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DNA Synthesis in Human Lymphocytes: Intermediates in DNA Synthesis, *in vitro* and *in vivo*

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Two *in vitro* systems, one using a whole cell lysate, and the other a suspension of washed nuclei prepared from a human lymphocyte line, are characterized with respect to their requirements for the synthesis of DNA and to the nature of the synthetic product.

Ribonucleoside triphosphates have a stimulatory effect on DNA synthesis that cannot be accounted for by their effect in maintaining deoxyribonucleoside-triphosphate levels. The rate of synthesis in the lysate is approximately 10% of the *in vivo* rate and is linear for 20 to 40 minutes; in those nuclei that are active, a maximum of 1% of the cellular DNA is newly synthesized.

A class of fragments, with a mean chain length of 120 nucleotides, can be distinguished on polyacrylamide gels and is the earliest labeled intermediate in the whole cell lysate. These short fragments grow to several hundred nucleotides and then attach to high molecular weight DNA. The *in vitro* synthetic product consists of continuous strands several million daltons in size. The *in vitro* fork movement rate, and probably also the individual chain growth rate, are approximately 10% of the corresponding *in vivo* rates.

Washed nuclei make 120-nucleotide intermediates similar to those of the lysate; nuclei, however, do not synthesize high molecular weight DNA, the largest product being around 10 S. If cytoplasmic extract is added back to nuclei some of the ability to synthesize large DNA is restored.

In intact exponentially growing cells labeled with radioactive thymidine, a similar 120-nucleotide DNA fragment has been identified.

1. Introduction

The study of DNA synthesis in cell-free systems offers possibilities for certain biochemical and physical studies which, in intact cells, are made impossible by the barrier imposed by the cellular membrane. With prokaryotes, one approach to cell-free synthesis has been to permeabilize the cell membrane by treatment with organic solvents or non-ionic detergents, or by plasmolysis. In eukaryotes the replicative apparatus is assumed to be in the nucleus, which is stable and permeable after isolation, and has been investigated for its ability to carry on replicative synthesis *in vitro* (Friedman & Mueller, 1968; Lynch *et al.*, 1972; Kidwell, 1972; Probst *et al.*, 1972; Lazarus, 1973; Hershey *et al.*, 1973; Hallick & Namba, 1974). Another approach has attempted to maintain the cell intact and to introduce nucleotides by injection (Waqar & Huberman, 1973) or by permeabilization with DEAE-dextran (Fox, Mynderse & Goulian, unpublished results), or by keeping cell structures partially

intact with Ficoll (Burgoyne, 1972). However, the injection method can apply only to a few multinucleate single-celled organisms, and the available permeabilization techniques may be limited in their ability to add or remove macromolecules.

In the studies to be described here, broken cell systems were developed to permit the study of DNA synthesis in animal cells. Two systems are described, both of which are prepared from a cultured line of human lymphocytes. One consists of a whole cell lysate in which the outer membranes are dissolved by detergent treatment while the nucleus remains intact, whereas the other uses isolated washed nuclei. Both systems have been used to characterize requirements for synthesis and the nature of the synthetic product, particularly its earliest intermediates.

A detailed characterization of the products of cellular DNA synthesis will reveal attributes that may be useful in establishing the relationship between *in vitro* and *in vivo* reactions. One useful characterization, which is employed here, contrasts the large size of new synthesis with the short regions of repair synthesis (Cooper & Hanawalt, 1972; Lehmann, 1972). Another is the discontinuous nature of nascent DNA, originally described by Okazaki *et al.* (1968) for micro-organisms. The size of the nascent DNA is also examined in the intact cultured lymphocyte, to provide a basis for comparison with *in vitro* synthesized products.

2. Materials and Methods

(a) *Cells*

Human lymphocyte cell line 8866 (from Dr R. Lerner, Scripps Clinic, La Jolla, CA.; Fox *et al.*, 1973) was grown in suspension at 37°C in Modified Eagles Medium for suspension cultures (Flow Laboratories Ltd.), containing 2 mM-sodium pyruvate, 1% Antibiotic-Antimycotic Solution (Gibco), 1% Non-essential Amino Acid Solution (Flow), 0·5% Vitamin Solution (Flow), 1·4% $NaHCO_3$, 2 mM-glutamine, and 10% fetal calf serum (Flow). Cells were maintained at 1 to 20×10^5 cells/ml, and harvested at 6 to 9×10^5 cells/ml for experiments.

(b) *Chemicals*

Nonidet P-40 was from Shell, and Sarkosyl (sodium dodecyl sarcosinate) from Geigy. Unlabeled nucleotides were from P-L. *N*-ethylmaleimide, dithiothreitol, and PEP† (tri-sodium) were from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were obtained from Kodak, and agarose and Pronase from Calbiochem. [*methyl*-^{3}H]dThd, [*methyl*-^{3}H]dTTP, [*methyl*-^{14}C]dThd, and [5-^{3}H]dCTP were purchased from Schwarz-Mann. BrdUTP was synthesized by bromination of dCTP followed by deamination with nitrous acid (Bessman *et al.*, 1958). Ara-CTP was synthesized by S. Goulian (Yoshikawa *et al.*, 1967; Sowa *et al.*, 1971).

(c) *Solutions*

Tris-EDTA buffer contains 10 mM-Tris·HCl (pH 7·4), 5 mM-EDTA. Buffer I contains 30 mM-KCl, 60 mM-Na-morpholinopropane sulfonate (pH 7·6), 0·5 mM-$CaCl_2$, 10 mM-$MgCl_2$, 2 mM-potassium phosphate (pH 7·6), 10 mM-glucose and 0·1 mM-dithiothreitol. Sarkosyl lysis buffer contains 1% Sarkosyl, 50 mM-EDTA, 20 mM-Tris·HCl (pH 7·4); phosphate-buffered saline contains 0·9% NaCl, 10 mM-potassium phosphate buffer (pH 7·4). Toluene scintillation fluid contains 8 g PPO and 100 mg dimethyl-POPOP and 3·8 l toluene. Triton X100 scintillation fluid contains 1 l Triton X100 (Rohm and Haas), 16 g PPO, 200 mg dimethyl-POPOP, and 3 l toluene. Alkaline lysis buffer contains 1 M-NaOH, 0·05 M-potassium pyrophosphate, 0·05 M-potassium tripolyphosphate, 1 mM-EDTA.

† Abbreviations used: PEP, phosphoenolpyruvate; ara-CTP, 1-β-D-arabinosylcytosine-5'-triphosphate; BrdUTP, bromodeoxyuridine triphosphate.

(d) In vivo *labeling of DNA with* [^3H]*deoxythymidine*

To cells that had been prelabeled overnight (18 h) with [^{14}C]dThd (1 nCi/ml, 33 mCi/mmol), and which were growing logarithmically at 8×10^5/ml, was added [^3H]dThd (20 μCi/ml, 50 Ci/mmol). The suspension was gently mixed at 37°C, and samples removed at the indicated times and pipetted rapidly into an equal volume of cold ethanol. Unlabeled cells diluted in ethanol were added to samples to give an equivalent amount of DNA in all samples, and all were centrifuged (4000 *g*, 10 min). The cell pellets were resuspended in 1 ml of 0·9% NaCl, 2 mg Pronase/ml, 50 mM-EDTA, 10 mM-potassium phosphate (pH 7·4), 20 mM-Tris·HCl (pH 7·4). Sarkosyl was added to 1% and digestion allowed to continue overnight at 45°C, before centrifugation in alkaline sucrose gradients.

(e) In vitro *synthesis of DNA*

Cells were collected and washed once with cold phosphate-buffered saline, with centrifugation each time at 120 *g* for 10 min. The packed cells (5×10^8/ml) were resuspended in an equal volume of buffer I containing 1 M-sucrose, then made 0·25% Nonidet P-40 and the resulting "lysate" kept at 0°C for at least 5 min before using for incorporations.

Nuclei were prepared by centrifugation of the lysate at 500 *g* for 5 min, followed by two washes in 1 M-sucrose–buffer I and finally resuspended in an equal volume of 1 M-sucrose–buffer I. The supernatant from the first centrifugation of lysate is referred to as cytoplasmic extract.

Except where stated otherwise the standard incorporation mixture (0·15 to 0·3 ml) contains 80 mM-sucrose, 20 mM-KCl, 0·5 mM-$CaCl_2$, 9 mM-$MgCl_2$, 1·5 mM-potassium phosphate (pH 7·6), 20 mM-glucose, 45 mM-morpholinopropane sulfonate (pH 7·6), 20 mM-PEP, 0·1 mM-dithiothreitol, 2·5 mM-ATP, 0·2 mM each of GTP, CTP, UTP, and the three unlabeled dNTPs, 0·02 mM of the labeled deoxynucleotide (0·8 Ci/mmol) (dTTP unless stated otherwise), and lysate or nuclei equivalent to 4×10^7 or 8×10^7 cells/ml, respectively. Standard incubation was at 37°C and was carried out with gentle agitation in a shaker water bath.

