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STUDIES ON DNA REPLICATION IN ANIMAL CELLS

(Annual Report)

by

C. PAOLETTI, N. DUTHEILLET-LAMONTHEZIE, Ph. JEANTEUR
and A. OBRENOVITCH
(Institut Gustave Roussy)

1966

Report prepared by the Biochemistry and Enzymology Laboratory
Institut Gustave Roussy — Villejuif, France

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In DNA extracted longer after this pulse, the part of radioactive material which shows up such characteristics becomes progressively less important and in 2 hours DNA the behaviour of labelled material and bulk DNA is nearly identical. The best explanation of these data is the existence of structurally modified DNA, correlated to its replication.

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FIG. 4 — Density gradient centrifugation in CsCl at alkaline pH.

FIG. 5 — Specific activities of total DNA's extracted by the Schmidt and Thannhauser procedure at various times after thymidine — ^{3}H injection (25 μc/mouse) and chase by 2 μM of cold thymidine 5 min. after the label injection.
For each time, ascites cells from 15 mice were pooled.

FIG. 6 — Repartition of radioactivity in the different chromatographic fractions versus time.

STUDIES ON DNA REPLICATION IN ANIMAL CELLS (*)

1 — INTRODUCTION

The semi-conservative scheme of DNA replication has been demonstrated for bacteria (Meselson and Stahl, 1958) and extended to mammalian cells (Simon, 1961); it implies the existence of an intermediate structure between those of the mother and the two daughters molecules in which each parental single-strand is bound to both parental and daugther complementary single-strands; such a structure may be called replicative form of DNA; it has been inferred to have a Y like shaped structure associated with some specific physico-chemical properties.

Autoradiographic (Cairns, 1963), genetic (Yoshikawa and Sueoka, 1963), biophysical (Baldwin and Shooter, 1963; Rolfe, 1963; Hanawalt and Ray, 1964; Lark and Lark, 1964; Rosenberg and Cavalieri, 1964; Guérineau, Dechambre and Paoletti, 1966) data have been accumulated in support of such a scheme for bacteria. DNA replicative forms have also been demonstrated into bacterial viruses (Frankel, 1963; Kozinski and Lin, 1965; Smith and Skalka, 1966). In animal systems, data are more scanty (Ben Porat, Steere and Kaplan, 1962; Rosenberg and Cavalieri, 1964, Cairns, 1966). Nevertheless, the existence of replicative forms in DNA may be reasonably assumed. The present paper brings some experimental arguments in favour of this hypothesis.

The basis of the experimentation was as following : mouse Ehrlich ascites cells were given a pulse of a radioactive precursor of DNA (^{3}H thymidine); the label of a DNA extracted soon after this pulse must be found in molecules (newly labelled DNA) which had just been synthesized. Would replicative forms exist, they should be labelled at that time. On the other hand, when DNA was extracted later on, the label must be into molecules synthesized since a long time and consequently any eventual replicative form would not be labelled.

In case these forms have some specific features, the behaviour of radioactive material from each DNA should be different when subjected to technics which differentiate DNA's according to their molecular size, shape or structure, such as chromatography on methylated albumine, zone centrifugation in sucrose, buoyant density centrifugation in CsCl and Cs_2SO_4, and alkaline denaturation. The results displayed such differences and were in support of the existence in animal cells of transient macro-molecular forms of DNA related to its duplication.

2 — MATERIALS AND METHODS

2.1 — Chemicals

Sucrose was an Analar product. Bovine albumin (fraction V) was obtained from Armour Company. Non radioactive thymidine and venom phosphodiesterase were Sigma Chemical products. CsCl was purchased from American Potash Chemicals and recrystallised before use. Cs_2SO_4 was a Merck product. Trypsin, chymotrypsin, pancreatic DNase

(*) Manuscript received on January 14, 1966.

and RNase were all products of Worthington Biochemical Corporation. RNase was always boiled ten minutes in acetate buffer 0.15 M, pH 5 before use.

Thymidine methyl ^3H (6.7 C/mM) was purchased from New England Nuclear Corp. and tested for radiochemical purity by paper chromatography before use (90 % of (^3H) were with thymidine spot).

2.2 — Counting of radioisotopes

Determinations of (^3H) were done in a Tricarb Liquid Scintillation Spectrometer. Aqueous samples of 0.5 ml to 1 ml were put on Kleenex papers (8 cm × 3 cm) fitted to the inside wall of counting vials. After drying, the papers were impregnated by 0.8 ml of a scintillation solution : 4 g of 2-5 diphenyloxazole and 100 mg of 1.4-bis-2-(4 methyl-5-phenyloxazolyl benzene dissolved in 1 litre toluene. Efficiency of counting was 20 %.

2.3 — Biological material and technique of labelling

Ehrlich adenocarcinoma cells regularly transplanted into the peritoneum of C_{57} black mice homogeneous with respect to age and sex have been used throughout this work. Four or five days after the graft of 25×10^6 cells, the mice were intraperitoneally given 25 μc of (^3H) thymidine in 1 ml of physiological saline under sterile conditions. Krebs-2 ascites cells have also been used. They were transplanted into the peritoneum of inbred Swiss mice. The animals were injected with ^3H thymidine seven days after the graft of 20.10^6 cells. Animals were killed two minutes before the indicated time of labelling and the ascitic fluid withdrawn from the peritoneum into a tube containing a few ml of SC (*) at 37° C. At the indicated time, the tubes were frozen at — 80° C. 5 minutes was chosen as a time compatible with an easy handling of mice and obtention of a newly labelled DNA of a sufficiently high specific activity for accurate counting; 7 hours was chosen in consideration of the cellular and mitotic cycle of Ehrlich ascites cells (Edwards, 1960) to rule out the possibility that DNA synthesized during the pulse would enter into a new replication process at the extraction time; intermediate times were selected to obtain kinetic data.

