Ligation and Synthesis of Chromatin Deoxyribonucleic Acid *in vitro* in Neuronal, Glial and Liver Nuclei Isolated from Adult Guinea Pig

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Neuron-rich and glial nuclear preparations and liver nuclei were isolated from adult guinea pigs. These nuclei were incubated to carry out DNA-ligation and -synthesis reactions. Before and after incubation, the sizes of single-stranded DNA and DNA-synthesis patterns in single strands were analysed by using alkaline sucrose-density-gradient centrifugation. Isolation of nuclei by cell-fractionation technique shortened chromatin DNA and decreased markedly the number-average molecular weight of DNA strands. Chromatin DNA in neuronal and glial nuclei was ligated at the nicks during incubation in a reaction mixture containing ATP, Mg^{2+} , dithiothreitol and four deoxyribonucleotides. The number-average molecular weights were estimated to increase 1.1- and 2.1-fold in neuronal and glial nuclei respectively. DNA strands in liver nuclei were shortened during incubation, but elongated under conditions that inhibit deoxyribonuclease. Since the endogenous deoxyribonuclease activity was conspicuously higher in liver nuclei than in neuronal and glial nuclei, the shortening and elongation were thought to depend on the balance between DNA ligase and deoxyribonuclease reactions. DNA synthesis occurred at the gaps in chromatin DNA and about 50% of the total synthesized DNA was found in the shorter strands having 6 to 297 bases in all species of nuclei. Based on these results, it was concluded that in nuclei isolated from non-dividing cells (neurons) and slowly dividing cells (glial and liver cells) DNA-ligation and -synthesis reactions proceeded in parallel at the breaks in single-stranded DNA, which was produced mainly by endogenous deoxyribonuclease during isolation and incubation processes.

The mature neuron is known to lose the capacity for cell division (Altman, 1969) and thought to be unable to renew damaged DNA in terms of replication. Therefore it can be inferred that chromatin DNA must be repaired continuously to maintain the cell function during its long life span. Stambolova *et al.* (1973, 1974) and work from our laboratory (Inoue *et al.*, 1976) showed that DNA is synthesized *in vitro* in nuclei of mature neurons and the enzyme mainly responsible for the DNA synthesis is DNA polymerase- β , whose biological role is presumed to be related to the repair of chromatin DNA (Waqar *et al.*, 1971; Grisham *et al.*, 1972; Hübscher *et al.*, 1977). In a preliminary report (Inoue & Kato, 1976), we showed in addition that DNA ligase, another enzyme expected to participate in repair of DNA, is present in mature neuronal nuclei.

To discover whether or not the enzymes described above repair chromatin DNA in nuclei of a typical non-dividing cell, we incubated neuronal nuclei isolated from mature brain with and without substrates for DNA synthesis, and analysed alterations in the size of chromatin DNA after incubation by

using alkaline sucrose-density-gradient centrifugation. The same analysis was done with nuclei isolated from glial and liver cells, which are thought to be slowly dividing cells. Simultaneously DNA-synthesis patterns in DNA strands of various sizes obtained by the above centrifugation technique were also studied for neuronal, glial and liver nuclei. Ligation of DNA *in vitro* was found to proceed in parallel with DNA synthesis at the single-stranded breaks that were produced in chromatin DNA during isolation of these nuclei and were also formed by endogenous DNAase during incubation for DNA-ligation and -synthesis reactions.

Experimental

Nuclear preparations

Neuronal and glial nuclei were isolated from adult female guinea pigs (300g) by the method described previously (Kato & Kurokawa, 1967, 1970). The nuclear preparation (P_{51}) contained 51% neuronal nuclei and the remainder consisted mainly of nuclei of oligodendroglial origin. The P_{55} preparation,

Abbreviation used: DNAase, deoxyribonuclease.

consisting of 55% neuronal and 45% glial nuclei (Inoue *et al.*, 1976), was obtained under the same conditions as those for isolating the glial nuclear preparation (P_3), which contained 3% neuronal and 97% oligodendroglial nuclei. Liver nuclei were isolated by the same procedure as that for the P_{51} preparation. The isolated nuclei were finally suspended in 0.32M-sucrose solution at a concentration of $(3-30) \times 10^7$ nuclei/ml and stored at -80°C . Sizes of chromatin DNA and the activities of DNA polymerase, DNA ligase and DNAase did not change for 3 months at -80°C . All experiments were performed within 2 months after isolation of cell nuclei.

Cell suspensions

The cerebral cortex of a guinea pig was minced with scissors and the cerebral cells were suspended in 50mM-Tris/HCl, pH7.5, by repeated suction and ejection of minced tissue blocks with a Pasteur pipette. The suspension was filtered through stainless-steel mesh ($125\mu\text{m} \times 125\mu\text{m}$) to remove clumps of cells and connective tissue. The liver-cell suspension was prepared in the same solution after sieving through bolting nylon by the method of Branster & Morton (1957). The cell concentration was approx. 10^6 cells/ml, determined by counting unstained cells in a haemocytometer.