For measurement of total incorporation, reactions were stopped by mixing with 2 ml alkaline lysis solution; when nucleic acids are to be analyzed further the samples are diluted with 2 ml Sarkosyl lysis buffer.

In incubations where BrdUTP replaced dTTP, the rate and extent of incorporation were the same (within 10%) as parallel incubations with dTTP. In all pulse–chase experiments, there was less than 10% increase of label incorporation during the chase.

(f) *Preparation of samples*

To prepare DNA from incubation mixtures for subsequent analysis (e.g. CsCl, sucrose gradient, etc.) the sample in Sarkosyl lysis buffer was digested overnight at 45°C with Pronase (2 mg/ml), then recovered by ethanol precipitation. Fractions from alkaline sucrose gradients that were to be analyzed further (e.g. by polyacrylamide gel electrophoresis) were pooled, brought to pH 5 to 6 with acetic acid and precipitated with ethanol. Precipitates were redissolved in Sarkosyl lysis buffer, digested with Pronase, and reprecipitated with ethanol.

Ethanol precipitations of nucleic acid were carried out by adding 2·5 vol. ethanol to a sample or fraction that was 0·3 to 1·0 M in Na^+, K^+ or Li^+, pH 5 to 6, and contained at least 20 μg nucleic acid/ml (carrier RNA or DNA added, if necessary). The mixture was kept overnight at −20°C, and the precipitate collected by centrifugation (12,000 *g*, 30 min, 4°C), and redissolved in Tris-EDTA buffer, unless stated otherwise.

Acid insoluble counts were determined by precipitation with 1 M-HCl at 0°C for 5 min, in the presence of 250 μg carrier DNA (herring sperm), 2 mg potassium pyrophosphate, and 2 mg potassium tripolyphosphate, followed by filtration onto glass fiber filters (GF/C, Whatman). The latter were washed extensively with 0·01 M-HCl (25 ml), then with ethanol, dried, and counted in toluene scintillation fluid.

Assays done directly on incubation mixtures containing cell lysate or nuclei required a double precipitation to reduce background counts. Samples were first heated in alkaline lysis solution (2 ml) at 100°C for 5 min, then were precipitated in the cold (10 min) with

3 ml 2 M-HCl. The precipitate was collected by centrifugation (12,000 *g*, 10 min), redissolved in 2 ml alkaline lysis solution, heated at 100°C, 5 min, then reprecipitated in the cold with 3 ml 2 M-HCl, and collected and counted on GF/C filters.

When Sarkosyl was present in samples to be acid precipitated (e.g. top fractions of sucrose gradients in which sample applied to gradient contained Sarkosyl), 2 vol. ethanol were added to the acid precipitation mixture to avoid interference by the Sarkosyl with acid precipitation of nucleic acid.

(g) *Velocity sedimentation*

Alkaline sucrose gradients were 5% to 20% sucrose, 0·3 M-KOH, 0·7 M-KCl, 1 mM-EDTA. Samples were brought to 0·3 M-KOH, 0·7 M-KCl, 1 mM-EDTA and held at 25°C for 1 h before applying to the alkaline gradient.

Neutral gradients were 5% to 20% sucrose, 1 M-KCl, 2 mM-EDTA, 10 mM-Tris·HCl (pH 7·4).

All fractions were collected from the bottom of the centrifuge tube. The size of BrdUTP DNA was estimated from sedimentation velocity in 3·8 ml neutral sucrose gradients in a SW56 rotor (Spinco) at 45,000 rpm for 90 min at 20°C, with internal 30 S (phage fd DNA) marker. For low molecular weight material, a 12 S (minicircular) DNA marker was used, and the time of centrifugation was increased to 4 h.

Other conditions for centrifugations are included in Figure legends.

(h) *Polyacrylamide gel electrophoresis*

Gels (8 cm × 0·7 cm) were 3% acrylamide (19:1, acrylamide/methylenebisacrylamide), 0·5% agarose, 6·5 M-urea, 0·04 M-sodium acetate (pH 5), 1 mM-EDTA, 0·05% tetramethylethylenediamine, 0·075% ammonium persulfate. Electrophoresis buffer contained 7 M-urea, 0·04 M-sodium acetate (pH 5), 1 mM-EDTA (Heyden *et al.*, 1972). Samples were concentrated by ethanol precipitation, washed with ethanol, dried, taken up in 0·15 ml 7 M-urea, 20% sucrose, and heated to 80°C for 5 min before applying to the gels. Electrophoresis was carried out at 3 mA/tube. Bromophenol blue (0·01%) was included in the sample, as a visual marker. Gels were cut into 2-mm fractions with a Mickle gel slicer (Brinkmann), digested with 0·5 ml H_2O_2 (30%) overnight at 60°C, and counted in 5 ml Triton X100 scintillation fluid.

(i) *Nucleic acids*

Sedimentation markers of fd DNA (Tseng & Marvin, 1972) and *Escherichia coli* 15 minicircular DNA (Cozzarelli *et al.*, 1968) were prepared according to published procedures. S values were calculated as a linear function of distance sedimented relative to the markers and were related to molecular weight by empirical equations (Studier, 1965).

Markers for electrophoresis were (1) [^{32}P]transfer RNA purified by electrophoresis from the Hirt (1967) supernatant from ^{32}P-labeled *E. coli*; (2) 140-nucleotide [^{32}P]RNA marker from T4 infected cells, a gift from J. Abelson (Paddock & Abelson, 1973); (3) DNA markers prepared from sonicated ^{32}P-labeled *E. coli* DNA fractionated by velocity sedimentation, and confirmed for size by total to terminal ^{32}P with bacterial alkaline phosphatase.

Lymphocyte [^{32}P]DNA marker for equilibrium gradient centrifugation was prepared from ^{32}P-labeled lymphocytes (0·1 mCi/ml medium) by digestion with RNase A (0·2 mg/ml; 3 h, 37°C) and Pronase (2 mg/ml; 16 h, 45°C) in the presence of 1% sodium dodecyl sulfate, and then by extraction with phenol, and equilibrium centrifugation in CsCl.

A sample of lymphocyte DNA was prepared for analysis of cellular DNA content by Sarkosyl–Pronase digestion, phenol extraction and digestion with 0·3 M-NaOH (37°C, 18 h), followed by precipitation with alcohol and re-solution in 10 mM Tris·HCl (pH 7·5), 5 mM-$MgCl_2$, 0·1 mM-EDTA. Using 44 μg/ml as the concentration of DNA corresponding to an absorbance of 1·0 O.D. at 260 nm (Schaller *et al.*, 1969), and a correction for the cells in S phase (taking the latter as 30% of the generation time, and assuming that cells in S phase have an average of 1·5 × diploid cell content of DNA), the resulting figure was 7·7 pg/diploid cell. This is similar to the published figure of 7 pg/cell (Métais & Mandel, 1950) for human lymphocytes.

3. Results

(a) *DNA synthesis in whole cell lysates of lymphocytes*

(i) *Overall kinetics and requirements for incorporation*

The whole cell lysate used in these experiments is made by treating cells with 0·25% Nonidet P-40 which results in the dissolution of the outer cell membrane leaving the nucleus intact with associated cytoplasmic membranes. In the presence of all four dNTPs and salts, the lysate incorporates [^{3}H]dTTP into acid-insoluble material linearly for 20 minutes (Fig. 1), and at a diminishing rate for an additional 10 to 20 minutes. With the generation time of 15 hours (37°C) and cell DNA content of 7·7 pg the overall *in vivo* rate of synthesis in a random population of cells is 280 pmoles total nucleotide/min per 10^7 cells. The *in vitro* incorporation corresponds to about 35 pmoles total nucleotide/min per 10^7 cells or approximately 12% of the *in vivo* rate. In those cells that are active in this system, presumably the one-third of cells in S phase at the time of harvesting, approximately 1% of the cellular DNA is synthesized during the course of a 30-minute incubation.