The disappearance of radioactivity from the peritoneal cavity is very rapid : after 2 minutes, 25 % of the injected tritium was recovered in the ascitic fluid and only 13 % after 5 minutes. Consequently, one can consider that a physiological pulse actually took place. Nevertheless, in kinetic experiments, 2 μ moles of unlabelled thymidine were given 5 minutes after administration of the label.

Pleuropneumonia-like organisms could not be found in forteen different ascites cells samples, except for one case (Klieneberger.-Nobel E., 1962). Bacteria were never found.

2.4 — DNA extraction

Labelled DNA was prepared according to the procedure of Dutheillet-Lamonthézie and Guérineau (1965). Frozen cells stored at — 80° C were thawed at 0° C, washed three times with SC and broken in SC by ultra-Turrax (3 seconds) or teflon-pestled Potter.

(*) Abreviations :

— MAK : methylated albumin coated Kieselguhr.
— SC : saline citrate: 0.14 M NaCl containing 0.05 M sodium citrate.
— 1/10 SC : SC diluted tenfold.
— 5 min DNA and 7 h DNA refer to DNA's extracted from cells withdrawn 5 minutes and 7 hours after label injection.
— SA : specific activity expressed as cts/min/OD unit.
— dAT : double stranded copolymer of A and T deoxyribonucleotides.
— dG — dC : homopolymers of G and C deoxyribonucleotides, hydrogen bounded together.

Centrifugation 15 minutes at 30,000 g was run to remove the bulk of cytoplasmic RNA while the pellets were essentially made up of nuclei. These pellets were washed and centrifuged three times before being resuspended by ultra-Turrax (3 seconds) or Potter in a sufficient amount of SC to have a final DNA concentration of 400 to 800 μg/ml. Phenol solution (phenol : 9 volumes; EDTA 10^{-3} M : 1 volume; concentrated KOH up to pH 9.0) was slowly added to the suspension with shaking at room temperature. After one hour shaking, the viscous, milky suspension was centrifuged for 30 minutes at 30,000 g. The upper aqueous layer was then carefully withdrawn and subjected to a second phenol treatment during 15 minutes. After centrifugation as above, 10 volumes of the aqueous layer containing DNA received one volume of 1.5 M sodium acetate buffer pH 5.5. Phenol was then extracted by shaking ten times with three volumes of peroxide free ether containing acetic acid (1/750 V/V). Ether was finally removed by nitrogen bubbling.

The last two steps were RNase treatment (10 μg/ml) according to Rolfe (1963) and ethanol precipitation by adding two volumes of ethanol saturated with potassium acetate followed by redissolution in 1/10 SC. The last steps were often purposely omitted because newly labelled DNA molecules might be supposed to have such a structure as to be modified by ethanol precipitation.

Sometimes, proteolytic enzymes prior to phenol extraction were used. The pellet containing nuclei was lysed by treatment with a 6 M NaCl solution which resulted in a dramatic increase of viscosity. The saline concentration was lowered by dialysis against SC to about 0.2 M. One half of the solution was extracted by phenol and the other one treated by chymotrypsin (10 μg/ml) and trypsin (10 μg/ml) for three hours at 37° C, before phenol treatment.

2.5 — Methylated albumin column chromatography

Two methods were concomitantly used :

2.5.1 — Continuous elution method. The experimental procedure of Mandell and Hershey (1960) was followed, the amount of the three layers being halved. The water-jacketted column (1.8 cm × 12 cm) was loaded with 10 to 21 OD units of extracted material (about 0.4 OD unit/ml) in 0.4 M NaCl 0.05 M pH 6.8 phosphate buffer. An exponential NaCl gradient between 0.4 and 1.0 M in 0.05 M pH 6.8 phosphate buffer was first applied followed by a pH gradient in 1.0 M NaCl up to pH 10.5 with 1.0 M NaCl 1.5 N NH_4 OH solution (Lacks, 1962). NaCl concentration of the gradient was measured by refractometry.

2.5.2 — Stepwise elution procedure as described by Sueoka and Cheng (1962*a*). The same amounts of material as above were loaded in 0.5 M NaCl — 0.05 M pH 6.8 phosphate buffer and eluted by 0.1 M increase of salt concentration in 0.05 M pH 6.8 phosphate buffer; for each step, four 5 ml fractions were collected. Additional elution step was done by 20 ml of 1.0 M NaCl — 1,5 M NH_4 OH pH 10.5 solution.

In both chromatographic procedures, temperature was kept constant at 20 ± 2° C.

2.6 — Sucrose gradients

On top of a 20 ml preformed linear 15 — 5 % sucrose gradient in 0.14 M NaCl (Britten and Roberts, 1960) were layered 4 to 5 ml of labelled material solution (1.7 to 6.4 OD units in 0.14 M NaCl). Gradients were spun at 20,000 rev/min for 5 hours at 20° C in the SW 25 rotor of model L2 Spinco Ultracentrifuge and ten drop fractions collected by piercing the bottom of the tube. These fractions, usually 25 to 30, were then tested for DNA content and radioactivity.

2.7 — Buoyant density gradients

2.7.1 — At neutral pH, CsCl stock solution was 62 % (W/W) in distilled water. Labelled material (1 to 5 OD units) in neutral buffer or SC solution was added along to make density around 1,690 g/ml 3 ml of such a solution were run in the SW 39 rotor of a L2 Spinco preparative Ultracentrifuge at 25° C for 50 hours at 35,000 rev/min One or two drop fractions were collected after piercing the bottom of the tube, diluted with 1 ml of distilled water and assayed for OD and radioactivity.

Cs_2SO_4 stock solution was 45 % (W/W) in distilled water. Labelled material solution (1 to 5 OD units) and saline were added along up to a density around 1,430 g/ml 2.5 ml of such a solution were run for 40 hours at 31,000 rev/min as above and dropwise collected. Densities were derived from refractive index.