Conditions for ligation reaction of chromatin DNA strands and DNA-synthesis reaction

Approx. 10^7 nuclei (60 μg of nuclear DNA) were incubated at 37°C for 30 min in a mixture (200 μl) consisting of 50mM-Tris/HCl, pH7.5, containing 5mM-MgCl₂, 5mM-dithiothreitol, 0.2% (w/v) bovine serum albumin, 1mM-ATP, 0.5mM each of deoxyribonucleotides (dATP, dGTP and dCTP), and 10 μM -[^3H]dTTP (10 μCi). These were the same conditions as for the DNA-synthesis reaction used previously (Inoue *et al.*, 1976), except that the dTTP concentration was increased 10-fold in the mixture. In some experiments, these four deoxyribonucleotides were omitted to see if the ligation reaction proceeded separately from the DNA-synthesis reaction. To induce the conditions that would inhibit DNAase activity in isolated nuclei, 50mM-Tris/HCl, pH7.5, was replaced by 200mM-Tris/HCl, pH8.0, and 2.5mM-cadaverine, 2% (w/v) dextran (type T-500), and 1mM-EGTA were added to the reaction mixture (Lynch *et al.*, 1972). DNA ligation and synthesis were stopped by cooling to 0°C , and 0.5ml of ice-cold 50mM-Tris/HCl, pH7.5, was added to dilute the suspended nuclei to a concentration of 1.4×10^7 nuclei/ml. The diluted suspension was analysed by alkaline sucrose-density-gradient centrifugation. The nuclear preparation not incubated was diluted in the same way and used as a control.

Alkaline sucrose-density-gradient centrifugation

A lysing solution (0.5ml; 0.5M-NaOH and 0.1M-disodium EDTA) was layered on 34ml of a sucrose density gradient [5 to 20% (w/v) in 0.1M-NaOH, 0.9M-NaCl, and 3mM-disodium EDTA in a 38.5ml centrifugation tube] cushioned by 1.5ml of a 25% sucrose solution. Cell or cerebral nuclear suspension (0.5ml) was gently mixed with lysing solution on top of the gradient and incubated at 29°C for 3h. Liver nuclear suspension (0.3ml) was analysed to decrease the amount of nuclear DNA, since tetraploid nuclei were included (Johnston *et al.*, 1968). Centrifugation was carried out at 12°C by using an SW-27 rotor in a Beckman model L3-50 ultracentrifuge (Ono & Okada, 1973) at 82500g for 165 min to obtain DNA-sedimentation profiles for cell suspensions, and was carried out for 7h in the case of nuclear-preparation analysis. Fractions (2ml) were collected serially from the top of the gradient by introducing a 50% sucrose solution through a syringe needle that pierced the bottom of the centrifugation tube. Half of each fraction was used for fluorimetric determination of DNA (Kissane & Robins, 1958). Trichloroacetic acid (1ml; 10%, w/v) and 50 μl of a bovine serum albumin solution (1mg/ml) were added to the other half of each fraction. The precipitated DNA was collected on a glass-fibre disc (2.4cm in diameter) and then washed successively with 15ml of 5% trichloroacetic acid, 5ml each of 95% ethanol and diethyl ether. The disc was placed in a vial for scintillation counting and dried completely at 60°C . The dried precipitate on the disc was solubilized in 1ml of Soluene-350 (Packard Instrument Co., La Grange, IL, U.S.A.) by incubation at 50°C for 2h, and the radioactivity in the solubilized precipitate was determined in a scintillation spectrometer (Packard Tri-Carb model 3380), with toluene as scintillation fluid, containing 0.4% (w/v) 2,5-diphenyloxazole and 0.025% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. The counting efficiency was 44.5%. The molecular weight corresponding to each fraction was calibrated by centrifuging DNA molecules purified from T4 bacteriophage (DNA mol.wt. 6.5×10^7) and λ phage (DNA mol.wt. 1.7×10^7). The number-average molecular weight was calculated from the sedimentation profiles obtained in the fractions, except for the first and last fractions (Ono & Okada, 1973; also see Figs. 1-4).

DNAase assay

The activity was determined by measuring the acid-soluble radioactivity released from ^3H -labelled substrate DNA (5900d.p.m./ μg of DNA) by isolated nuclei incubated in the assay reagent. The reagent (40 μl) consisted of 50mM-Tris/HCl, pH7.5, containing 5mM-MgCl₂ and 250 μg of substrate DNA/

ml. The substrate DNA was purified by the method of Marmur (1961) from L5178Y cells, which were grown for four generation times (40h) in Fischer's medium containing 10% horse serum and [^3H]-thymidine (40 nM, 0.2 $\mu\text{Ci/ml}$). The isolated nuclei (about 10 μg of nuclear DNA) were incubated at 37°C for 30 min and the reaction was stopped by cooling the mixture to 0°C. Then 10 μl of a solution of calf thymus DNA (3.8 mg/ml) and 20 μl of liver nuclear preparation were added as co-precipitants to the cooled mixture. Trichloroacetic acid (500 μl ; 5%, w/v) was added successively and the whole mixture was centrifuged at 1000g for 15 min in the cold to separate the supernatant. Then 500 μl of the supernatant was added to 10 ml of toluene scintillation fluid containing 33.3% (w/v) Triton X-100, 0.55% 2,5-diphenyloxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, and the radioactivity was determined. The counting efficiency was 39.5%. The DNAase reaction rate was linear for at least 30 min and was proportional to the amount of nuclei assayed.