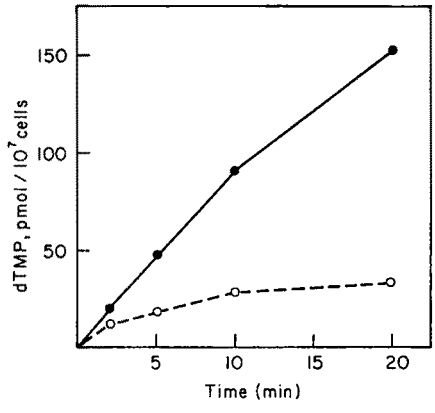

FIG. 1. DNA synthesis in lymphocyte lysate and nuclei.
Parallel incubations (0·15 ml) under standard conditions (see Materials and Methods), of lymphocyte whole cell lysate and washed nuclei, for the times indicated. Background levels in parallel incubations containing EDTA (30 mM) were less than 1 pmol and essentially constant during the course of incubation. —●—●—, lysate incorporation; --○--○--, nuclei incorporation.

Requirements for synthesis are listed in Table 1. All four deoxyribonucleoside triphosphates must be present; synthesis is decreased 60 to 70% by eliminating one nucleotide. ATP and PEP are also necessary; breakdown of nucleotides is extremely rapid in their absence. The dependence upon ATP and PEP will be discussed further below. Synthesis is inhibited by *N*-ethylmaleimide and also by ara-CTP, the nucleoside of which is a potent inhibitor of DNA synthesis *in vivo* (Roy-Burman, 1970). Incorporation is not *via* the nucleoside as DNA is not labeled when [^{3}H]dThd is substituted for [^{3}H]dTTP (not shown). The apparent K_m for dTTP in this system is 4 to 6×10^{-6} M. Incorporated counts are found in the nucleus when it is separated from cytoplasmic debris by centrifugation; this indicates that the DNA synthesis observed is not mitochondrial or in fragments of DNA released from damaged nuclei.

TABLE 1

Requirements for DNA synthesis in lymphocyte lysate

Incubation mixture	Activity (%)
Complete	100
−dATP	37
−dGTP	27
−dCTP	31
−dATP, −dGTP, −dCTP	12
−ATP	57
−PEP	14
−ATP, −PEP	5
−GTP, −UTP, −CTP	95
$-Mg^{++}$	1
$-Mg^{++}$, $+Mn^{++}$ (1 mM)	46
+ara-CTP (0·3 mM)	8
+*N*-ethylmaleimide (1 mM)	4
−glucose	82
−dithiothreitol	89

Standard conditions (Materials and Methods) for incubations (0·3 ml) of whole cell lysate, carried out for 15 min at 37°C, with [^{3}H]dTTP.

(ii) *Rate and size of synthesis measured by BrdUTP incorporation*

Studies of DNA synthesis have led to the observation that replicative synthesis is extensive while repair synthesis occurs predominantly in limited regions (Cooper & Hanawalt, 1972). Substitution of the density label BrdUTP for dTTP during synthesis allows a distinction to be made between *in vitro* product and pre-existing DNA on the basis of density.

The product of the incorporation was followed as a function of time in order to measure the progressive change in density. The nucleic acids were isolated after 5 or 30-minute incorporation times and centrifuged to equilibrium in a CsCl gradient. Within the time period used only 50% substitution is expected for semi-conservative synthesis, i.e. with one new and one old strand in duplex molecules.

The density change found in the *in vitro* synthesized DNA indicated a progressive increase in substitution of dTMP by BrdUMP, from 5% after 5 minutes (Fig. 2(a)) to a broad range with an average of 20% substitution after 30 minutes (Fig. 2(b)). With an average size estimated, by velocity sedimentation (Materials and Methods), at 25×10^6 daltons for the double-stranded DNA (Fig. 2(a) and (b)), the size of the substituted DNA would correspond to $1{\cdot}25\times10^6$ daltons after 5 minutes and 5×10^6 after 30 minutes of synthesis. The growth rates calculated from the 5 and 30-minute values are 800 and 500 nucleotides/minute (average of 650), respectively. To calculate fork movement rate requires, in addition, an estimate of the number of forks contributing to the synthesis of a 25×10^6 dalton fragment (Fig. 2(a),(b)). This can be approximated by a comparison of the fragment size to the average inter-fork distance. Huberman & Riggs (1968) demonstrated, by autoradiography, bidirectional replication from origins spaced 30 μm apart (60×10^6 daltons, double strand), although this may be a slight overestimation (Hand & Tamm, 1974). The average inter-fork distance of the replicative units or "replicons" would be half the average inter-origin distance, or 30×10^6 daltons. Since the isolated fragments (25×10^6 daltons)

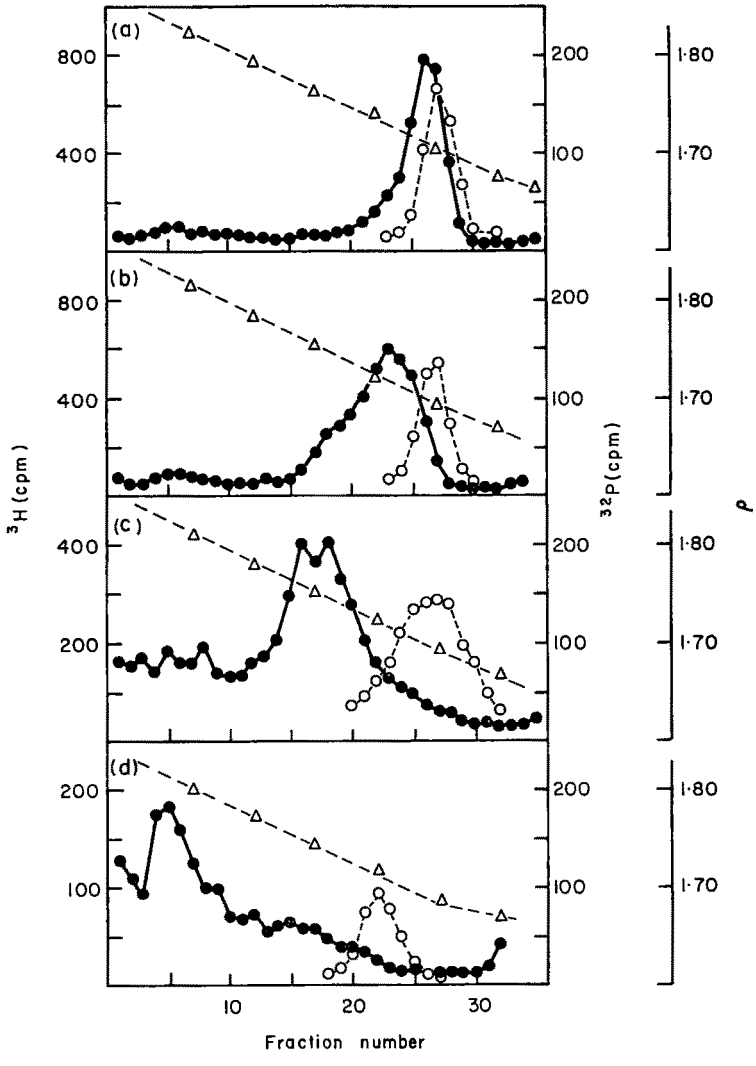

FIG. 2. Equilibrium density gradient centrifugation of DNA labeled with BrdUTP in lymphocyte lysate.

Standard conditions (see Materials and Methods) were used for incubation of lymphocyte lysate except that the labeled dNTP was [^{3}H]dCTP (0·06 mM), and BrdUTP was substituted for dTTP. Incubations (0·3 ml) were carried out at 37°C for 5 and 30 min. Incorporation was stopped with 2 ml Sarkosyl lysis buffer, it was sheared by 4 passages through a 21G needle and digested with Pronase. DNA was recovered by ethanol precipitation and resuspended in Tris-EDTA buffer. Equilibrium density gradient centrifugation was carried out in a mixture of 7·2 g Tris-EDTA buffer and 10 g CsCl (ρ = 1·75) containing a portion of the sample and a [^{32}P]DNA (lymphocyte) marker, in a type 65 (Spinco) rotor at 45,000 rpm for 48 h at 25°C.

(a) 5-min sample; (b) 30-min sample; (c) 5-min sample, sonicated before banding (1 min at 40 W, Branson Microtip); (d) 30-min sample, heat denatured (100°C, 5 min) before banding.

—●—●—, ^{3}H; --○--○--, ^{32}P; --△--△--, ρ (determined by pycnometry).

containing the newly synthesized regions are approximately the same size as the average inter-fork distance (30×10^6 daltons), the fragments would contain between one and two growing points. Therefore, 1·5 forks would contribute to the overall rate for a strand and the average rate would then be about 400 nucleotide-pairs/min per fork. The range for rates of synthesis measured by autoradiography (Huberman & Riggs, 1968) is 0·5 to 1·2 μm/minute. An average rate of 1 μm/minute *in vivo* corresponds to synthesis of 3000 nucleotide-pairs/min per fork. The measured rate for fork movement *in vitro* is, therefore, about 15% of the *in vivo* rate. The similarity of the latter to the corresponding figure for overall synthesis (see previous section) suggests that there is a slowdown in the *in vitro* activity of individual replicative units but that the total number of active replicative units is close to normal.