2.7.2 — At alkaline pH reproducible pH measurements could be obtained using a radiometer PHM 4 C pH meter standardized with saturated $Ca(OH)_2$ solution prepared just before use in freshly distilled water and filtered through a sintered-glass, pH of this solution was 12.45 at 25° C and all determinations were performed in a water bath at constant temperature (25° C). To 3 ml of CsCl stock solution were added 0.2 ml of the extracted material solution (1 to 5 OD units) in 0.14 M NaCl then 0.3 ml of 0.4 M potassium phosphate buffer pH 10.95. Saturated KOH solution, diluted to 1/50 before use was added with a micropipette up to pH ranging between 11.35 and 11.48. Centrifugations were as above for 50 hours.

2.8 — Miscellaneous techniques

OD measurements were done with a Zeiss PMQ II spectrophotometer. Dosage of nucleic acid components in cells was performed according to the Schmidt and Thannhauser (1945) procedure. DNA and RNA contents were measured by diphenylamine reaction for deoxyribose (Burton, 1956) and orcinol reaction for ribose (Moule, 1953). Alkaline treatment of phenol extracted DNA or chromatographic fractions was made by 0.5 N KOH 16 hours at 37° C. Protein determinations were performed after exhaustive dialysis of phenol with the procedure of Lowry, Rosenbrough, Farr and Randall (1951), bovine albumin being taken as a standard. DNAse treatment was performed by 10 μg/ml enzyme at pH 7.5 and 10^{-2} M. Mg^{++} 1 hour at 37° C. RNase treatment was accomplished according to Rolfe (1963).

Electrophoresis of nucleotides was performed with the apparatus described by Markham and Smith (1952) in 0.02 M sodium citrate buffer pH 3.5. Ribonucleotides were obtained by alkaline hydrolysis (cf above) and deoxynucleotides by enzymatic hydrolysis with pancreatic DNase and venom phosphodiesterase. The technique of Wyatt (1951) was used for paper chromatography of radioactive thymidine and bases obtained by perchloric hydrolysis of DNA.

Boundary sedimentation measurements were carried out in the 12 mm. Kel-F centerpiece of a Spinco model E analytical ultracentrifuge at 44,770 rev/min at 20° C with 0.2 OD unit/ml, a concentration small enough to allow one to neglect the concentration effect.

3 — RESULTS

3.1 — Analytical properties of DNA and efficiency of extraction procedure

The yield of phenol extracted DNA, compared to the DNA content evaluated by the Schmidt and Thannhauser (1945) procedure was close to 100 %. The relative yield of (^3H) cts (compared to yield of U.V. absorbing material) gave quite different results with

5 min and 7 hrs DNA's : 40 to 70 % of the total radioactivity of 5 min. DNA was recovered by phenol extraction while nearly 100 % was obtained with 7 h DNA. Unrecovered radioactivity of 5 min DNA was localized in the aqueous phenolic interfacial layer from which it could be partially recovered by further treatment with proteolytic enzymes and phenol; it has already been reported that « nascent » DNA molecules are more resistant to extraction (Goldstein and Brown, 1961; Ben Porat, Steere and Kaplan, 1962).

When DNA preparations were analysed prior to RNase treatment and ethanol precipitation, they contained some RNA amounting to 20-25 % and 2-5 % proteins. Otherwise, they contained less than 2 % of RNA or proteins.

Sedimentation coefficients observed with different lots of DNA were between 24 and 32 S. No difference between ultra-Turrax and hand-teflon-pestled Potter extracted DNA's was observed.

3.2 — Chromatographic behaviour of 5 min. DNA and 7 hrs DNA

3.2.1 — *Definition of fractions* :

Three fractions were conventionally defined :

— 0.7 M fraction : bulk of DNA eluted before 0.8 M NaCl elution.

— 0.8 M fraction : material eluted between 0.8 M and 1.0 M NaCl at pH 6.8.

— pH fraction : material eluted during pH increase at 1.0 M NaCl.

Moreover, a part of radioactive material cannot be eluted from the column, either under the above conditions or by NaOH N, HCl N, 5 M NaCl, proteolytic enzymes, Na dodecyl sulfate 0.5 % in NaCl 0.01 M; it has been called NE (non elutable) part of radioactive DNA. Since then, this radioactive part has been eluted from the column by a 0.1 M PO_4Na_3 solution.

Some U.V. absorbing material, presumably hydrolyzed or S - RNA was not adsorbed on MAK columns. It did not contain any significant amount of radioactivity.

3.2.2 — *Elution patterns of 5 min and 7 h DNA* (Fig. 1).

With 7 h DNA, profiles of U.V. absorbancy and radioactivity obtained by both elution procedures were closely superimposed, indicating a homogeneous distribution of labelled molecules in all fractions. On the contrary, 5 min DNA exhibited quite different patterns of radioactivity and U.V. absorbancy and displayed two remarkable characteristics : (*a*) the so-called 0.8 M and pH fractions showed a much higher specific activity than 0.7 M one; (*b*) the NE part is relatively important. Quantitative evaluation is given in Table I. Earlier chromatographic fractions eluted near 0.6 M NaCl are enriched up to 20 % in satellite band as described by Cheng and Sueoka (1963).

Evidence that all the radioactive label was associated with a deoxyribonucleic material was obtained :

— When 5 min DNA was subjected before chromatography to DNase degradation, less than 5 % of radioactivity was adsorbed on MAK columns and eluted all over salt gradient.

— Radioactive material in CsCl density gradient at neutral pH was totally localized at the density of bulk DNA with both 5 min (Fig. 3) and 7 h DNA's. Such bandings were also found with 0.7 M fraction alone and with 0.8 M fraction run with unlabelled DNA. In all these cases, DNase pretreatment resulted in complete disappearance of banding.

TABLE I

Distribution of UV absorbing material and radioactivity among the three chromatographic fractions and the NE part of 5 min DNA

	Stepwise elution (*)		Continuous elution (**)	
	OD	(^{3}H)	OD	(^{3}H)
0.7 M fraction	75 ± 7	30 ± 8	78	25
0.8 M fraction	14 ± 4	26 ± 5	11	35
pH fraction	9 ± 4	15 ± 3	10	20
NE part	—	29 ± 10	—	20

Expressed as percentage of OD units or radioactivity adsorbed on the MAK columns.

