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Deoxyribonucleic Acid Synthesis in Mammalian Systems

PERMEALYSED EHRLICH ASCITES CELLS *IN VITRO* FIRST LABEL OKAZAKI-TYPE LOW-MOLECULAR-WEIGHT DEOXYRIBONUCLEIC ACID AND THEN HIGH-MOLECULAR-WEIGHT DEOXYRIBONUCLEIC ACID

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During the evaluation of a method of preparing permealysed Ehrlich ascites cells, short-term labelling experiments were carried out with d[^{3}H]TTP. In the first minute the bulk of the label appeared as low-molecular-weight pieces of DNA. Subsequently the label appeared in DNA of much higher molecular weight. A brief description of the preparation procedure and the properties of the product is provided. Evidence is presented to show that the nucleotide was incorporated directly without intermediate conversion into dTMP or thymidine.

Previous studies in this laboratory with isolated nuclei from rat liver have been on preparations that showed, to variable degrees, two different types of behaviour—one in the presence of calcium and one in its absence.

DNA synthesis in nuclei prepared by one procedure (Burgoyne *et al.*, 1970*a*,*b*) was almost completely dependent on a calcium-dependent endonuclease activity. This enzyme cleaved a large number of phosphodiester bonds in the primary structure of the DNA. These acted as priming sites for the low-molecular-weight DNA polymerase (Wallace *et al.*, 1971). The result of this was an incorporation of label that consisted of stretches 1–3 nucleotides long added to a large number of termini (Waqar, 1972; M. A. Waqar & L. A. Burgoyne, unpublished work). Thus in an alkaline sucrose gradient the sedimentation pattern of the label almost corresponded to the sedimentation of the bulk of the DNA or the label sedimented somewhat slower than the bulk DNA (Burgoyne *et al.*, 1970*a*,*b*; Waqar, 1972). The lower the molecular weight of the bulk DNA, the more nearly did the peak of DNA correspond to the peak of radioactivity. These observations are explained when the following points are considered.

The ratio of 3′-OH terminus nucleotides relative to internal, non-terminus nucleotides is inversely proportional to molecular weight with single-stranded DNA molecules. As the newly synthesized DNA was a very small proportion of the total DNA and consisted of short lengths attached to the 3′-OH termini of the old DNA (Waqar, 1972) it had very little effect on the sedimentation or diffusion rate of the resultant molecules. Thus the ratio of newly synthesized DNA, relative to total DNA, will decrease as the sedimentation coefficient of the resultant molecules increases down a heterogeneous peak.

This effect would not be as pronounced at very low molecular weights because the resolution of lower-molecular-weight classes is low.

The second type of behaviour could be observed in the absence of the calcium activation; in this case nucleotide incorporation was greatly decreased but the very small amount of DNA that was labelled had a lower molecular weight than the bulk DNA and it did not get transferred into the high-molecular-weight class (Waqar, 1972).

Thus permealysed cells were used in an attempt to get a preparation intermediate between normal cells and nuclei, which synthesizes high-molecular-weight DNA *in vitro* in a similar manner to that of whole cells. This paper describes results obtained by using such a preparation obtained from Ehrlich ascites cells.

Experimental

Materials

The preparation procedure relied on the use of high-density Ficoll solutions. These are viscous and difficult to pipette accurately and thus all accurate measurements and dilutions of these solutions were made by weight.

Ficoll (mol.wt. 400000) was supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. All weights were uncorrected for water content as the water content was low and relatively constant (approx. 1.8% in our batches).

Standard 54% Ficoll. To make 20ml of solution, 10.8g of Ficoll was placed in a 100ml centrifuge tube, then 2ml of the appropriate buffer (10×concentrated) and 11.07ml of water or water containing other additives was added. The centrifuge tube was incubated at approx. 50°C for 5min with gentle shaking and centrifuged at approx. 10000*g* for 30min, with the temperature set at approx. 20°C. After this procedure, solution of the Ficoll was almost complete. The solution was finally mixed with a thin glass-rod.