Enzymes and chemicals

Deoxyribonuclease 5'-oligonucleotidohydrolase (EC 3.1.4.5, DNAase) from bovine pancreas, deoxyribonucleic acid from calf thymus (type I), all four deoxyribonucleoside 5'-triphosphates and adenosine 5'-triphosphate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; [$\text{Me-}^3\text{H}$]thymidine 5'-triphosphate (sp. radioactivity 53.7–54.3 Ci/mmol) and [$6\text{-}^3\text{H}$]thymidine (sp. radioactivity 5.0 Ci/mmol) were supplied by New England Nuclear, Boston, MA, U.S.A. Cadaverine (pentane-1,5-diamine) was obtained from Nakarai Chemicals, Tokyo, Japan. Other chemicals were of analytical grade.

Results

Size of single-stranded DNA in chromatin of cells and isolated nuclei

The DNA-sedimentation profile for cerebral cells showed a sharp peak and narrow distribution compared with that of liver cells (Fig. 1). The number-average molecular weight was higher in cerebral cells than in liver cells, as indicated in the legend to Fig. 1, which implies DNA strands were broken into shorter pieces in liver cells. When cell nuclei were isolated by using the cell-fractionation technique, single strands of DNA were shortened conspicuously in both cerebral and liver nuclei (Fig. 2). The number-average molecular weight was decreased to less than one-hundredth with cerebral nuclei, and to about one-fortieth with liver nuclei (see legend to Fig. 2 and Table 1).

Compared with the neuron-rich nuclear preparation (P_{51}), the DNA-sedimentation profile for the glial nuclear preparation (P_3) was located on the heavier side and the difference in molecular weight was statistically significant ($P < 0.025$; Fig. 2 and Table 1). This comparison was feasible although P_3 nuclei were isolated in more concentrated sucrose solution than

Fig. 1. Sedimentation profiles of chromatin DNA of suspended-cell preparations

Cell suspensions (5×10^5 cells) from cerebral cortex ($\bullet$) and liver ($\blacktriangle$) were analysed by alkaline sucrose-density-gradient centrifugation for 165 min. The number-average molecular weights calculated from the profiles were 9.8×10^7 for cerebral cells and 1.3×10^7 for liver cells.

Fig. 2. DNA-sedimentation profiles for isolated nuclei. The neuron-rich nuclear preparations, P_{51} (51.7 μg of DNA, $\circ$ — $\circ$) and P_{55} (44.5 μg of DNA, $\circ$ --- $\circ$), glial preparation P_3 (39.7 μg of DNA, $\circ$ $\circ$) and liver nuclei (40.9 μg of DNA, $\circ$ — $\cdot$ — $\circ$) were analysed by alkaline sucrose-density-gradient centrifugation for 7 h. The number-average molecular weights calculated from the profiles were: P_{51} , 9.6×10^5 ; P_{55} , 10×10^5 ; P_3 , 15×10^5 ; liver nuclei, 4.5×10^5 .

that used for isolating P_{51} nuclei, since P_{55} nuclei obtained by the similar procedure as for isolating P_3 nuclei had a molecular weight of similar magnitude to P_{51} nuclei. DNA strands of liver nuclei were distributed in a broader range of the lower-molecular-weight fractions and their number-average molecular weight was lowest among the nuclear preparations (Fig. 2 and Table 1).

Ligation reaction of DNA strands

While DNA synthesis proceeded *in vitro*, single strands of DNA in P_{51} and P_3 nuclei were ligated, hence elongated, and the sedimentation patterns skewed to the left (Figs. 3a and 3b). Even though four deoxyribonucleotides were omitted from the reaction mixture, the DNA-sedimentation patterns were similar to those obtained by the complete mixture. Thus the elongation in P_{51} and P_3 nuclei occurred independently of the DNA synthesis *in vitro* in those nuclei. The average molecular weights were increased 1.4- and 2.0-fold with P_{51} and P_3 nuclei respectively as a result of the ligation reaction (Table 1).