(*) 19 experiments; $\sigma = \sqrt{\frac{(x - m)^2}{n - 1}}$

(**) 7 experiments - arithmetical mean.

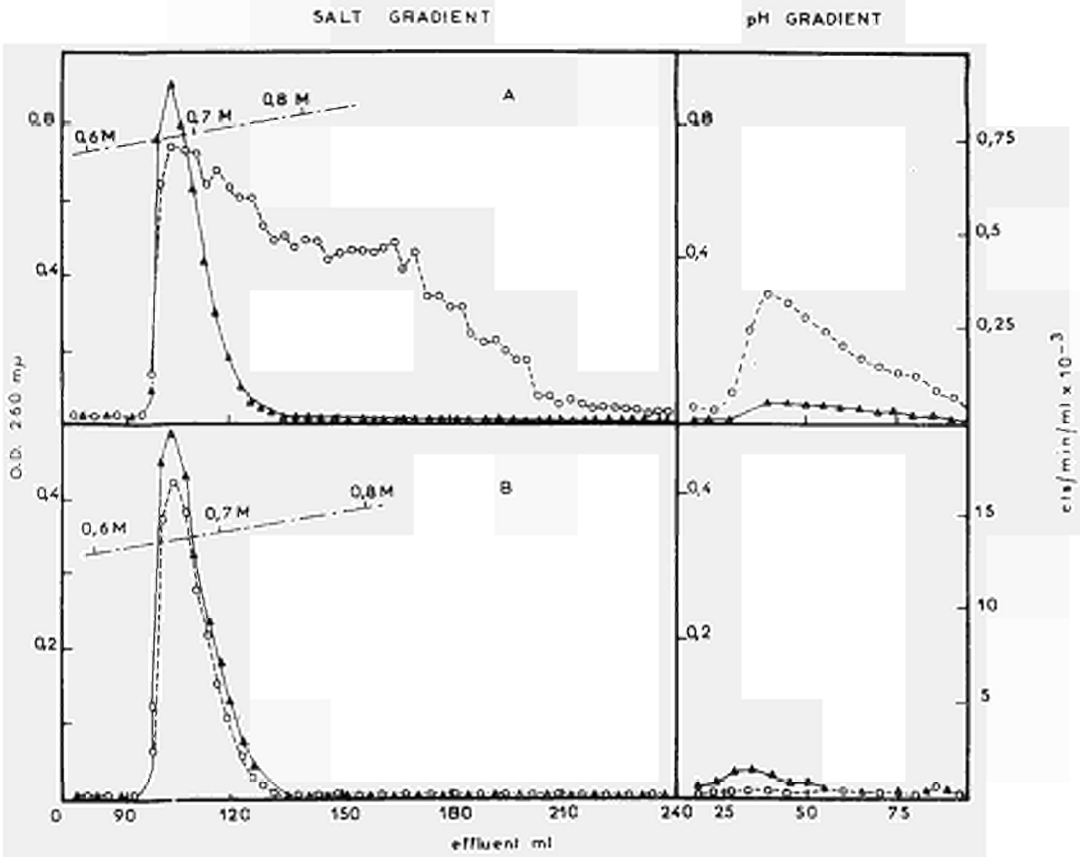

FIG. 1 — Methylated albumin chromatography with gradient elution of 5 min and 7 h DNA's.

a) 5 min DNA : 19.1 OD units, 81,900 cts/min
Recovery : OD : 101 %; (^{3}H)cts : 82 %.

b) 7 h DNA : 10.8 OD units, 286,280 cts/min
Recovery : OD : 104 %; (^{3}H) cts : 95 %.

OD —▲—▲—▲— ; (^{3}H) - - o - - o - -.

— Existence of radioactive RNA was disproved by the following data. Neither chromatographic patterns nor banding in CsCl were affected by RNase (10 μg/ml). 95 % or more of the radioactivity remained in the acid precipitable form after alkaline treatment of total and 0.8 M fraction of 5 min DNA; the small amount of alkali-sensible material in 5 min DNA could not be found in 7 h DNA, and after electrophoresis together with alkali-hydrolyzed RNA yielded no radioactivity associated with usual ribonucleotides (not even with ribothymidylic acid). Paper chromatography of perchloric hydrolysate of total DNA allowed 90-95 % of (^{3}H) counts to be recovered on thymine spot-Likewise, 90-95 % of radioactivity was found associated with thymidylic acid after electrophoresis of enzymatic hydrolysate of 5 min DNA.

Three main criteria are involved in DNA fractionation by MAK column : G-C content, molecular weight and extents of hydrogen bonding (Sueoka and Cheng, 1962*a*). Fractionation according only to a difference in base composition cannot explain our results because a nearly pure dAT (crab satellite DNA) is eluted before 0.8 M NaCl (Sueoka and Cheng, 1962 *b*) while most of the radioactive material concerned here is still adsorbed at this molarity. Furthermore, based on the assumption of a maximum enrichment (100 %) in A-T base pairs, the specific activity of radioactive fractions could not exceed 1.7 times that of bulk DNA (1.7 being the ratio of 100 % to 58 % A-T content of mouse DNA). Higher ratios actually were observed.

3.2.3 — *Influence of protein contamination*

CsCl centrifugation eliminates the most important part of proteins remaining with DNA after extraction. Since radioactive material, in a previously centrifuged DNA, showed chromatographic diagrams similar to those obtained with non centrifuged DNA, proteins could not explain the high affinity of this material for MAK. Nevertheless, the existence of small amounts of proteins strongly bound to DNA could not be ruled out and was demonstrated for phage P_1 transducing particles DNA (Ikeda and Tomizawa, 1965).

Consequently, the effect of trypsin-chymotrypsin on a 5 min DNA was tested, the enzymes being removed by a phenol treatment. In this case also, no significant modification was observed when chromatographic diagrams before and after treatment were compared (Table II).

