of the label is incorporated into the binding molecule, and the amount of sonication-resistant material

Fig. 5. Effect of sonication on the affinity of pulse-'chased' DNA for nitrocellulose filters

Cells were grown in medium containing [^{14}C]thymidine for 3 days, pulsed for 1 min with [^3H]thymidine, and the label was then 'chased' with ETC₁₀ medium + 10 μM -thymidine for the indicated times: (a) 0 min; (b) 2 min; (c) 5 min; (d) 8 min; (e) 10 min; (f) 15 min; (g) 20 min; (h) 30 min. After extraction of the DNA its affinity, before and after sonication, for nitrocellulose was measured. $\blacktriangle$, ^3H ; $\bullet$, ^{14}C .

will depend on the exact nature of the binding site and the effect that sonication has on it. The fact that the material pulsed for 0.5, 1.0 and 3.0 min exhibited similar degrees of binding after shear shows that it is not simply a matter of removing native regions from

the binding site, as is the explanation of the pulse-'chase' results. If the lowest molecular weight that is produced by shear corresponds to a length synthesized in about 2 min, then in a 3 min pulse one-third of the ^3H will be in a position in the DNA that is outside the

binding molecule, and hence the amount of sonication-resistant material would fall by one-third. The results do show a fall of this order.

From these results it appears the growth rate of nascent DNA is not less than $0.5 \mu\text{m}/\text{min}$.

Column chromatography on BND-cellulose

BND-cellulose has been used in studies on the replication of the DNA of polyoma virus (Bourgaux-Ramoisy, 1971) and for studies on DNA repair systems in *E. coli* (Iyer & Rupp, 1971). We have found this material to be of considerable value in studying DNA synthesis in BHK-21/C13 cells. Nascent DNA which was pulse-labelled for 0.5 min with [^3H]-thymidine behaves very differently from the bulk (native ^{14}C -labelled) of the DNA as shown in Fig. 6(a). Of the bulk DNA 40.6% was eluted with 10 ml of 1 M-NaCl-Tris-EDTA, 41.7% in the 0–2% caffeine gradient and 17.7% in the 6 M-guanidine thiocyanate fraction. In contrast very little of the nascent DNA, only 7.4%, was eluted in the 1 M-NaCl-Tris-EDTA, 61.5% in the caffeine gradient and 31.1% in the guanidine thiocyanate fraction. This reflects the pattern observed when heat-denatured DNA was chromatographed on the BND-cellulose, whereby 38.2% was recovered in the caffeine gradient and the rest in the 6 M-guanidine thiocyanate fraction. It has been reported that partially single-stranded DNA was eluted in the caffeine gradient (Iyer & Rupp, 1971). Our controls show that 6 M-guanidine thiocyanate removes fully denatured DNA.

DNA pulse-labelled for 1.0 and 3.0 min showed similar elution profiles. A difference might have been expected, but this was not observed. We have shown, however, that the binding properties of the DNA are, to some extent, dependent on the size of the molecule. For example, after sonication the proportion of material in the caffeine gradient and guanidine thiocyanate fractions decreases appreciably, whereas that in the NaCl-Tris-EDTA fraction increases substantially (Fig. 6b). This reflects the results obtained with nitrocellulose.

Column chromatography on hydroxyapatite

Single-stranded DNA is eluted from hydroxyapatite at about 0.16 M-potassium phosphate buffer, pH 6.8 (Bernardi, 1969a,b), and native DNA at about 0.25 M. Fig. 7 shows that some of the nascent DNA was eluted before the bulk of the DNA and was thus behaving as single-stranded or partially single-stranded material. We have found, however, that hydroxyapatite does not yield consistent results, and in some experiments there was very little separation of nascent from bulk DNA.

Column chromatography on MAK

A number of physical and chemical parameters are important in the fractionation of DNA on MAK (e.g. size, G+C content), but of interest in this work is its ability to separate native and denatured DNA.

Fig. 6. BND-cellulose column chromatography of pulse-labelled DNA

(a) About $100 \mu\text{g}$ of DNA in 0.3 M-NaCl-Tris-HCl-EDTA was adsorbed on to BND-cellulose columns. (b) Samples of DNA were sonicated for 30 s before chromatography. Arrows indicate commencement of elution with: (1) 0.3 M-NaCl-Tris-HCl-EDTA; (2) 1 M-NaCl-Tris-HCl-EDTA; (3) caffeine gradient, 0–2% (w/v) caffeine in 1 M-NaCl-Tris-HCl-EDTA; (4) 6 M-guanidine thiocyanate. $\blacktriangle$, ^3H ; $\bullet$, ^{14}C .