To estimate the changes of average molecular weights of DNA in neuronal and glial nuclei respectively from the above results obtained for the mixed nuclear preparations, the following calculation was performed. The number-average molecular weight ($\bar{M}_n$) is usually expressed as:

$$\bar{M}_n = \frac{\sum_i (d_i/M_i)M_i}{\sum_i (d_i/M_i)}$$

where d_i is the amount of DNA recovered in the i th fraction and M_i is the mean molecular weight of DNA strands in the i th fraction. When the amount of DNA is given as a percentage (x_i) of the total recovered DNA (see Figs. 1-4), $\bar{M}_n$ can be expressed as:

$$\bar{M}_n = \frac{1}{\sum_i (x_i/M_i)}$$

Therefore $\bar{M}_n$ of chromatin DNA in the nuclear preparation containing $100 \times p\%$ neuronal nuclei and $100 \times q\%$ glial nuclei ($\bar{M}_{n(p)}$) can be calculated as:

$$1/\bar{M}_{n(p)} = p/\bar{M}_{n(N)} + q/\bar{M}_{n(G)}$$

where $p + q = 1$, and $\bar{M}_{n(N)}$ and $\bar{M}_{n(G)}$ represent $\bar{M}_n$ of chromatin DNA in neuronal and glial nuclei respectively. From the values in Table 1 and the neuronal and glial nuclear populations in P_{51} and P_3 preparations, it was calculated that $\bar{M}_{n(N)} = 4.9 \times 10^5$ and $\bar{M}_{n(G)} = 8.7 \times 10^5$ before the ligation reaction. After the reaction $\bar{M}_{n(N)}$ and $\bar{M}_{n(G)}$ increased to 5.4×10^5 and 18×10^5 respectively. These calculations signify that single strands of chromatin DNA in neurons were ligated and the molecular weight was

Fig. 3. Ligation of single strands of chromatin DNA and DNA-synthesis patterns in isolated nuclei

DNA-sedimentation profiles ($\circ$, amount of DNA in each fraction as a percentage of the total recovered DNA) and DNA-synthesis patterns ($\bullet$, dTMP incorporation in each fraction as a percentage of the total incorporation) were obtained by alkaline sucrose-density-gradient centrifugation for 7 h with P_{51} (a), P_3 (b), and liver nuclei (c). The curves indicate the average patterns and the open and filled circles on the curves represent average values from the same samples in Table 1: eight samples for P_{51} nuclei; six samples for P_3 nuclei; and five samples for liver nuclei. The broken lines give the profiles resulting from the DNA-ligation reaction and the solid lines ($\circ$ — $\circ$) show the control profiles for non-incubated nuclei. For simplicity, standard deviations are not indicated on the curves, but the difference in DNA amount in each fraction is tested statistically between the profile after the ligation reaction and that for control nuclei and the significance of the results was as follows: at $P < 0.01$, P_{51} (no fraction), P_3 (fractions 3, 4, 5, 6 and 12), and liver nuclei (fractions 1-15); at $P < 0.05$, P_{51} (fractions 4, 10 and 11), P_3 (fractions 10, 11, 13 and 15). The coefficient of variation for each mean value in Table 1 ranged from 0.13 (in the case of control P_3 nuclei) to 0.40 (in the case of incubated P_3 nuclei).

Table 1. *Number-average molecular weights of chromatin DNA in isolated nuclei*

The molecular weights were calculated from the DNA-sedimentation profiles, whose averaged patterns are shown in Fig. 3. The numbers of preparations used to obtain the average values are given in parentheses. To cancel the fluctuation of the molecular-weight value in each performance of centrifugation and to check only its increased portion, the ratio of molecular weight for incubated nuclei to that for control nuclei was calculated. The averages of these ratios are given in the right-hand column with standard deviations ($\pm$ S.D.). The paired comparison with these values (Guttman & Wilks, 1965) was performed by using Student's *t* test. The significance of the results was as follows: * $P < 0.01$; † $P < 0.025$; ‡ $P < 0.05$; and § $P < 0.075$ compared with control nuclei; || $P < 0.01$ and ¶ $P < 0.025$ compared with P_{51} nuclei.

Nuclei	$10^{-5} \times \text{mol.wt. } (\pm \text{ S.E.M.})$		Ratio of mol.wt. of incubated nuclei/mol.wt. of control nuclei
	Control (not incubated)	Incubated at 37°C for 30 min	
P_{51} (8)	6.2 ± 0.70	8.2 ± 0.69 §	$1.4 \pm 0.26^*$
P_3 (6)	8.5 ± 0.44 ¶	17 ± 2.8 †, ¶	2.0 ± 0.94 ‡
Liver (5)	3.3 ± 0.27 ¶	0.79 ± 0.08 *,	$0.24 \pm 0.05^*$

increased 1.1-fold. The increase in molecular weight of glial chromatin DNA was 2.1-fold and was much greater than the increase in neuronal nuclei.

In comparison with neuronal and glial nuclei, DNA strands in liver nuclei became shorter during incubation and the molecular weight was decreased to about one-quarter of the original value (Fig. 3c and Table 1). Elimination of four deoxyribonucleotides had no effect on the DNA-sedimentation profile. In this case, DNA strands were thought to be shortened enzymically during incubation, and therefore with liver and cerebral nuclei DNAase activities were determined against naked DNA as substrate, to estimate the activities in these nuclei in the degradation of nuclear DNA complexed to proteins.