The continuity of new synthesis assumed in the above calculations can be demonstrated in two ways. First, after sonication of the 5 and 30-minute samples to $0{\cdot}25 \times 10^6$ daltons (estimated by velocity sedimentation), the densities of both samples were found to be fully hybrid (Fig. 2(c), 5-min sample shown). Since sonication results in random breakage of the isolated DNA, and most of the label is at hybrid density, the size of the hybrid region before sonication must have been several times the sonicated size. The material that bands in the heavy region of the gradient after sonication (Fig. 2(c)) may be due to denaturation during sonication; the amount did not increase with incubation time. Another demonstration of the continuity of *in vitro* synthesis can be made through measurement of the single strand size and density of the product. After heat denaturation of the isolated DNA, the predominant portion of the product (70%) after a 30-minute synthesis is at the density of fully substituted single strands (Fig. 2(d)). The single strand size (measured by velocity sedimentation) ranges from 0·5 to 5×10^6 daltons with an average value of 2×10^6 daltons. From these two types of evidence it may be concluded that the *in vitro* product consists of continuous strands of at least several million daltons.

(iii) *Discontinuous synthesis*

To test whether synthesis in the lysate proceeds through a discontinuous intermediate, the product of synthesis after very short labeling times, with and without subsequent dilution of label, was analyzed in alkaline sucrose gradients. In a 10-second pulse, over 60% of the incorporated label sediments as a discrete peak around 4 S (Fig. 3(a)). This pattern cannot be the result of an initial burst of synthesis, since similar labeling patterns are obtained if the radioactive label is added at various times after the beginning of synthesis (data not shown). In other experiments, the slowly sedimenting species has been shown to have a broad distribution with an average size of approximately 4 S. When unlabeled dTTP is added after 10 seconds of [^{3}H]dTTP incorporation, the counts are "chased" into high molecular weight material within a few minutes (Fig. 3(b) to (d)), during which time the peak also grows from a 4 S size to a broader distribution around 8 S.

(iv) *Polyacrylamide gel analysis of discontinuous synthesis*

The discontinuous 4 S size DNA can be resolved further on polyacrylamide gels in order to examine the growth of the nascent chains. For these experiments the incubation temperature was lowered to 26°C, to slow the growth rate and allow a more accurate kinetic analysis. A [^{3}H]dTTP pulse of 25 seconds was followed by excess unlabeled dTTP; synthesis was stopped after different chase times, and the

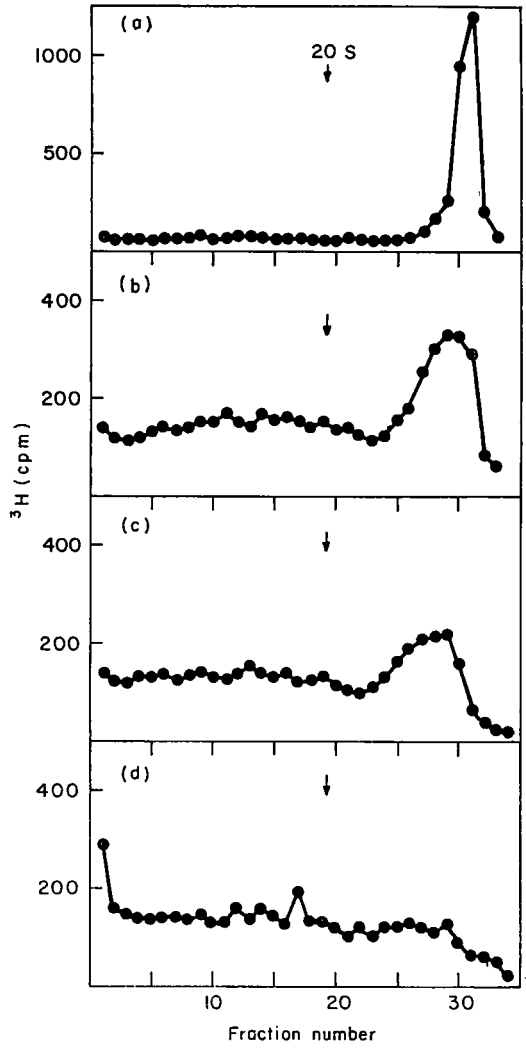

FIG. 3. Alkaline sucrose gradient sedimentation analysis of DNA from pulse–chase experiments in lymphocyte lysates.

Separate incubations (0·3 ml) of lymphocyte lysate prepared as in standard conditions except for [^{3}H]dTTP at 0·006 mM (18 Ci/mmol), incubated at 37°C for 10 s before addition of unlabeled dTTP to a concentration of 1 mM. Incubations were terminated with Sarkosyl lysis buffer after 10 s labeling, and 1, 2 and 5 min after beginning the chase with unlabeled dTTP. After treatment with Pronase and ethanol precipitation, samples (0·3 ml) were sedimented through 12-ml alkaline sucrose gradients, in a SW40 rotor (Spinco) at 40,000 rpm for 5 h at 20°C. Recoveries were 80 to 90% from all samples. The position of an internal 20 S marker (fd [^{14}C]DNA) is indicated by the arrow.

(a) 10-s pulse-label (no chase); (b) 10-s pulse-label, 1-min chase; (c) 10-s pulse-label, 2-min chase; (d) 10-s pulse-label, 5-min chase.

products were electrophoresed in 3% polyacrylamide–0·5% agarose gels, in a buffer that contained a high concentration of urea in order to reduce or eliminate secondary structure (Heyden *et al.*, 1972).

After the 25-second labeling period about 70% of the incorporated counts is included in the gel, with the peak at approximately 120 nucleotides and a range of about 70 to 200 nucleotides (Fig. 4(a)). The chase shows that this material is a

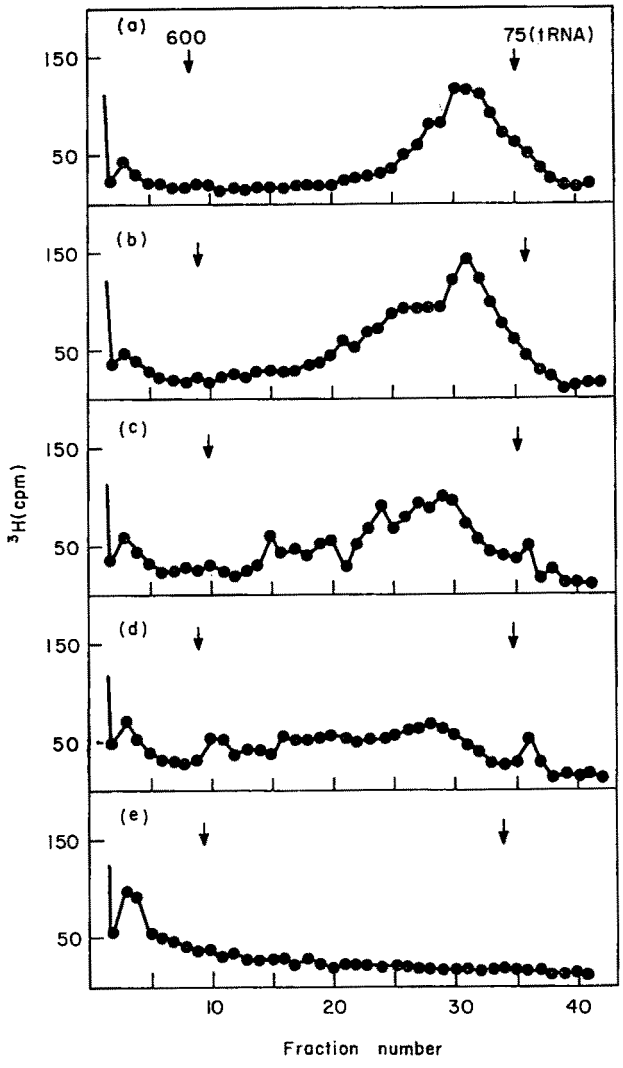

FIG. 4. Polyacrylamide gel electrophoretic analysis of DNA from pulse–chase experiment with lymphocyte lysate.