TABLE II

Distribution of radioactivity in the different chromatographic fractions and the NE part in 5 min DNA's before and after CsCl centrifugation or enzyme treatment.

	CsCl centrifugation		Enzymes treatment	
0.7 fraction	32.4	37.2	12.7	12.3
0.8 fraction	25.0	29.7	20.0	26.7
P fraction	18.2	10.7	11.0	13.5
NE part	24.4	22.4	56.3	47.5

The results are expressed in percentage of radioactivity adsorbed on the MAK columns.
The centrifuged DNA and enzyme treated DNA were extracted from two different lots of animals.

3.3 — Behaviour of radioactivity of 5 min and 7 h DNA in sucrose gradient centrifugation

Similar results were obtained when 10 μg or 200 μg of DNA were layered on top of sucrose gradients. Consequently, we generally used 200 or 300 μg of DNA to avoid too low U.V. absorbancy. In a 7 h DNA, U.V. material and radioactivity (Fig. 2*b*) showed

the same rate of sedimentation while, in a 5 min DNA (Fig. 2*a*), the radioactivity sedimented more slowly than the U.V. material. RNAse pretreatment or alcohol precipitation did not modify significantly the centrifugation patterns.

Difference in molecular size alone could not explain these results: if radioactive material differed only by a lower molecular size from bulk DNA, it should have a reduced affinity for MAK which was not observed.

Contamination by proteins which would make the radioactive material lighter should also be ruled out because of the results obtained with a CsCl gradient centrifuged DNA (Fig. 2*c*).

Difference between macromolecular conformation of bulk and newly labelled DNA is the best hypothesis to explain these data.

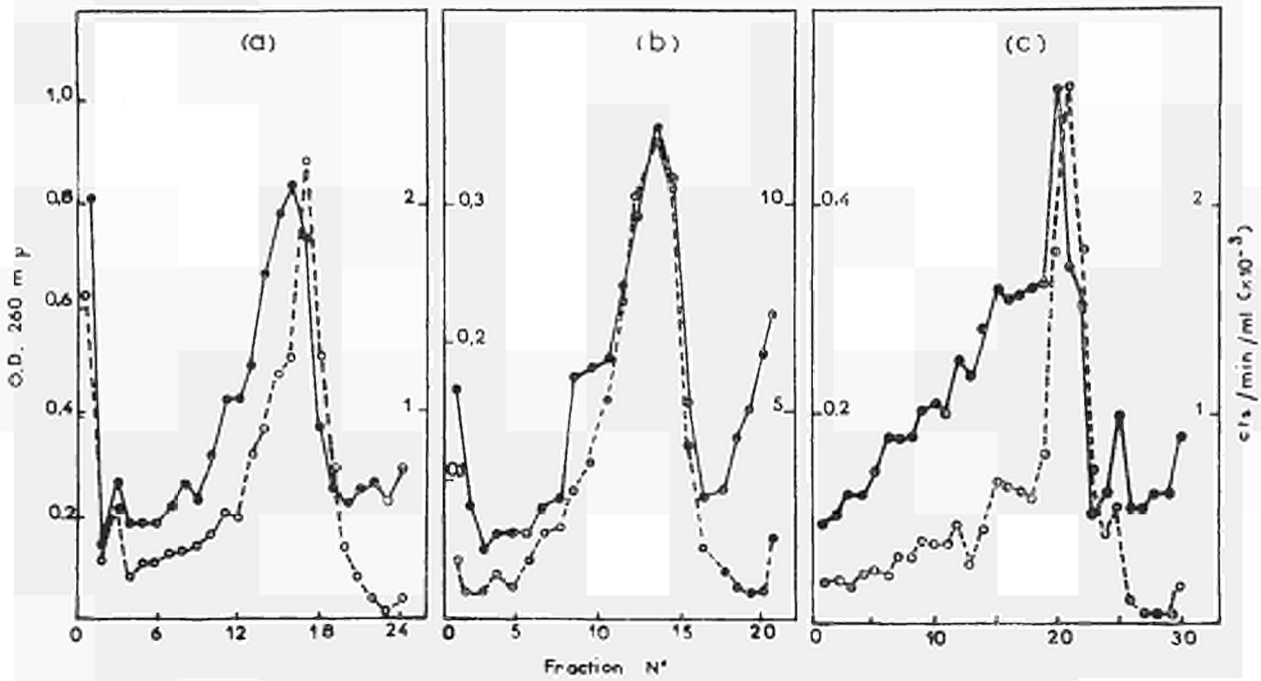

FIG. 2 — Sucrose gradient centrifugation (15-5 %) of radioactive DNA

a) 5 min DNA : 6.4 OD units, 21.000 (^{3}H) cts/min.

b) 7 h DNA : 3.9 OD units, 74.000 (^{3}H) cts/min.

c) 5 min DNA after previous banding in CsCl density gradient : 5.1 OD units, 16.000 (^{3}H) cts/min.

OD — . — . — . — : (^{3}H) - - o - - o - -.

3.4 — Behaviour of radioactivity of 5 min and 7 h DNA in density gradients

Pneumococcal DNA eluted at alkaline pH from MAK columns was reported by Lacks (1962) to be single-stranded. Consequently, single-stranded DNA could be expected to be found in 5 min DNA, which gave a relatively large amount of radioactive pH fraction. Density gradients in which denatured DNA is shifted towards higher density by 0.017 g/ml in CsCl (interpolated to 42 % G-C content : Vinograd, Morris, Davidson and Dove, 1963) and by 0.026 g/ml in Cs_2SO_4 (Erikson and Szybalski, 1964) gave clear evidence that the difference between newly labelled and bulk DNA did not involve single-stranded polynucleotidic chains or even denatured forms which would have been characterized by buoyant density variations. It is worth noting that the one drop collection of the gradient allowed

the detection of the mouse satellite band in a preparative ultracentrifuge (Fig. 3). It could be inferred from such a result that a difference of about 0.003 g/cm³ in buoyant density between U.V. absorbing and radioactive material would have been detected.