DNAase activities in isolated nuclei

As shown in Table 2, the DNAase activity on the basis of nuclear DNA or protein was highest in liver nuclei and approx. 10-fold more active than that of cerebral nuclei. This indicates that the amount and the concentration of DNAase were highest in liver

nuclei. Glial nuclear preparation (P_3) contained the lowest amount of DNAase, but the enzyme concentration on a protein basis was higher in P_3 nuclei than in P_{51} nuclei. From the neuronal and glial nuclear populations in both preparations, it was estimated that neuronal nuclei contained 1.6-fold more DNAase than glial nuclei.

Ligation reaction of DNA strands in conditions that inhibit DNAase

To minimize the effect of DNAase on elongation of DNA strands, the ligation reaction was carried out in the mixture as described in the Experimental section. The DNAase activity in liver nuclei was inhibited by 60% in the medium containing cadaverine and other components. The DNA-sedimentation profile for liver nuclei obtained after incubation in this mixture shifted to the left, and the molecular weight increased further as the incubation period was extended (Fig. 4). This suggested that the rate of the ligation reaction exceeded the rate of the DNAase reaction.

To confirm this suggestion, chromatin DNA in P_{51} nuclei was nicked by pancreatic DNAase and the shortened strands were incubated in the DNA-ligation and -synthesis reaction mixture as follows.

Pancreatic DNAase was added to 280 μ l of the DNAase assay reagent at a concentration of 87 ng/ml with P_{51} nuclei (436 μ g of nuclear DNA/ml). After the mixture was incubated for 30 min at 37°C, a 100 μ l portion was cooled to 0°C and kept as DNAase-treated nuclei. Separately 160 μ l from the same mixture was added to 95 μ l of 530 mM-Tris/HCl, pH 8.0, containing concentrated components for DNA-ligation and -synthesis reactions and for inhibiting DNAase activity. The final concentrations of these components were adjusted to those under the conditions for both reactions indicated in the Experimental section. DNAase activity of P_{51} nuclei was inhibited by 41% and pancreatic DNAase

Table 2. *DNAase activities of isolated nuclei*

Results are expressed as means $\pm$ S.E.M. The significance of the results was as follows: *different from P_3 ($P < 0.01$); †different from P_{51} ($P < 0.05$); ‡different from P_{51} , P_{55} and P_3 ($P < 0.01$).

Nuclei	Number of preparations	DNA hydrolysed (ng/30 min at 37°C)	
		Per μ g of DNA	Per μ g of protein
P_{51}	6	$1.35 \pm 0.06^*$	$0.272 \pm 0.010^*$
P_{55}	6	$1.85 \pm 0.15^{*,\dagger}$	0.331 ± 0.035
P_3	6	1.07 ± 0.06	0.375 ± 0.026
Liver	6	14.3 ± 2.9 ‡	3.81 ± 0.73 ‡

Fig. 4. Ligation of liver chromatin DNA strands in conditions that inhibit endogenous DNAase in isolated nuclei

The ligation reaction was carried out with liver nuclei (80.5 μ g of DNA) for 1 h ($\circ$ — $\circ$) or for 3 h ($\circ$... $\circ$) in the mixture containing the components to inhibit DNAase and those for DNA-ligation and -synthesis reactions (see the Experimental section). DNA-sedimentation profiles are given for both the nuclei after the reaction and the nuclei not incubated ($\circ$ — $\circ$) as a control. dTMP-incorporation patterns of the nuclei incubated under DNAase-inhibiting conditions were not markedly different from the pattern of the nuclei incubated in the normal reaction mixture (not indicated in the Figure). The number-average molecular weights were 3.1×10^5 for the control nuclei, 3.4×10^5 for the nuclei incubated for 1 h, and 3.6×10^5 for the nuclei incubated for 3 h.

was completely inactivated in the above mixture. The ligation reaction of nicked chromatin DNA was performed at 37°C for 1 h; then the DNA-sedimentation profile was obtained by alkaline sucrose-density-gradient centrifugation. The profiles were obtained simultaneously with the DNAase-treated nuclei as above and the original non-incubated nuclei as a control. In this experiment, the molecular weight of chromatin DNA in P_{51} nuclei was decreased from 6.3×10^5 to 1.8×10^5 by DNAase digestion. However, the molecular weight increased to 2.0×10^5 when the DNAase-treated nuclei were incubated in the above DNA-ligation-reaction mixture. This further supports the conclusion that elongation and shortening of DNA strands resulted from the balance between the rates of the DNA ligation and the DNAase reactions.

DNA synthesis in single DNA strands fractionated by alkaline sucrose-density-gradient centrifugation

DNA synthesis *in vitro* in cerebral and liver nuclei was observed predominantly in the shorter strands of chromatin DNA (Figs. 3a–3c). Of the total

radioactivity incorporated 52% was found in DNA molecules smaller than mol.wt. 9.2×10^4 (fraction 3) with P_{51} nuclei and 47% with P_3 nuclei. The same shorter strands in liver nuclei incorporated 59% of the total dTMP incorporated.