Fig. 7. *Hydroxyapatite column chromatography of pulse-labelled DNA*

DNA, which was pulse-labelled for 1 min with [^3H]thymidine, was adsorbed on to hydroxyapatite columns in 0.05M-potassium phosphate buffer, pH 6.75. Elution was carried out with a gradient of potassium phosphate buffer. Δ , ^3H ; $\bullet$, ^{14}C .

Fig. 8. *MAK column chromatography of pulse-labelled DNA*

DNA, which was pulse-labelled for 5 min with [^3H]thymidine, was adsorbed on to MAK. The first and second arrows indicate respectively commencement of elution with 0.75M-guanidine thiocyanate and 1% (v/v) NH_3 in 1.6M-NaCl-0.5M-potassium phosphate buffer, pH 6.7. Δ , ^3H ; $\bullet$, ^{14}C . For explanation of the lettering of peaks see the text.

Native DNA is eluted at 0.6–0.75M-NaCl and denatured DNA at 0.8–0.95M-NaCl. However, recovery of the latter, though only about 50%, can be increased by raising the pH of the eluting buffer (Sueoka & Cheng, 1967); guanidine thiocyanate has a similar effect (Ellem & Rhode, 1969).

The results we obtained (Fig. 8) are similar to those of Harrison (1971). DNA, pulse-labelled for 5min, showed four peaks on chromatography on MAK, P_1 , P_2 , β_1 and β_2 , although P_2 was not particularly well defined. The P_1 fraction is presumably native DNA, fraction P_2 is partially denatured DNA and fractions β_1 and β_2 the material which is not normally eluted with 0.95M-NaCl. After a 3 min pulse the elution profile was similar except that peak P_1 was relatively smaller, and after a longer pulse (10min) peak P_1 was relatively larger, suggesting that fractions P_2 , β_1 and β_2 are precursors of fractions P_1 . The large amounts of ^{14}C -labelled material in the denatured P_2 , β_1 and β_2 peaks is probably caused by properties of the molecule similar to those that enable 25% of native DNA to bind to nitrocellulose.

Discussion

We have established that synthesis of DNA in BHK-21/C13 cells is a discontinuous process and that the anomalous properties of the replicating region of the molecule are probably due to single-stranded regions of the parental strand. We were unable to obtain more than 50% of the counts from [^3H]thymidine-pulsed cells in 'Okazaki fragments', which would have proved that both strands of the DNA replicate discontinuously. However, the amount of pulse label recovered in 'Okazaki fragments' varies from one reported system to another (see, e.g., Schandl & Taylor, 1969; Probst *et al.*, 1971). This is because discontinuous replication involves three separate reactions; initiation, chain growth and ligation of fragments. The rates of these reactions no doubt vary from one organism to another, and hence the number of fragments present, the lifetime of these fragments, and the degree of asymmetry of the reactions on each strand (Denhardt, 1972; Ginsberg & Hurwitz, 1970) could account for the differences in results obtained by different workers. In BHK-21/C13 cells the lifetime of an 'Okazaki fragment' is considerably less, in at least one of the strands, than 15s at 20°C, as a large proportion of the material incorporated during this pulse-time is associated with molecules of about 30S. We were not able to give a shorter pulse of [^3H]thymidine because the incorporation of label was then too low to give meaningful experimental results. This being so, and if the only denatured part of the DNA molecule is associated with the 'Okazaki fragment' within the replicating region, then the size of

that part of the nascent DNA molecule which produces its special properties must be very small.

The relatively constant sonication-resistant proportion of ^3H material is explained by the smallest molecules produced by shear being about as large as a piece of DNA that is synthesized in 2min. We were unable on this basis, however, to explain the fact that sonication decreases the degree of binding of the DNA pulsed for 0.5min and 1.0min.

No separation of nascent from bulk DNA was obtained on CsCl-density gradients (see also Probst *et al.*, 1971), which shows that the amount of denatured material is very small. Probst *et al.* (1971) suggested, because of this, that the apparently single-stranded properties of nascent DNA on nitrocellulose and MAK results from the formation of complexes with other cell components, presumably membrane-like structures. However, treatment of our DNA with Pronase, ribonuclease and amylase produced no change in the observed properties.

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