Moreover, a dissymetric distribution of (^{3}H) counts compared to OD peak, which could mean a difference in base composition of radioactive fractions, was not observed.

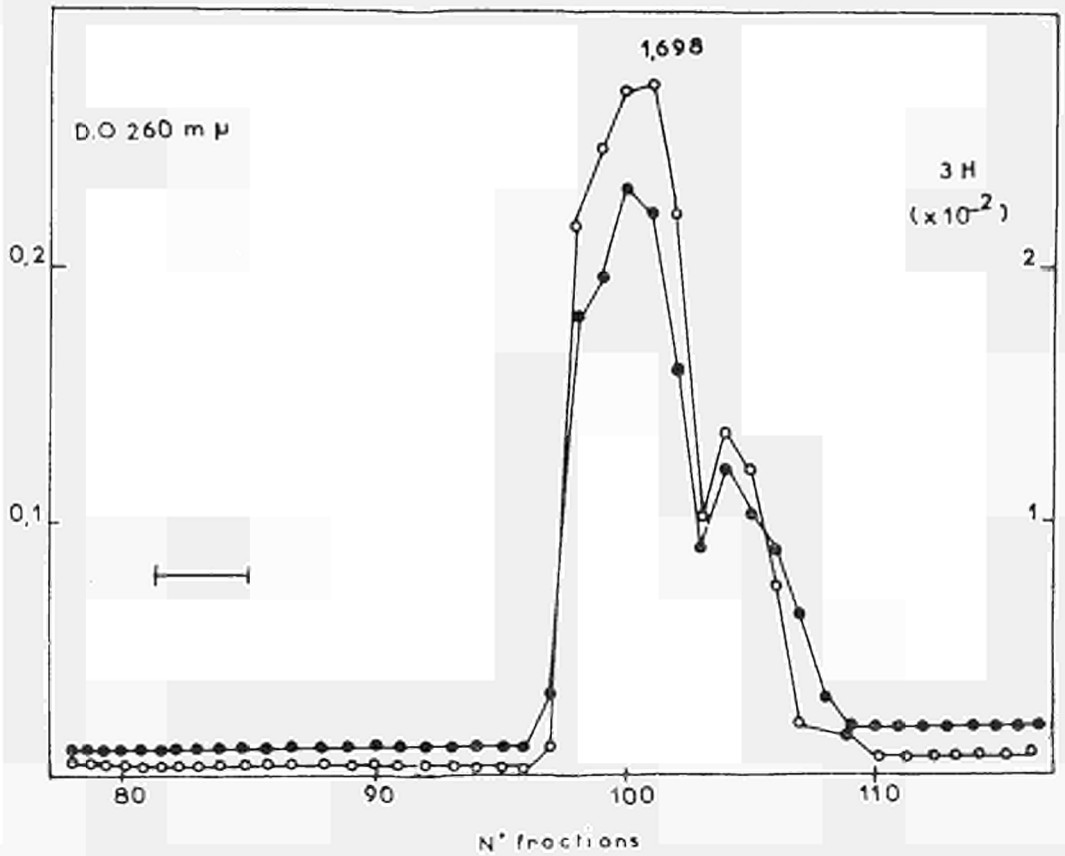

FIG. 3 — Neutral CsCl density gradient of a five min. DNA - Total number of fractions 196 with a density gradient of about 0.001 g/cm³ between two drops.

The bracket shows where denatured DNA could be expected.
OD : . — . — . ^{3}H — o — o —.

3.5 — Increased sensitivity to alkaline denaturation of radioactive material of 5 min DNA

In the course of alkaline denaturation of 7 h DNA, the ratio of (^{3}H) counts to U.V. absorbing material remained unchanged throughout the process, pointing to a homogeneous distribution of radioactivity among DNA molecules (Fig. 4*c*, *d*).

5 min DNA exhibited a different behaviour as the alkaline transition of the radioactivity was seen to occur at lower pH than that of the U.V. absorbing material (Fig. 4*a*, *b*). Two explanations might account for such data : slight structural modifications responsible for increased sensitivity of newly labelled DNA molecules to alkali titration or lower G-C content of highly radioactive molecules; it has been established that melting pH of dAT in 0.5 M Na^+ (Inman and Baldwin, 1962) is lower than melting pH of dG : dC in 0.1 M Na^+ (Radding, Josse and Kornberg, 1962) (10.9 versus 11.4). Such a relationship can probably be extended to higher molarities. Nevertheless, the G-C content difference — if any — should be smaller than 2-3 % due to our inability to find up any difference in the CsCl preparative ultracentrifugation gradient between OD and radioactivity patterns (Fig. 3).