All other Ficoll solutions were made by appropriately diluting weighed lots of the standard 54% Ficoll solutions with weighed lots of buffers.

Buffers. Phosphate saline. The 10×concentrated stock solution contained 100mM-Na_2HPO_4 and 8.5% (w/v) NaCl, adjusted to pH7.4 with HCl. Buffer G. The 10×concentrated stock solution contained 2mM-spermidine, 0.1M-hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethane sulphonic acid] and 0.7M-tris, adjusted to pH7.4 with HCl. EDTA and EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] were kept as 100mM stock solutions of their sodium salts at pH7.4. Ascites cells. Ehrlich ascites tumour cells were maintained in Swiss albino mice and transferred at intervals of 4–6 days, so as to maintain high growth rates. Preparations were made from cells that were grown 4–6 days in their last host.

Methods

Procedure. Operations A–C were done at approx. 20°C and were to remove any dense debris, such as erythrocytes, from the ascites suspension. All Ficoll concentrations are w/w.

A. Heparin (100 units) was given intraperitoneally to an infected mouse and the cells were harvested.

B. Each ml of ascites suspension had mixed into it 1.5g of 54% Ficoll buffered with phosphate saline. This was placed in a 5ml centrifuge tube and overlaid with approx. 1.5ml of 27% Ficoll buffered with phosphate saline and 1ml of 16% Ficoll buffered with phosphate saline. This was then centrifuged in a swing-out rotor for 20min at 75000*g*.

C. The cells that migrated up to the interphase between the 27% Ficoll and the 16% Ficoll were collected.

D. The cells were dispersed in cold (0–4°C) 40% Ficoll in buffer G, containing 4mM-EDTA, 4mM-EGTA, 15mM-2-mercaptoethanol, and were homogenized in an ordinary Teflon–glass tissue homogenizer.

E. The homogenate was centrifuged at 25000*g* for 30min at 12°C.

F. All subsequent operations in this procedure were done at 0–4°C. The pellet from step E was dispersed in approx. 1ml of 15% Ficoll in buffer G, containing 0.1mM-EDTA, 0.1mM-EGTA and 15mM-2-mercaptoethanol, and was layered on to a discontinuous gradient in a 5ml centrifuge tube as follows: 0.7–0.8g each of 37% Ficoll, 41% Ficoll, 45% Ficoll, 50% Ficoll, 54% Ficoll, all buffered with buffer G, containing 15mM-2-mercaptoethanol, 0.1mM-EDTA and 0.1M-EGTA.

G. This solution was centrifuged at 75000*g* for 45min at 0–4°C in a swing-out rotor [MSE SW, 3×5ml tubes; $r_{av.}$ 7.39cm (2.906in)].

H. The permealysed cells were in a layer just above the bottom of the centrifuge tube. They were removed carefully to avoid contamination with the upper layers in the preparation tube. This was most conveniently effected by a stepwise aspiration of the upper layers of the gradient. After the removal of each layer the exposed walls of the tube were washed with water and then the debris-laden water was aspirated before the aspiration of the next layer.

I. The preparation was washed by centrifugation at 10000*g* for 15min from 15% Ficoll in buffer G, containing 15mM-2-mercaptoethanol, 0.1mM-EDTA and 0.1mM-EGTA. It was finally suspended in the same solution.

Assays. The usual assay mixture contained: 3mM-ATP, 2mM-phosphoenolpyruvate, 9mM-$MgCl_2$, 100μmol each of dCTP, dATP, dGTP, 2mM-EGTA, 0.2M-EDTA and 8.9μmol of d[*Me*-^{3}H]TTP (The Radiochemical Centre, Amersham, Bucks., U.K.; 11.3Ci/mmol) in 15% Ficoll in buffer G. The standard assay had a volume of 0.05ml. Variations from these conditions are noted when they occur.