To compare the patterns and rates of DNA synthesis in single strands of various sizes in different nuclear species, an average amount of deoxyribonucleotides incorporated per single strand in each fraction was calculated from the results given in Fig. 3 and plotted in Fig. 5. The average incorporation per single strand of liver chromatin DNA increased linearly from lower- to higher-molecular-weight fractions. In sharp contrast, the incorporation

Fig. 5. Average deoxyribonucleotide incorporation into single-stranded DNA fractionated by alkaline sucrose-density-gradient centrifugation

From the amount of DNA and dTMP incorporated for 30 min in each fraction, the average amounts of deoxyribonucleotides (mol) incorporated per mol of DNA were calculated in each fraction with P_{51} ($\circ$), P_3 ($\square$), and liver nuclei ($\triangle$ — $\triangle$) with respect to molecular weights corresponding to the fractions and on the assumption that dTMP incorporated approximates to one-quarter of the total deoxyribonucleotide incorporated into newly formed DNA. The ordinate scale is equivalent to the number of bases of newly formed DNA/single strand of DNA. The averaged incorporation patterns are shown in Fig. 3 for the same samples analysed here. From the results in Fig. 4, similar curves were obtained for liver nuclei incubated for 1 h ($\triangle$ — $\triangle$) and for 3 h ($\triangle$... $\triangle$) under conditions that inhibit DNAase, in addition to the curve for the averaged incorporation in liver nuclei incubated in the normal reaction mixture. The plots for fractions 14–18 were omitted because the amounts of DNA and dTMP incorporated in these fractions were too small to be measured accurately and the ratios between the two values were not reliable.

patterns for P_{51} and P_3 nuclei were similar and the average incorporation curves reached the plateau levels at fraction 6. The amount of incorporation into cerebral nuclei was larger in shorter strands (fractions 1–6) than that in liver nuclei, and P_{51} nuclei showed more incorporation in all fractions than P_3 nuclei. In larger molecules of DNA strands (fraction 10–13), the average incorporation was markedly larger in liver nuclei than in P_{51} and P_3 nuclei.

The average DNA-synthesis activities of the same samples analysed to obtain these patterns were determined simultaneously by measuring [^3H]dTMP incorporation and DNA amount of nuclei in a portion of the reaction mixture for DNA-ligation and -synthesis reactions. The activities were: 30.5 ± 4.0 units (where 1 unit represents 1 fmol of dTMP incorporated/ μg of nuclear DNA per 30 min $\pm$ s.d.) for eight samples of P_{51} nuclei; 11.7 ± 4.9 units for six samples of P_3 nuclei; and 50.1 ± 10 units for five samples of liver nuclei. These values were about 5.5-fold greater with cerebral nuclei, but 2.5-fold greater with liver nuclei, when compared with the activities in our previous results (Inoue *et al.*, 1976). This difference was thought to be reasonable, based on the results of the following experiment; namely the concentration of dTTP was varied from 1 to $10 \mu\text{M}$ at several levels in the reaction mixture and it was found that DNA-synthesis activities in isolated nuclei were increased with the increasing concentration of dTTP, but the increase was not linear and levelled off, as indicated above, to different extents depending on cerebral and liver nuclei (detailed results not shown).

In conditions that inhibit DNAase, DNA-synthesis activities in P_{51} and liver nuclei were decreased by 78 and 73% respectively. The DNA-synthesis patterns of liver nuclei in these conditions approached similar patterns as for P_{51} and P_3 nuclei, which had lower DNAase activities than liver nuclei. However, the average incorporation rates per single strand were decreased markedly corresponding to inhibited DNA-synthesis activities, even when liver nuclei were incubated for 3 h, except in three lower-molecular-weight fractions (Fig. 5). The DNA-synthesis pattern of P_{51} nuclei in the same conditions did not alter significantly (results not shown).

Discussion

The number-average molecular weight of nuclear DNA obtained from the cerebral cell suspension (Fig. 1) agrees well with the values reported on cerebellar granular cells of dog (Wheeler & Lett, 1972) and mouse thymocytes (Ono & Okada, 1974). As observed on incubation for the DNA-ligation and -synthesis reactions (Fig. 3c), highly active DNAase in liver nuclei (Table 2) acts on chromatin

DNA during preparation of the cell suspension, shortening the DNA strands. As it seems difficult to prevent this shortening of DNA by endogenous DNAase during preparation, an accurate determination of size of DNA *in vivo* might be generally impossible in any species of cells, if DNA size is analysed by using cell samples prepared by mincing or homogenizing tissue.