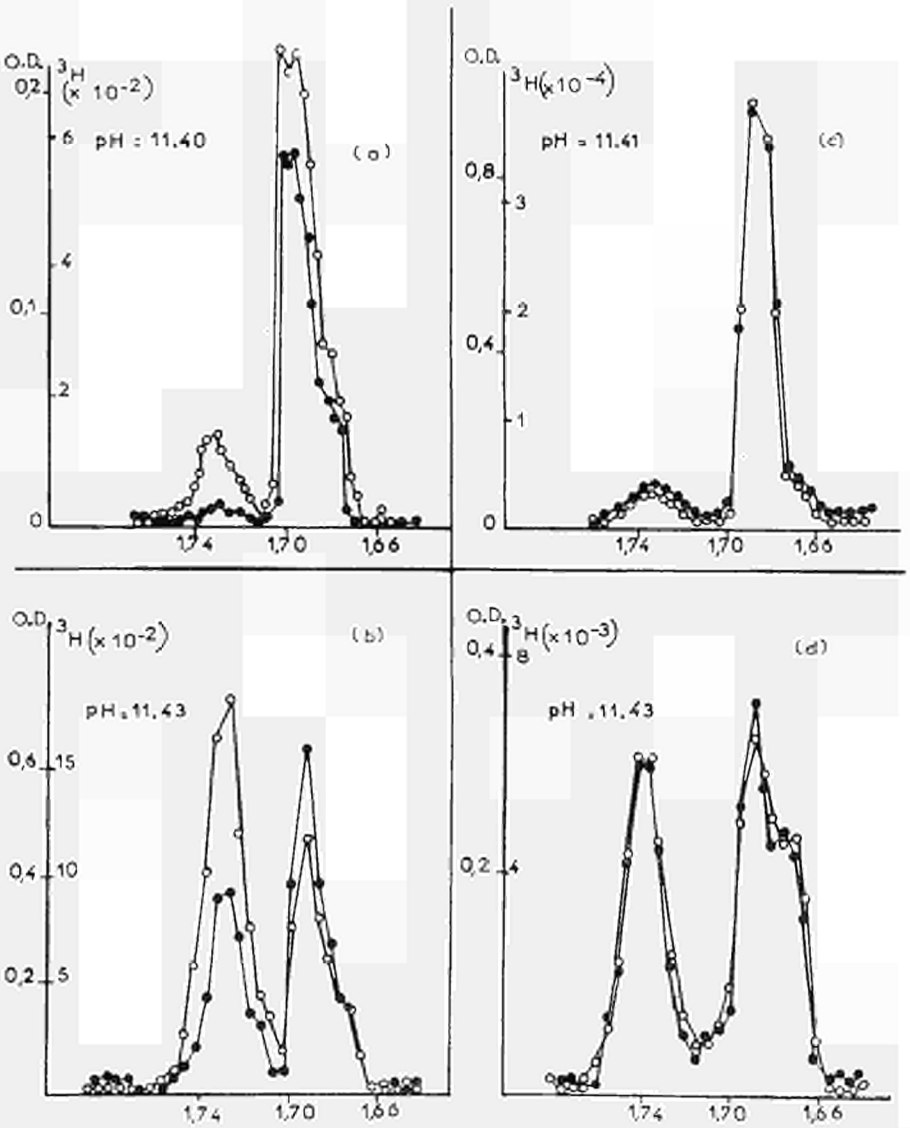

FIG. 4 — Density gradient centrifugation in CsCl at alkaline pH.
Left hand diagrams (*a* and *b*) are for 5 min DNA and right hand ones (*c* and *d*) for 7 h DNA.
OD — . — . — . ; ^{3}H — o — o — o —.

3.6 — Kinetic data

Significant decrease of the specific activities of total cell DNA was not observed at six various times between 5 min and 7 h after label injection and chase by cold thymidine at 5 minutes (Fig. 5). These data are not consistent with the hypothesis of a part of DNA having a different turn over from bulk DNA.

Although the specific activity of total DNA rapidly reached a constant level at about 10-20 minutes after injection of thymidine ^{3}H, the radioactivity repartition in chromatographic fractions was time depending over a two hours post injection period (Fig. 6).

At 5 minutes, the specific activity of 0.7 M fraction was only one third that of the unfractionated DNA; it then increased regularly over the first two hours after labelling to reach a value nearly equal to that of total DNA.

The 0.8 M fraction was rapidly labelled; at 5 minutes, its specific activity was two to five times greater than that of total DNA towards which it then decreased, remaining nevertheless slightly higher at 2 and 7 hours.

The P fraction was also rapidly labelled but one must be cautious about calculated S. A. values due to its very low concentration and to possible release into it of column

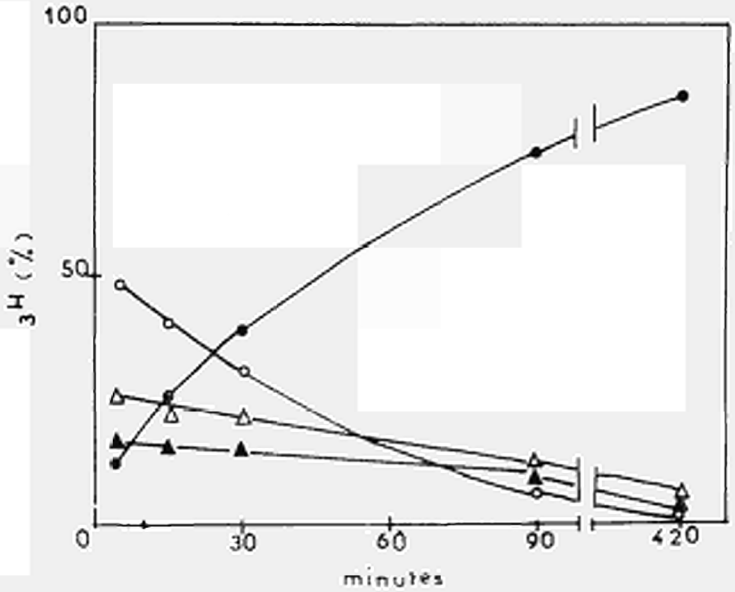

FIG. 5 — Specific activities of total DNA's extracted by the Schmidt and Thannhauser procedure at various times after thymidine ^{3}H injection (25 μc/mouse) and chase by 2 μM of cold thymidine 5 min after the label injection.

For each time, ascites cells from 15 mice were pooled.

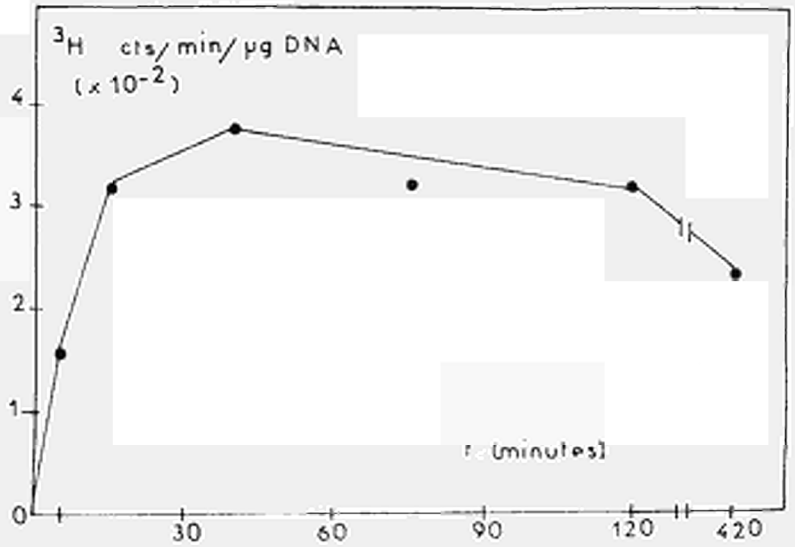

FIG. 6 — Repartition of radioactivity in the different chromatographic fractions versus time

● 0.7 M fraction
△ 0.8 M fraction
▲ pH fraction
○ NE part.