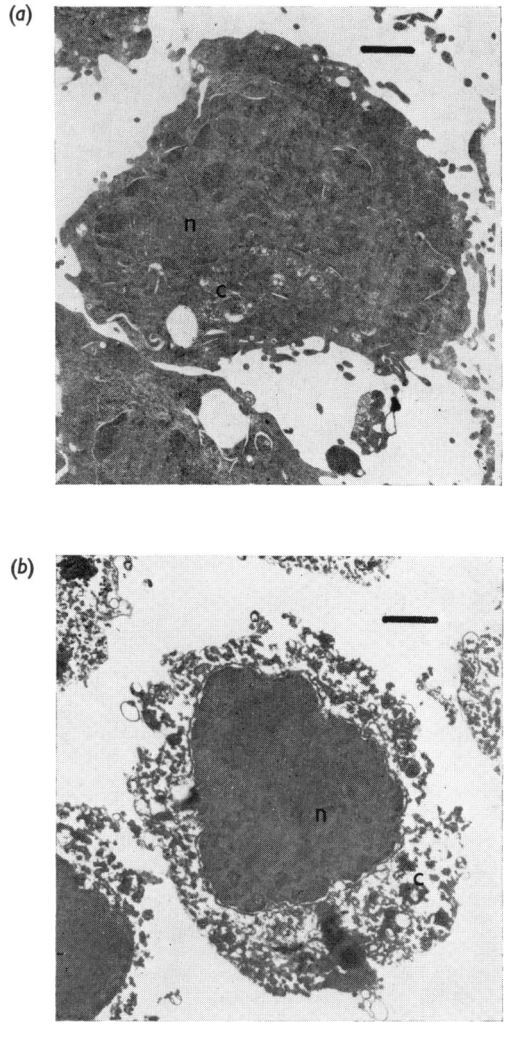
Samples were deposited on Oxoid membrane discs (2.25cm diam., grade 0.45), then given 13 1-h washes in baths of cold 0.12M-H_2SO_4, 0.05M-H_3PO_4 and 0.25M-Na_2SO_4 followed by a brief wash in 1% trichloroacetic acid, neutralized by being exposed to NH_3 vapour, and then were dried and counted in a scintillation counter.

Time-course of DNA synthesis. At the cell densities in the assays reported in this paper, DNA synthesis usually continued for approx. 10–15min with decreasing rates in the last few minutes. At decreased cell densities, relatively linear rates have been observed for up to 30min.

Results

Permealysed cells

Examination of the product in visible light showed it to consist of particles that look, superficially, like nuclei and which stain differently from normal, whole cells. However, low-power (approx. magnification× 10000) electron micrographs show that much cytoplasmic material was still observable although clearly modified (Plate 1). The fixation and staining procedure used for Plate 1 stained normal cells, nuclei and cytoplasm to almost the same degree, but the nuclei and cytoplasmic remnants in the permealysed



EXPLANATION OF PLATE 1

Electron micrographs of intact Ehrlich ascites cells compared with permealysed cells

Material was fixed in 2% (v/v) glutaraldehyde at 0–4°C for 1h, post-fixed with 1% osmium tetroxide, dehydrated, and embedded in Araldite via an acetone series. Sections were stained with methanolic uranyl acetate and then 0.2% lead citrate. The bar indicates 1μm. (*a*) shows whole cells in 0.85% NaCl and 10mM-potassium phosphate buffer, pH7.4, during fixation. (*b*) shows permealysed cells fixed as in (*a*). n, Nuclear material; c, cytoplasmic material.

cells reacted differently in the fixation and staining procedure.

The product had a different density from that of intact cells in Ficoll solutions and it incorporated d[^{3}H]TTP directly into its DNA without prior conversion into nucleoside (see below).

Proof that d[^{3}H]TTP is not being incorporated as thymidine by intact cells

Omission of dATP, dGTP and dCTP from the standard assay mixture caused more than 90% inhibition of d[^{3}H]TTP incorporation during 15min.