The cell-fractionation procedure introduced further breakage in DNA of cerebral and liver nuclei (Figs. 1 and 2). The breakage of DNA strands is thought to be caused not only by endogenous DNAase, but also by shearing forces upon centrifugation and homogenization, and other factors in fractionation processes (Hyodo & Ono, 1970; Burgoyne *et al.*, 1970a,b; Kaufman *et al.*, 1972). These various factors act on chromatin DNA in different ways and to different extents depending on the nuclear species and isolation conditions. As a result, the effects of these factors were averaged and each nuclear preparation had a characteristic value of molecular weight with a relatively small fluctuation from preparation to preparation (Table 1). Thus isolated nuclei can provide DNA strands with relatively regular sizes as good starting materials to investigate the DNA-ligation and -synthesis reactions *in vitro*. However, it is biologically meaningless to compare simply the DNA sizes, artificially resulting from the isolation conditions, among different kinds of nuclear preparations.

As described in the Results section, the elongation or shortening of chromatin DNA during incubation reflected the balance between ligation and degradation of original DNA strands (Fig. 3). Endogenous DNAase was the main factor responsible for this degradation (Table 2 and Fig. 4).

The ligation of DNA has been thought to play an important role in replication and repair of DNA. The elongation of nascent DNA strands *in vitro* in isolated nuclei has been a means of analysing the replication mechanism in proliferating cells (Kidwell & Mueller, 1969; Lynch *et al.*, 1972; Hallick & Namba, 1974; Otto & Reichard, 1975; Tseng & Goulian, 1975). Ligation of short pieces of nascent DNA, catalysed by DNA ligase, is thought to cause elongation as part of the replication mechanism (Klein & Bonhoeffer, 1972). As an example of DNA ligation caused by the repair mechanism, isolated nuclei from Ehrlich ascites tumour cells are known to rejoin X-ray-induced single-stranded breaks of chromatin DNA (Matsudaira & Furuno, 1972). In this case of dividing cells, the cytoplasmic factor was required for the elongation, which occurred to a markedly greater extent compared with the present results.

In contrast with these dividing cells, the mature neurons are well known as typical non-dividing cells (Pelc, 1964), since mitotic structures have not been

found in them (Allen, 1912; Bryans, 1959). Glial cells were reported to carry out mitosis (Smart & Leblond, 1961; Mori & Leblond, 1969, 1970), and liver cells also continue to synthesize DNA for cell division in the adult animal (Grisham *et al.*, 1972; Lynch *et al.*, 1972; Stambolova *et al.*, 1973). These cell species do not proliferate rapidly and are generally thought to be slowly dividing cells. The present paper demonstrates for the first time an elongation of chromatin DNA in non-dividing cells *in vitro* and the similar elongation pattern in slowly dividing cells, especially in liver nuclei in DNAase-inhibiting conditions. The ligation reaction of DNA strands that causes this elongation was independent of growth of DNA strands based on DNA synthesis *de novo*. The present ligation reaction did not require cytoplasmic components. However, in one experiment, in which the cytoplasmic fraction from cerebral cortex was added to the reaction mixture by the method of Matsudaira & Furuno (1972), the molecular weight of DNA in P_{51} nuclei increased by 28% of that in the same nuclei incubated in the reaction mixture without the cytoplasmic fraction (results not shown).

The joining of short strands is thought to be caused by DNA ligase in the present elongation reaction of chromatin DNA. Since elongation of DNA strands in neuronal and glial nuclei was estimated from DNA-sedimentation profiles for the mixture of neuronal and glial nuclei (P_{51} preparation), there is a possibility that DNA ligase was not present in neuronal nuclei and the ligase in glial nuclei was solubilized into the reaction mixture and so catalysed a ligation reaction in neuronal nuclei. This possibility can be eliminated because much more DNA ligase is contained in neuronal nuclei than in glial nuclei (Inoue & Kato, 1976), and more than 80% of the enzyme is retained in neuronal nuclei at the ionic strength in the reaction mixture. From the higher content of DNA ligase in neuronal nuclei, it seems likely that the ligation of DNA would proceed to a greater extent in neuron-rich P_{51} nuclei than in P_3 nuclei composed mainly of glial nuclei. But the elongation was more predominant in P_3 nuclei (Table 1 and Fig. 3). This result is thought to be due to the higher activity of DNAase in neuronal nuclei than in glial nuclei (Table 2).

DNA synthesis *in vitro* in the isolated nuclei was marked in the shorter strands (Fig. 3). The similar dTMP incorporation was observed previously in rat liver nuclei (Burzio & Koide, 1973). The average number of bases in the shorter strands collected in fractions 1, 2 and 3 were 6.2, 68 and 279 respectively, based on the average molecular weight (330) of each deoxyribonucleotide component. The short strands are thought to be produced by endogenous DNAase as shown by Burgoyne and his co-workers (Hewish & Burgoyne, 1973; Burgoyne *et al.*, 1974). From their results, DNAase is known to make single-stranded