U.V. absorbing material. Nevertheless, one can state that its maximum S. A. was higher than that of bulk DNA over the 5-15 minutes range and then decreased.

The NE part was very important into 5 min DNA; nearly half of the radioactivity could not be eluted from the column. On the other hand, into 2 and 7 h DNA's, NE part is strongly reduced.

The overall pattern of these kinetic data surprisingly pointed to the rather long life span of newly labelled forms of DNA; one must last for more than two hours after pulse labelling to get a DNA devoid of radioactive material displaying a different behaviour from total U.V. material.

4 — DISCUSSION

When DNA was extracted from cells withdrawn after a short *in vivo* (^{3}H) thymidine pulse, most of the radioactive label was found in a deoxyribonucleic material which differed from bulk DNA by several criteria : slower sedimentation rate in sucrose gradient; higher affinity for columns of methylated albumin: higher sensitivity to alkaline denaturation, i.e. lowering of the pH at which began the alkaline induced helix-coil transition (alkaline CsCl density gradients). However, at neutral pH its behaviour in Cs_2SO_4 and CsCl density gradients did not differ from that of bulk DNA.

As shown by the results, such a behaviour could not be accounted for only by a difference in base composition or in molecular size of newly labelled DNA, therefore it should be related to structural modifications.

DNA intra-helical rearrangement leading to intermediate structural states between helix and coil may be evoked; it could involve breakage or weakening of some hydrogen bonds or modification of Van der Waals and hydrophobic forces. These naturally occuring forms may have some analogy with intra-helical rearranged structure experimentally observed by quite different physical techniques : microspectrophotometry (Chamberlain and Walker, 1965); spectrophotometry and viscosimetry (Freund and Bernardi, 1963; Tikhonenko, Perevertaylo and Dobrov, 1963); oscillopolarography (Palecek, 1965); circular dichroism (Brahms and Mommaerts, 1964) and low angle X-ray scattering (Luzzati, Mathis, Masson and Witz, 1964). Moreover, the existence of DNA metastable forms related to replication has been postulated in bacteria as well as in mammalian cells by Rosenberg and Cavalieri (1964).

One can point out the remarkable stability of the replicative structures presently described : they withstood mechanical disruption of cells and nuclei and, after extraction, did not disappear after such treatment as ethanol precipitation, CsCl centrifugation and proteolytic enzymes treatment. This contrasts with the fragility of replicative forms of bacterial DNA (Rolfe, 1963; Hanawalt and Ray, 1964).

An other explanation for the maintenance of the modified molecular structure can be offered by sticking of small amounts of non DNA material related to unknown substances or specific proteins like those demonstrated for bacteria (Jacob, Brenner and Cuzin, 1963; Lark and Lark, 1964) which seem to be involved in the physiological function of DNA.

A point of importance is to establish whether these replicative forms characterize the nuclear DNA or whether they are restricted only to a part of the DNA, either endogenous (mitochondrial) or from symbiotic organisms.

DNA-like fibers have been visualized through electron microscope inside mitochondrial bodies of Ehrlich cells (Nass and Nass, 1964) and characterized into mice tissues (Corneo, Moore, Sanadi, Grossman, Marmur, 1966). However, this deoxyribonucleic material

is quantitatively very small and can probably be neglected unless thymidine incorporation is preferential at this cytoplasmic level; although such an eventuality is not supported by well known autoradiographic data, it has recently been reported that rat liver mitochondrial DNA is much more rapidly labelled than nuclear DNA (Schneider and Ruff, 1965).

The replicative fractions of 5 min DNA cannot be accounted for by DNA of bacteria or PPLO which were not, or very rarely, found in our ascites cells. The hypothesis of a viral DNA cannot be completely ruled out but two lines of evidence made it quite unlikely : permanence of observations made on different lots of animals and different types of cells (Ehrlich and Krebs ascites) which could not be expected from viral cycles due to their biological variability and host cells specificity; identical buoyant density values of radioactive and bulk DNA which implicates the same GC content for each material in a 2-3 % range.

Finally, the best explanation for the kinetic data is that newly replicated DNA is a macromolecular transient form of metabolically stable DNA into which it is transformed by structural change. It is not possible to establish whether such forms actually do exist or not in the cells; their appearance from replicating DNA during cell disruption and DNA extraction through an enzymatic equipment or any other process, remains an open possibility.

The persistance of DNA replicative structures long after incorporation of thymidine into polynucleotidic chains is quite an interesting fact when correlated with the results obtained by Cairns (1966) through autoradiographic studies on DNA replication into HeLa cells. This observation is in support of Cairns' conclusion that DNA synthesis speed is lower in animal cells than in bacterial ones. However, if this measured speed was, at any replicative point, the same and constant all over the S period, i.e. 0.5 μ/mn, our replicative forms would not last more than 5 to 10 minutes. The only way to explain our much longer duration is to postulate either the non constancy of speed for each replicative point or a variation of speed from one point to another one depending on its location on peculiar chromosome(s) or on some peculiar part(s) of given chromosome(s). Asynchronous speed for DNA synthesis observed at the chromosome level has already been reported (Moorhead and Defendi, 1963; Petersen, 1964; Galton and Holt, 1965).

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