Procedure was as described for Fig. 3 except that the mixtures (0·15 ml) were incubated at 26°C for 25 s before the chase with unlabeled dTTP. After Sarkosyl–Pronase digestion the samples were precipitated with ethanol 4 times to remove labeled substrate, ^{32}P-labeled markers of tRNA and 600-nucleotide DNA were added and, after heat denaturation with urea (see Materials and Methods), were analyzed on polyacrylamide gels. Fractions (2 mm) were counted after digestion with H_2O_2. The first fraction (not plotted) contained for (a), 826 cpm; (b), 1076 cpm; (c), 1223 cpm (d), 1515 cpm; (e), 2500 cpm.

(a) 25-s pulse (no chase); (b) 25-s pulse, 20-s chase; (c) 25-s pulse, 40-s chase; (d) 25-s pulse, 60-s chase; (e) 25-s pulse, 180-s chase.

precursor for larger sizes (Fig. 4(b) to (e)). Essentially all of the counts are excluded from the gel 3 minutes after the beginning of the chase (Fig. 4(e)). Chain growth from the 120-nucleotide class is not synchronous and a measurement of the average chain elongation rate can be made by displaying the data from the experiment in Figure 4 as the integrated fraction of total counts *versus* fraction number (chain length) (Fig. 5). The point corresponding to 120 nucleotides on the 25-second pulse curve

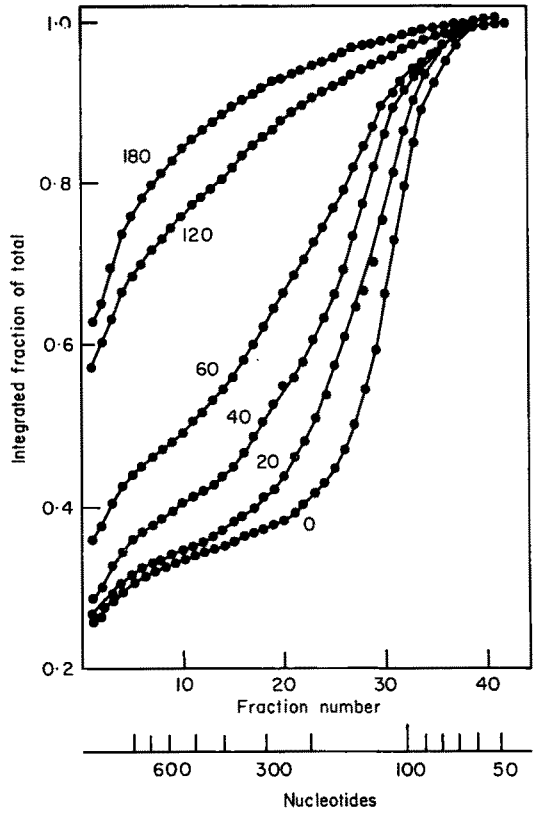

FIG. 5. Integrated fraction of label as a function of chain length, for *in vitro* pulse–chase with lymphocyte lysates.

The counts in Fig. 4 (along with 120-s chase sample not illustrated in Fig. 4) are plotted as an integrated fraction of the total counts relative to migration position. The chain lengths are obtained from a semilog plot of chain length *versus* migration distance using as standards, tRNA, 140-nucleotide RNA, 300 and 600-nucleotide DNAs (Materials and Methods). Chain lengths for the ends of the gel should be considered as approximate due to anomalous migration rates at the gel extremities (Dingman *et al.*, 1974). The numbers on the graph refer to the length of chase in seconds, after a 25-s pulse.

(0-min chase) is the 0·65 fraction level. Following this fraction of counts from the pulse curve to the 1-minute chase curve gives an estimate of 150 nucleotides/minute (26°C) for the average chain elongation rate. Label appears in the longer chains faster than expected from the average elongation rate (Fig. 5) so that this value is a minimum estimate.

The chain elongation rate of 150 nucleotides/minute is approximately $\frac{1}{8}$ the fork movement rate measured from BrdUTP incorporation (400 nucleotide-pairs/min per fork) (see previous section). The difference is due to the different temperatures at which these measurements were made; a minimum chain extension rate at 37°C was also measured on acrylamide gels and was 300 nucleotides/minute (data not shown).

(b) *Discontinuous synthesis of DNA by cells in culture*

Predominant synthesis *in vitro* of a 120-nucleotide size class could be due to an artifact favoring labeling of this size. Therefore evidence was sought for the existence of this discontinuous class *in vivo*. True steady-state labeling conditions are difficult

to achieve but were approached by using very low concentrations of [^{3}H]dThd (0·4 μM; 20 μCi/ml) (Bjursell & Reichard, 1973). Incorporation was rapidly terminated by dilution of the pulse-labeled culture in cold ethanol, and the labeled DNA was analyzed by velocity sedimentation in alkaline sucrose gradients, and by gel electrophoresis of the low molecular weight materials.

The size distribution of the product in alkaline sucrose gradients indicates a low molecular weight class at around 4 S, clearly distinguishable from the larger sizes, for labeling periods up to 2 minutes (Fig. 6). With increasing length of labeling period, the 4 S discontinuity is masked by a "tail" of labeled DNA from the larger sizes. A broad peak (2 to 10×10^6 daltons) appears at about 1 minute and persists for up to 20 minutes of labeling but it, too, is masked with longer labeling times (Fig. 6(c) to (f)). It is unclear at present whether the 2 to 10×10^6 dalton material represents replicon intermediates (Huberman & Riggs, 1968), or results from breakage of larger DNA sizes (Lehmann & Ormerod, 1970). The former alternative seems more likely since

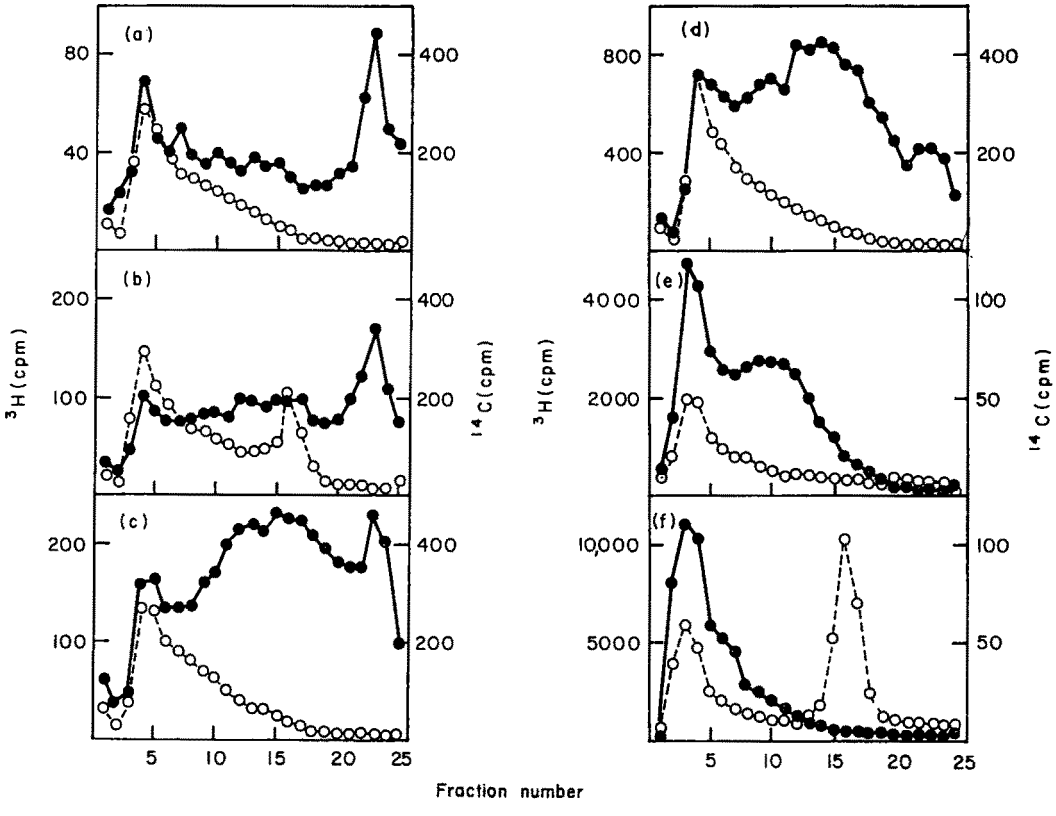

FIG. 6. Alkaline velocity sedimentation of DNA labeled with [^{3}H]dThd in intact cells for varying lengths of time.

Cultured lymphocytes prelabeled with [^{14}C]dThd were labeled at 37°C with 20 μCi [^{3}H]dThd/ml (50 Ci/mmol) for 20, 40 and 60 s, and 2, 20 and 40 min. Portions (10 ml for time points 2 min and less, and 2·5 ml thereafter) were processed as described in Materials and Methods and centrifuged in 31-ml alkaline sucrose gradients (over 6-ml saturated sucrose shelf) in a SW27 rotor (Spinco) at 25,000 rpm for 15 h at 2°C. Fractions of 1·5 ml were collected, of which 0·4-ml portions were assayed for acid-precipitable radioactivity for the 20-s to 2-min samples, and 0·2 ml for the 20 and 40-min samples. A 20 S marker (fd [^{14}C]DNA) was included in the 40-s and 40-min samples.