Addition of an excess of non-radioactive thymidine (2mM final concn.) to the standard assay mixture caused approx. 5% inhibition of d[^{3}H]TTP incorporation over 15min. This small inhibition could have two explanations: (*a*) 5% of the d[^{3}H]TTP is being incorporated by whole cells in the form of [^{3}H]-thymidine produced by d[^{3}H]TTP breakdown; (*b*) a very small amount of the non-radioactive thymidine is being converted by a kinase into dTTP, which is diluting the substrate d[^{3}H]TTP.

Thus a standard assay was set up containing 10μCi of [^{3}H]thymidine (19Ci/mmol) instead of the d[^{3}H]TTP. The preparation showed a low, but significant, incorporation of [^{3}H]thymidine. However, this incorporation was completely dependent on the mixture of phosphoenolpyruvate, ATP and the deoxyribonucleotides. This requirement for phosphorylating agents showed that the small thymidine effects were not due to direct uptake by intact cells but rather were due to low concentrations of kinases converting a little thymidine into dTTP.

Short-term labelling experiments

Over a time-period of approx. 2min the presence of 100μM-dTMP did not significantly dilute or otherwise inhibit the incorporation of 8.9μM-dTTP, but 100μM-dTDP effectively suppressed incorporation of radioactivity in less than 20s (Fig. 1). As the rate of synthesis was almost independent of nucleotide concentration between the limits 8–100μM-dTTP, the effect of dTDP is probably due to rapid dilution of the substrate nucleotide.

In the first 1–2min of labelling with d[^{3}H]TTP up to 60% of the radioactivity was found in short lengths of DNA (Fig. 2). However, as labelling continued the bulk of the radioactivity was found in higher-molecular-weight regions.

Similar results were also obtained if the incubation had a large amount of non-radioactive dTTP added after 2.5min (Fig. 3). In both of these experiments the assay mixture (see the Figure legends) contained 2mM-non-radioactive thymidine to thoroughly exclude any possibility that significant radioactivity uptake was occurring via non-phosphorylated thymidine.

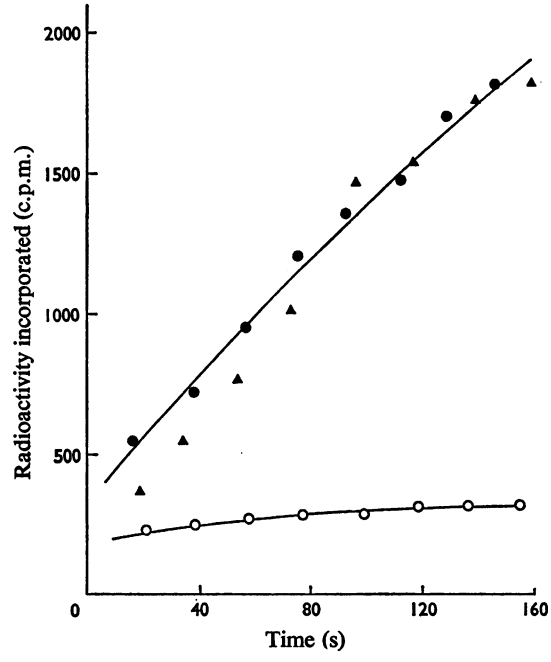


Fig. 1. *Competition of dTMP and dTDP with the substrate d[^{3}H]TTP in permealysed cells*

The assay mixture was as described in the Experimental section except that the final volume was 0.25ml, the d[^{3}H]TTP had specific radioactivity 23.9Ci/mmol, and all assays contained 1mM-non-radioactive thymidine and cells equivalent to 71μg of DNA. Samples (0.025ml) were taken at the times indicated and the reaction stopped by dispersion in a solution containing 0.025ml of water and 0.01ml of 1.8M-KOH and 100mM-EDTA. The alkaline solutions were dried on to Oxoid membranes and acid washed as described in the Experimental section. ●, No further additions; ▲, 100μM-non-radioactive dTMP added; o, 100μM-non-radioactive dTDP added.