breaks (nicks and gaps) in the bridging strands between spherical chromatin particles (ν bodies or nucleosomes; Olins & Olins, 1974; Olins *et al.*, 1975; Oudet *et al.*, 1975). Simpson & Whitlock (1976) showed with rat liver nuclei that the bridging strands composed of 40–50 base-pairs were more susceptible to endogenous DNAase and digested first. Extensive action of DNAase formed single-stranded nicks at 10 base intervals in DNA strands composed of 150–160 base-pairs in nucleosomes. From these results it is expected that in isolated nuclei, gaps are formed in bridging strands mainly by DNAase during the isolation process, and utilized as sites for DNA synthesis under the present reaction conditions. The breaks are formed in bridges and nucleosomes during isolation, but the incubation for the reaction is thought to introduce further gaps in the bridges, and in addition cause many more nicks in the bridges and nucleosomes by extensive action of DNAase. Thus, the shorter strands that have newly formed DNA are produced during incubation, and dTMP incorporation is found predominantly in DNA strands in fractions 1–3, which have a similar number of or fewer bases than chromatin units (200 base-pairs; Konberg, 1977).

Simultaneously the shorter strands with no newly formed DNA were produced by DNAase during incubation; therefore, due to these strands the shorter strands having newly formed DNA were diluted and the average amounts of newly formed DNA per single strand became smaller in the lower-molecular-weight fractions in cerebral and liver nuclei (Fig. 5). In P_{51} and P_3 nuclei, the elongation reaction overwhelmed the DNAase reaction and longer strands were formed. The longer strands are thought to have newly formed DNA pieces only on 3'-OH termini and not to have patches of synthesized DNA pieces in the midst of a strand, because the average deoxyribonucleotide incorporation per single strand does not increase linearly in proportion to strand length, but is at a constant level in the fractions whose molecular weights are greater than 1.0×10^6 (3090 bases; fraction 6). As described in the Results section, the elongation of strands occurred similarly whether or not four deoxyribonucleotides were present in the reaction mixture. This result further supports the proposal that newly formed DNA pieces were neither ligated nor incorporated in the middle of strands.

DNA-synthesis activity was higher in liver nuclei than in cerebral nuclei, as shown in the Results section. Hence many more or longer DNA pieces must be newly formed at single-stranded gaps in liver nuclei than in cerebral nuclei. Therefore the amount of newly formed DNA per single strand should be larger in strands of all lengths in liver nuclei as actually found in the longer strands, but it was smaller in the lower-molecular-weight fractions

(Fig. 5). In the case of liver nuclei, the curve of average DNA synthesis increases linearly with increasing length of the DNA molecule. This linear increase offers two possible modes of DNA formation at the single-stranded gaps: (1) the gaps were filled by the newly formed DNA pieces, and the nicks between the ends of growing strands and those of the neighbouring strands were sealed by DNA ligase to form longer strands; (2) the newly formed DNA was too short to fill gaps, and the unlabelled strands of various lengths without newly formed DNA were produced simultaneously by a highly active DNAase in such a way that the above linear relationship was obtained by diluting the shorter strands having newly formed DNA much more with these unlabelled strands. Both modes of DNA formation are thought to occur simultaneously. When DNAase was inhibited, the average-incorporation curves seemed to approach the patterns for cerebral nuclei, and to have plateaux in the higher-molecular-weight fractions (Fig. 5). This indicates that the gaps were not filled by newly formed DNA pieces in liver nuclei under the DNAase-inhibiting conditions, as observed in cerebral nuclei incubated in the reaction mixture in which DNAase was not inhibited.

The demonstration of the above joining of DNA strands *in vitro* and synthesis of DNA *de novo* at breakage gaps indicates that these reactions function *in vivo*, and probably supports the repair mechanism of chromatin DNA. These reactions, especially in the neuron, a typical non-dividing cell, are evidence for the presence of a repair mechanism that acts independently of the replication process in cell nuclei. Rejoining of DNA strands damaged by X-ray or γ -ray irradiation has been reported in special types of neurons (photoreceptor cells in retina and granular cells of cerebellum; Wheeler & Lett, 1972; Wheeler *et al.*, 1973), which also indicates the presence of a repair mechanism in non-dividing cells. In contrast with these findings, radioautography has failed until recently to detect [^3H]thymidine incorporation *in vivo* into mature neuronal nuclei in tissue slices (Altman, 1969). However, [^{14}C]thymidine incorporation, interpreted as a result from repair of chromatin DNA, has been demonstrated recently by a special radioautographic technique (activated radioautography) in neuronal nuclei isolated from rats that had been given the radioactive label intracisternally (Wintzerith *et al.*, 1977). This technique and the radioautography of labelled isolated nuclei are thought to improve the sensitivity to a very small amount of radioactivity incorporated into neuronal nuclei. On the basis of the present results, we suggest that the number of gaps in neuronal nuclear DNA is not large enough to observe [^3H]thymidine incorporation *in vivo* in ordinary radioautography. In this respect, the ligation and synthesis reactions of DNA in isolated nuclei, which have many more breaks of

DNA than chromatin DNA *in vivo*, are thought to offer a practical model for the investigation of the repair mechanism that is thought to function *in vivo* in cell nuclei.

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