(a) 20-s label; (b) 40-s label; (c) 60-s label; (d) 2-min label; (e) 20-min label; (f) 40-min label. —●—●—, ^{3}H; —○—○—, ^{14}C.

the intermediate region is labeled only after a lag (Fig. 6(a) to (c)). However, the size (2 to 10×10^6 daltons) is smaller than expected for the average inter-fork distance of 15 μm (15×10^6 daltons, single strand).

A more detailed size characterization of the low molecular weight fractions was carried out by gel electrophoretic analysis of the top six fractions (<12 S) from the gradients illustrated in Figure 6. In this case (Fig. 7), as with the *in vitro* labeled product (Fig. 4), a 120-nucleotide class is apparent, and in both it probably has the same functional significance, as the earliest identifiable intermediate in DNA synthesis. References to the 120-nucleotide class are intended to indicate a distinguishable broad peak centered around a size of about 120 nucleotides. The heterogeneity of the 120-nucleotide species is indicated by a width of 7 to 8 fractions compared to 1·5 fractions for a 140-nucleotide marker (Paddock & Abelson, 1973) (Fig. 7(a) and (b)).

Although the *in vitro* 120-nucleotide size class has been shown to be a precursor to high molecular weight products, it is difficult to make a similar demonstration for the *in vivo* product due to the substantial incorporation of radioactive precursors, presumably from intracellular substrate pools, even after addition of large dThd excess. However, an analysis of labeling kinetics of the *in vivo* 4 S peak is consistent with it being a precursor. The appearance of label in the 4 S region in the alkaline sucrose gradient (Fig. 6) is rapid, linear, and reaches a maximum between 3 and 10 minutes; the same is true for gel fractions smaller than 800 nucleotides (Fig. 7). The time required to reach maximum value probably reflects more the time needed to reach steady-state [^{3}H]dThd incorporation, which is approximately 5 minutes. That the 120-nucleotide region does not accumulate additional label after 5 minutes while bulk label continues to increase is consistent with its precursor role (Figs 6 and 7).

(c) *DNA synthesis in isolated nuclei*

Washed nuclei prepared from whole cell lysate also incorporate [^{3}H]dTTP into DNA although at a reduced rate and for shorter periods (Fig. 1). The initial rate of DNA synthesis by isolated nuclei is approximately 50% of that for lysates, and synthesis continues less than 10 minutes in isolated nuclei, compared to at least 20 minutes for lysates (Fig. 1). Sedimentation analysis of the DNA product from nuclei labeled for various times shows that a discontinuous class of molecules (<10 S) is the primary product (Fig. 8(a)). It may be inferred that the deficient synthesis of high molecular weight DNA in nuclei, in contrast to lysates, may result from removal of the cytoplasmic fraction. This is confirmed by comparison with a reconstituted mixture of nuclei and cytoplasmic extract, in which an increased fraction of the label appears in high molecular weight material (>20 S) (Fig. 8). Likewise, pulse label in nuclei is chased from the discontinuous peak to high molecular weight sizes with the addition of cytoplasmic extract but not without it (Fig. 9(a) to (c)). However, the rate and the net amount of synthesis in the reconstituted mixture are distinctly lower than that observed with the original whole cell lysate, presumably due to damage to nuclei during isolation and/or inactivation of the activity in the cytoplasmic extract.

Preliminary characterization of this activity in the cytoplasmic extract indicates that it is (1) non-dialyzable, (2) sensitive to trypsin (10 μg/ml, 10 min, 37°C) (suitable controls with soybean trypsin inhibitor, 30 μg/ml, were used), (3) destroyed by heat (50% inactivation at 60°C, 5 min), (4) not sedimentable at 100,000 *g* for 1 h, and (5) either present at low levels or readily inactivated by dilution. The activity causes

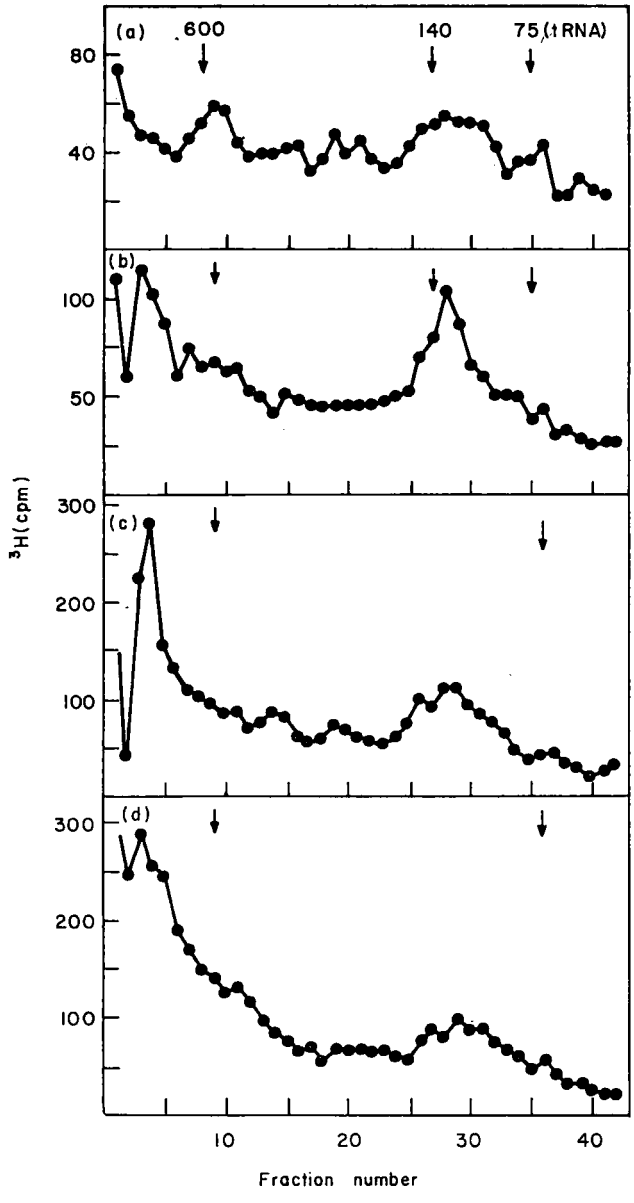

FIG. 7. Polyacrylamide gel electrophoresis of *in vivo* labeled low molecular weight DNA. Fractions 20 to 25 of each alkaline sucrose gradient from Fig. 6 were pooled and analyzed by polyacrylamide gel electrophoresis, along with a 600-nucleotide [^{32}P]DNA and (in (a) and (b)) 140-nucleotide [^{32}P]RNA (Materials and Methods). The marker migration positions are indicated by the arrows. The first fraction (not plotted) contained in (c), 350 cpm and (d), 3510 cpm. The 2 and 20-min samples are not illustrated.

(a) 20-s label; (b) 40-s label; (c) 60-s label; (d) 40-min label.

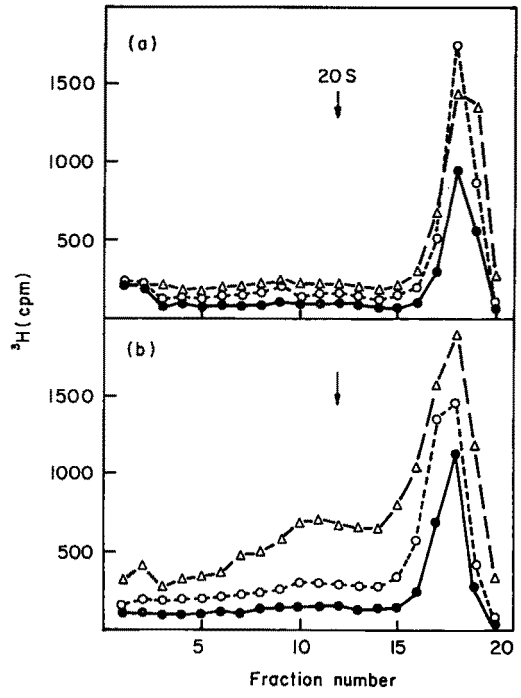

FIG. 8. Alkaline sucrose gradient analysis of DNA synthesized by lymphocyte nuclei and by nuclei supplemented with cytoplasmic extract.