Effects of detergent and urea on the preparation of permealysed cells

The preparation could be dispersed in buffer G containing 4mM-EDTA, with 1% Triton X-100 or 7M-urea without immediate disruption of the nuclei. Under these conditions the nuclei did not appear to lyse when observed with the light microscope.

A preparation was labelled with d[^{3}H]TTP for 22min and then washed once with the detergent or

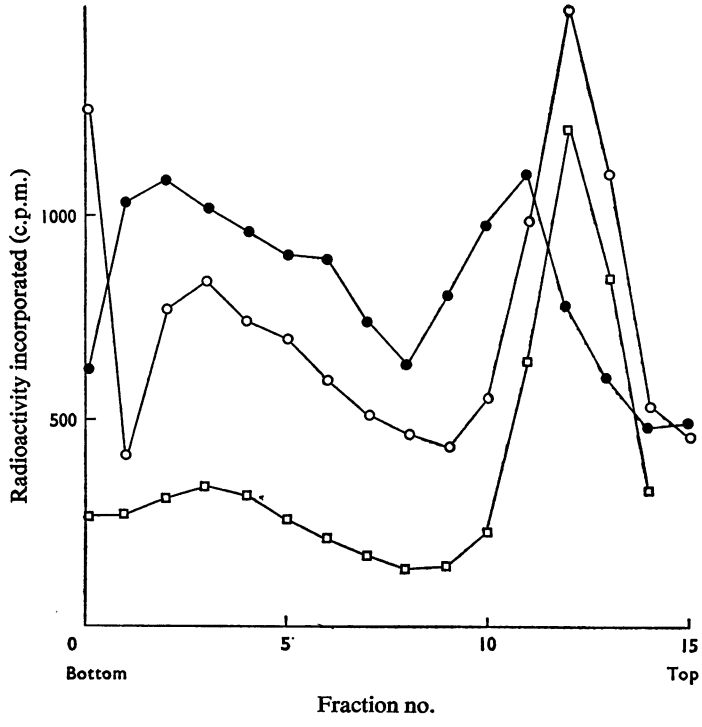


Fig. 2. *Alkaline sucrose-gradient analysis of product DNA after short-term labelling with d[^{3}H]TTP*

The assay mixture was as described in the Experimental section and contained 2mM-non-radioactive thymidine, and nuclei equivalent to 450μg of DNA in 0.5ml. At stated times, samples were taken and dispersed in buffer G, containing 4mM-EDTA at 0°C. The nuclei were centrifuged down, redispersed in a small volume of buffer G and lysed with one-fifth the volume of 1.8M-KOH and 100mM-EDTA. The wash with buffer G containing 4mM-EDTA had to precede lysis because Ficoll caused artifacts in the alkaline gradients even at considerably lower densities than those of the upper layers of the gradient. Lysate, equivalent to 77μg of DNA, was loaded on to alkaline-saline sucrose gradients (Olivera & Lehman, 1967) and centrifuged at 25000rev./min (52000$g_{av.}$) for 15.5h at 7.5°C. Fractions (0.35 ml) were collected, deposited on membrane filters by precipitation with 10% (w/v) trichloroacetic acid, fixed on filters by exposure to NH_3 and drying. The filters were then washed 13 times in cold acid and counted for radioactivity as described in the Experimental section. Incubation times: □, 1min; ○, 3min; ●, 6min.

urea solutions (as above) and compared with a sample washed with buffer G containing 4mM-EDTA alone. There was no significant loss of radioactivity in either case.

Discussion

The experiments with thymidine, dTMP and dTDP indicated that the ^{3}H incorporation was from dTTP or dTDP directly, and was thus not due to whole cells taking up [^{3}H]thymidine or even dTMP.

The rapid inhibition of incorporation by dTDP was intriguing but may simply reflect the presence of high amounts of nucleotide diphosphate kinase activity and, perhaps, a slightly higher rate of diffusion of dTDP into the structure in comparison with dTTP.