Incubation mixtures (0·15 ml) were prepared using standard conditions with lymphocyte nuclei, with and without one-tenth of the volume replaced by cytoplasmic extract. Separate incubations were carried out for 2, 5 and 15 min (37°C) and after treatment with Sarkosyl, Pronase and ethanol precipitation, the product was applied to alkaline sucrose gradients (11 ml over 1·5 ml saturated sucrose shelf) and centrifuged in a SW40 rotor (Spinco) at 40,000 rpm for 5 h at 20°C. The position of an internal 20 S marker (fd [^{14}C]DNA) is indicated by the arrows.

(a) Nuclei alone. (b) Nuclei with cytoplasmic extract. —●—●—, 2-min incubation; –-○--○--, 5-min incubation; —△—△—, 15-min incubation.

relatively little enhancement of net synthesis, in contrast to its pronounced effect on the maturation of nascent intermediates into high molecular weight DNA. The relationship of this activity to one that primarily stimulates DNA synthesis (Kidwell & Mueller, 1969; Hershey *et al.*, 1973) is unknown.

As synthesis in the nuclei is predominantly in the form of fragments of approximately 8 S size, the products are particularly suited for characterization by the polyacrylamide gels used here, which have an exclusion limit of approximately 800 nucleotides. The DNA labeled in 20 seconds at 26°C (Fig. 10(a)) appears smaller and may possibly be more heterogeneous than the product synthesized during a similar pulse length in the lysate (Fig. 4(a)). After a chase using unlabeled substrate, further chain growth (Fig. 10(b) and (c)) again shows the 120-nucleotide size class described previously, but few chains grow beyond the exclusion size of the gel (Fig. 10(d)). The results from gel electrophoretic analysis, using the same procedure as for the lysate (Figs 4 and 5), show that chain growth in the nuclei is slower than in the lysate, both up to the 120 nucleotide region and beyond. The rate of chain growth in nuclei is about one fifth to one tenth the rate observed in the lysate (data of Fig. 10).

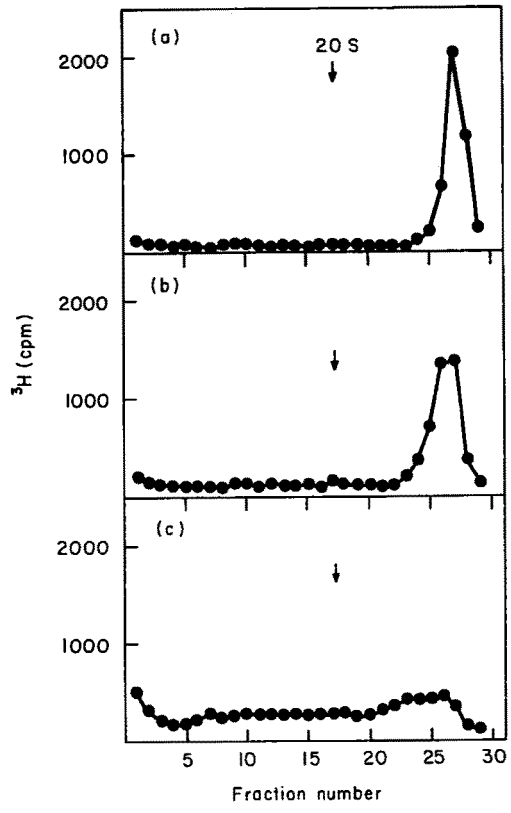

FIG. 9. Effect of cytoplasmic extract on pulse–chase in lymphocyte nuclei.
Incubation mixtures (0·3 ml) were prepared with lymphocyte nuclei according to the standard procedure except that the final volume was reduced by 1/12 (with proportional decrease in concentration of morpholinopropane sulfonate buffer, KCl, $MgCl_2$, $CaCl_2$, glucose and dithiothreitol, but not the remainder of incubation mixture components). The labeled substrate ([^{3}H]dTTP; 16 Ci/mmol) was at 0·006 mM. After incubation of all samples for 10 s, sample (a) was terminated (Sarkosyl lysis buffer); to sample (b) was added unlabeled dTTP (0·5 mM) along with 0·025 ml 1 M-sucrose–buffer I; to sample (c) was added unlabeled dTTP (0·5 mM), in addition to 0·025 ml cytoplasmic extract. After 30 min additional incubation, samples (b) and (c) were also stopped and, after Sarkosyl–Pronase treatment and ethanol precipitation, all were analyzed on alkaline sucrose gradients as in Fig. 8. The recoveries from all gradients were about 90%.

(d) *Stimulation of* in vitro *synthesis by ribonucleotides*

Several laboratories have reported stimulation by ATP of deoxyribonucleotide incorporation in eukaryote systems (Friedman & Mueller, 1968; Lazarus, 1973). Interest in this phenomenon stems in part from the demonstration in *E. coli in vitro* systems that ATP is a necessary component of replicative synthesis (Moses & Richardson, 1970; Vosberg & Hoffmann-Berling, 1971; Schaller *et al.*, 1972; Wickner & Hurwitz, 1972; Wickner & Kornberg, 1973; Ramareddy *et al.*, 1975). Nucleotide degradation is very rapid in the lysate and may represent the primary requirement for ATP and PEP, making it difficult to assess additional roles of ATP in DNA synthesis. For example, in 1 minute at 37°C, in the absence of ATP and PEP 70% of the [^{3}H]dTTP is degraded, primarily to dTDP. If ATP (2 mM) is present alone, 40% of the dTTP is degraded, whereas with PEP (20 mM) alone, 30% is degraded. When both are present, less than 2% degradation occurs in the same period of time.

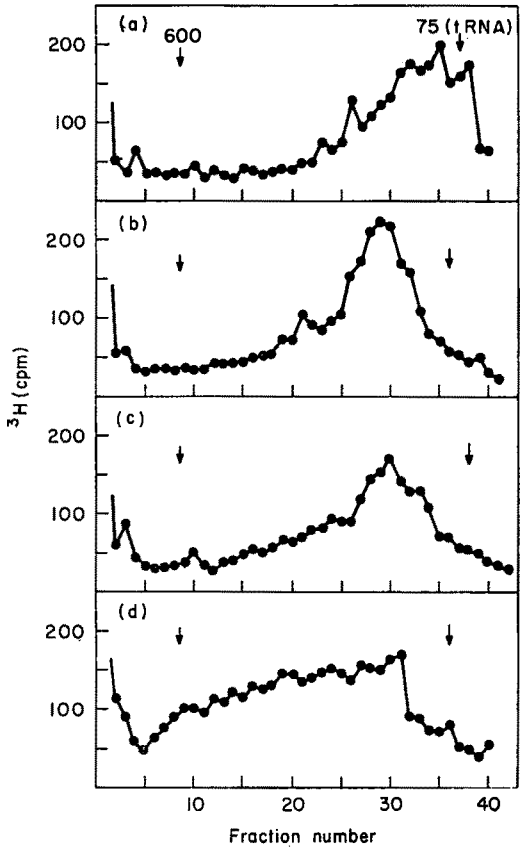

FIG. 10. Polyacrylamide gel electrophoretic analysis of DNA from pulse–chase experiment in lymphocyte nuclei.

Standard incubation mixtures (0·15 ml) were prepared with lymphocyte nuclei, containing the labeled dNTP ([^{3}H]dTTP; 16 Ci/mmol) at 0·006 mM. After incubation for 20 s at 26°C unlabeled dTTP was added to 0·7 mM and incubation was continued; reaction samples were terminated at the time unlabeled dTTP was added, and 1, 2 and 10 min thereafter. After Sarkosyl–Pronase treatment, and 4 ethanol precipitations to remove excess labeled substrate, samples were electrophoresed in polyacrylamide gels along with ^{32}P-labeled markers of tRNA and 600-nucleotide DNA. The counts for the first fraction are for (a) 726 cpm; (b), 891 cpm; (c), 918 cpm; (d), 1020 cpm.

(a) 20-s pulse, no chase; (b) 20-s pulse, 1-min chase; (c) 20-s pulse, 2-min chase; (d) 20-s pulse, 10-min chase.

In contrast, nuclei degrade less than 20% of the dTTP in 1 minute when both ATP and PEP are absent, presumably because most of the degradative enzymes are removed with the cytosol and the washing steps. Nuclei were therefore used for study of the possible function of ribonucleotides in DNA synthesis.