The preparation incorporated ^{3}H into DNA in a manner which, superficially at least, was very similar to that of normal replicating cells. The label first appeared in species of low molecular weight and then in much higher-molecular-weight species. This type of behaviour has been described during the replication of DNA in bacteria (Okazaki *et al.*, 1968), in whole mammalian cells (Schandl & Taylor, 1969) and in this particular strain of ascites tumour by Waqar (1972). Thus it seemed likely that the processes taking place in this preparation were closely related to the normal replication processes. However, it could be

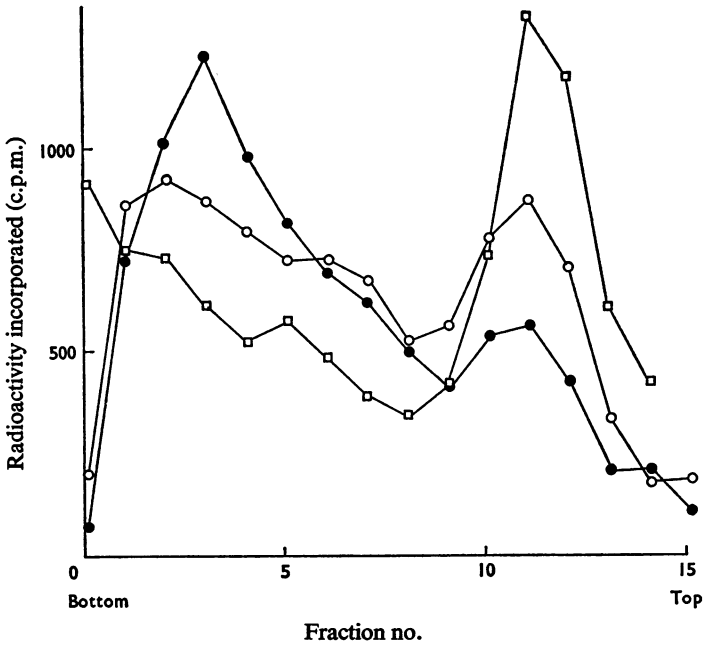


Fig. 3. *Alkaline sucrose-gradient analysis of product DNA after pulse labelling with d[^{3}H]TTP*

All assay conditions were as described in Fig. 1. However, at 2.5min non-radioactive dTTP was added to give a final concentration of 100μM-non-radioactive dTTP in addition to the initial 8.9μM-d[^{3}H]TTP. Centrifugation and gradient details were as in Fig. 1. Incubation times: □, 2min; ○, 6min; ●, 15min. At this nuclear density this is sufficient time for DNA synthesis to have ceased.

argued that the DNA synthesis observed *in vitro* was the repair of damage sustained during the isolation procedure, and it is difficult to exclude this possibility rigorously.

As washing a preparation with 7M-urea or 1% Triton X-100 failed to release measurable amounts of the incorporated ^{3}H it seemed unlikely that the ^{3}H was largely mitochondrial, and thus was most likely nuclear DNA.

The characteristics of DNA synthesis in the present preparation are different from the characteristics of DNA synthesis in the isolated nuclei prepared in this laboratory previously (Burgoyne *et al.*, 1970*a*,*b*; Wallace *et al.*, 1971). The nuclei in the present permealysed-cell preparation rapidly incorporate ^{3}H from dTTP into high-molecular-weight DNA and the preparation is not stimulated by calcium. This is in contrast to the previous nuclear preparations that do not produce a high-molecular-weight-DNA product and are usually measurably stimulated by calcium.

In attempts to gently remove the cytoplasmic sheath from around the nuclei of the permealysed cells the preparation was washed in buffer G containing EDTA without Ficoll. This did not remove the cytoplasmic sheath but it did cause the nuclei to produce some persistent low-molecular-weight labelled DNA rather than transient low-molecular-weight DNA.

In this respect it is noteworthy that necrotic cells in culture may continue to synthesize a low-molecular-weight DNA rather than the normal high-molecular-weight DNA (Williamson, 1970).

Thus I suggest that the correct co-ordination of the processes of DNA synthesis in mammalian cells may be particularly sensitive to the physical properties of the solutions in which they are taking place.

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