In the nuclear system, ATP is capable of stimulating DNA synthesis a maximum of two to three-fold (Table 2). This stimulation cannot be accounted for by an effect in sustaining the level of deoxyribonucleoside triphosphates since, even in the absence of ATP, in the 1-minute incubation more than 80% of the substrate survives as triphosphate, which is sufficient to sustain maximal rates of synthesis. The other three ribonucleoside triphosphates consistently stimulate an additional synthesis of 20 to 30% even though present in relatively low concentrations, in some instances

TABLE 2

Effect of ATP and low concentration CTP, GTP, UTP on DNA synthesis in lymphocyte nuclei

ATP (mM)	[^{3}H]dTMP incorporated (pmol in 1 min) CTP + GTP + UTP Absent	Present	dTTP remaining (%)
0	6·6	8·2	83
0·5	9·1	11·3	90
1	10·9	13·1	95
3	11·2	15·0	98
6	14·0	15·8	98
10	13·9	15·7	98

Incubations (0·15 ml) were carried out with washed lymphocyte nuclei under standard conditions, except that dATP, dCTP, dGTP, and [^{3}H]dTTP were all at 0·1 mM, and rNTPs were present only where indicated. When CTP, GTP, and UTP were present each was at 0·2 mM; ATP was varied as shown. After 1 min (37°C) incorporation was stopped by addition of 0·1 ml 0·2 M-EDTA and chilling to 0°C; the nuclei were sedimented by centrifugation 5 min at 150 *g*. A portion (0·002 ml) of the supernatant was applied to polyethyleneimine cellulose thin-layer plates (Brinkmann) and chromatographed in 1·0 M-LiCl. Per cent dTTP refers to the proportion of total radioactivity in the supernatant remaining as dTTP. The figures given for % dTTP are from the incubations without CTP, GTP, and UTP; the values for the corresponding incubations with CTP, GTP and UTP are, in all instances, nearly identical. Total acid-insoluble radioactivity was determined on the remainder of the incubation mixture.

less than 10% of the concentration of ATP. Experiments individually testing each of the other three dNTPs as labeled precursor give similar results (not shown) indicating that the stimulation by ATP is not specific for dTTP incorporation.

The specificity for ATP was further tested by substituting deoxyribonucleoside triphosphates and ribonucleoside triphosphates individually for ATP in the presence of low concentrations of the other three ribonucleoside triphosphates (Table 3). Stimulation resulted with each of the other three ribonucleoside triphosphates as well, but ATP was the most effective at all concentrations tested. Although the effects are small they are reproducible, and were much less pronounced or absent when the

TABLE 3

Effect of augmenting individual ribonucleoside triphosphates on DNA synthesis in lymphocyte nuclei

Ribonucleoside triphosphate	Concentration (mM) 2·2	4·2	6·2
ATP	9·4	11·5	12·3
CTP	8·7	8·6	9·0
GTP	8·7	9·8	9·0
UTP	8·0	9·3	9·3

Conditions for incubation with nuclei are as described in Table 2, except for rNTP concentrations. Concentration of individual rNTPs were increased as indicated, and in each case the other three rNTPs remained at 0·2 mM. Figures given are pmol [^{3}H]dTMP incorporated in 1 min at 37°C. Incorporation of 7·2 pmol resulted when all four rNTPs were present at 0·2 mM.

ribonucleoside triphosphates were replaced by deoxyribonucleoside triphosphates. The ATP analogue [α, β-methylene]ATP inhibits DNA synthesis (50% at 6 mM), presumably by competing with ATP, whereas [β, γ-methylene]ATP neither stimulates nor inhibits.

Thus, there is a clear cut stimulatory effect of ribonucleoside triphosphates on DNA synthesis in lymphocyte nuclei, unrelated to maintenance of deoxyribonucleoside triphosphates. Each of the four ribonucleoside triphosphates appears capable of functioning, although ATP is somewhat more effective than the other three, and the presence of all four together can always stimulate synthesis beyond that achieved with any single ribonucleoside triphosphate.

4. Discussion

DNA synthesis *in vitro* in lymphocyte lysates bears a close resemblance to replicative synthesis *in vivo* on the basis of (a) long continuous regions of new chains ($>$ 1 million daltons), (b) distinctive size of the nascent intermediate (120-nucleotide class), and (c) maturation of the low molecular weight nascent DNA to high molecular weight DNA. From the relatively close correspondence of the *in vitro* fork movement rate with the chain elongation rate of the nascent DNA fragments, it can be inferred that the number of fragments need not be more than one per arm per fork in order to account for the overall growth rate.

There are several indications that initiation of synthesis of the nascent chains occurs *in vitro*. First, since the lifetime of the 4 S material is less than 5 minutes (Fig. 3), predominant labeling of it by short pulses, at times up to 20 minutes after beginning the incubation, indicates that new initiations must have occurred (data not shown). Second, the *in vitro* synthetic product obtained when BrdUTP replaces dTTP has the density of fully substituted single strands. Both of the above could be accounted for, alternatively, by a large pool of incomplete fragments, especially if sufficiently short. However, there is a third type of evidence, based on the transfer of label from incorporated [^{32}P]dNTP to ribonucleotides after alkaline hydrolysis, which indicates that there is a RNA–DNA bond in each nascent DNA chain (Tseng & Goulian, 1975). Evidence from other systems that supports a role for RNA as primer for DNA synthesis (Brutlag *et al.*, 1971; Sugino *et al.*, 1972), together with experiments that relate the RNA–DNA junctions to DNA replication (Tseng & Goulian, 1975) permits the tentative conclusion that a new DNA chain is initiated at each RNA–DNA bond detected in the lymphocyte lysate, i.e. on each nascent chain.

The results of *in vitro* experiments reported here demonstrate the existence of a 120-nucleotide intermediate class that is a precursor to high molecular weight DNA (Fig. 4). The pattern of growth indicates that these fragments join to large DNA only after an increase in size, as seen both on gels (Fig. 4) and on gradients (8 S) (Fig. 3). Two mechanisms of growth may be considered for the extension of the 120-nucleotide size. In one, several 120-nucleotide fragments join together before being linked to high molecular weight DNA. Alternatively, the 120-nucleotide class undergoes elongation by nucleotide addition before joining to a large DNA molecule, and the distinct small class would then result from a slowing or pause in growth at this size. The present results do not distinguish these possibilities and experiments to resolve this are being undertaken. The chain elongation rate would be a measure of joining and/or nucleotide addition depending upon the mechanism outlined above.

The results for incorporation of [^{3}H]dThd in intact cells during steady-state growth also indicate a broad discontinuous intermediate around 120 nucleotides. Its similarity in size to the *in vitro* nascent DNA suggests a similar precursor role. It would appear that the 120-nucleotide region has a lifetime of 2 to 4 seconds *in vivo*, since 10% of the label after 20 to 40 seconds of labeling is in the 120-nucleotide range. Whether or not extension of the 120-nucleotide region occurs *in vivo* before joining to large DNA is not demonstrated. The material between 200 and 800 nucleotides (polyacrylamide gels) (Fig. 7) has kinetics of labeling that are similar to that of the 120-nucleotide class rather than to that of large DNA. This suggests that the 200 to 800-nucleotide sizes do not arise by some other mechanism, e.g. breakdown of bulk DNA.

Discontinuous synthesis, as originally described by Okazaki *et al.* (1968) for *E. coli*, does not appear to have any slow kinetic steps leading to synthesis of a completed 1000 to 3000-nucleotide fragment (Dingman *et al.*, 1974). The size of the discontinuous class for human lymphocyte cells agrees with other reported values, for a human heteroploid cell line (Nuzzo *et al.*, 1970), Chinese hamster ovary cells (Schandl & Taylor, 1969), and HeLa cells (Huberman & Horwitz, 1973). Several groups have also reported larger intermediates in synthesis (Schandl & Taylor, 1971; Sato *et al.*, 1972; Berger & Huang, 1974) and some smaller (Schandl & Taylor, 1971). The basis for the differences in sizes is still unclear at present. Studies of mammalian virus DNA synthesis have also indicated a 4 S fragment in synthesis of simian virus 40 (Fareed & Salzman, 1972) and polyoma DNA (Magnusson *et al.*, 1973; Francke & Hunter, 1974). It appears from these studies that viral and cellular synthesis may have in common a 4 S nascent intermediate in DNA synthesis.

The proportion of counts in the 120-nucleotide material at the earliest labeling times (70%) (Fig. 5) leaves open the question of whether that class of molecules is from only one strand of the fork (which would result in 50% of the label being in the 120-nucleotide region) or from both strands (which would have given 100%). Interpretation of the proportion obtained is clouded by the possibility, as yet difficult to test, that even the shortest pulses were too long to avoid maturation of some early intermediates. This question has been tested for the small animal viruses, simian virus 40 and polyoma 4 S, nascent fragments, however, with contradictory results (Fareed *et al.*, 1973; Pigiet *et al.*, 1973; Francke & Hunter, 1974).

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