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TWO-STAGE INCORPORATION OF THYMIDINE TRIPHOSPHATE INTO MAMMALIAN DNA AS INDICATED BY CHROMATOGRAPHY ON BENZOYLATED DEAE-CELLULOSE

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SUMMARY

Replicating DNA contains single-stranded regions as indicated by the extent of its recovery in the final fraction, following stepwise elution of sheared preparations from benzoylated DEAE-cellulose using 0.3 *M* sodium chloride, 1.0 *M* sodium chloride and caffeine solutions, respectively. In preparations of hepatic DNA, isolated up to 60 min after administration of [^{3}H]thymidine to rats subjected earlier to partial hepatectomy, the proportion of radioactivity contained in the caffeine-eluted fraction progressively decreased. This change was attributable to migration of incorporated radioactivity from replicating forms to mature (double-stranded) DNA, the latter being recovered from the column in 1.0 *M* sodium chloride. In terms of the same chromatograms, the relative size of the 0.3 *M* sodium chloride-eluted fraction also decreased, this fraction of radioactivity co-eluting from benzoylated DEAE-cellulose with thymidine triphosphate. The precursor—intermediate—product relationship between the three products separated by benzoylated DEAE-cellulose chromatography was confirmed using DNA isolated from cultured mammalian cells. Early eluting ratioactivity co-chromatographed with thymidine triphosphate on thin-layer cellulose, whilst the intermediate status of caffeine-eluted radioactivity was confirmed following pulse-chase labelling procedures. Utilizing stepwise chromatography of such DNA on benzoylated DEAE-cellulose, the effect of three inhibitors could thus be described as affecting either an early or late stage of DNA synthesis Such an approach offers a simple quantitative method of monitoring influences on DNA synthesis.

INTRODUCTION

Benzoylated or benzoylated naphthoylated DEAE-cellulose (BD- or BND-cellulose, respectively) may be used to fractionate DNA on the basis of secondary structure [1—5]. Double-stranded DNA is eluted in buffered sodium chloride while DNA having single-stranded regions may be recovered by addition of caffeine (or formamide or ethanol) to the salt solution [6]. In prepara-

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tions of DNA isolated from proliferating cells, newly incorporated radioactivity is detected in the single-stranded fraction before the double-stranded fraction is labelled [7]. Retention of replicating DNA by derivatized DEAE-cellulose may serve as an assay of repair, the latter being determined in terms of radioactivity immediately present in the double-stranded (non-replicating) fraction [8]. In "pulse-chase" experiments involving fractionation of DNA rapidly labelled by incorporation of [^{3}H]thymidine ([^{3}H]dThd), we noted that phenol-extracted DNA preparations contained radioactivity which was not bound to BD-cellulose. When such DNA preparations, in 0.3 *M* sodium chloride solution, were applied to BD-cellulose columns, acid-soluble radioactivity was recovered in the void volume. This paper concerns characterization of this activity as thymidine triphosphate (dTTP) and its ready separation from double-stranded DNA and DNA containing single-stranded regions. This simple separation provides a useful basis for assessing eukaryotic DNA synthesis in terms of a two-stage process.

EXPERIMENTAL

BD-cellulose was purchased from Boehringer (Mannheim, F.R.G.) and [methyl-^{3}H]dThd and [methyl-^{14}C]dThd (specific activity 25 Ci/mmol and 54 mCi/mmol, respectively) from Amersham Australia (Sydney, Australia). Female Wistar rats, maintained as previously described [9], were subjected to partial hepatectomy and [^{3}H]dThd (50 μCi) was injected 23 h after surgery.

CCRF-CEM cells, a human leukaemic T-cell line [10] were generously provided by Dr. Peter Slowiaczek (Ludwig Institute of Cancer Research, Sydney, Australia). These cells were maintained as static suspension cultures at 37°C in RPMI medium 1640 supplemented with N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer (20 m*M*), L-glutamine (2 m*M*), penicillin/streptomycin (100 U/ml) and 10% foetal calf serum (Commonwealth Serum Labs.). The cells were labelled by addition of [^{14}C]dThd (0.6 μCi/ml) to the medium 24 h before use. DNA was pulse-labelled by addition of [^{3}H]-dThd (final concentration, 10 μCi/ml) to the medium. After continuing incubation for various periods, cells were pelleted by centrifugation at 3000 *g* for 3 min and, if required, resuspended in fresh medium. Hydroxyurea, aphidicolin or 3-aminobenzamide (final concentrations, 2 m*M*, 2.95 μ*M* and 5 m*M*, respectively) were added either with [^{3}H]dThd or in the fresh medium following removal of the labelled precursor. For dThd incorporation periods less than 2 min (and to ensure minimal incorporation during centrifugation), cells were initially pelleted and resuspended using RPMI (1 ml) contained in plastic microcentrifuge tubes. After being maintained at 37° for 60 min, [^{3}H]dThd (25 μCi) was added and the cells kept at 37°C for a specified period before sedimentation by centrifugation for 5 s. When cells were permitted to incorporate dThd for periods of 2 min or longer, there was no difference in DNA labelling, following BD-cellulose fractionation, according to which of the above procedures was used. After either labelling procedure, the experiment was terminated by placing the pelleted cells in an ice—salt slurry. The cells were then washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS).

DNA was isolated by phenol extraction from liver [11] or cultured cells

[12] as previously described, the final solution being dialysed overnight at 4°C against 10 m*M* Tris—HCl—1 m*M* EDTA (pH 8.0) (TE buffer). Samples of DNA (100 μg, at least 20 000 dpm) were sheared and subjected to stepwise elution from BD-cellulose as previously described [13]. Usually, total radioactivity in collected fractions was determined by addition of scintillant (Instagel, Packard Instruments). Where indicated, acid-insoluble material was precipitated from collected fractions onto glass fibre discs and subjected to scintillation counting [13].

Thymidine phosphates were separated by thin-layer chromatography using 0.1-mm cellulose impregnated with polyethyleneimine. Ascending elution with 1.5 *M* sodium formate (pH 3.4) was continued until the solvent front had covered 50% of the plate at which time elution was continued with 3.0 *M* sodium formate. After drying, standards were located by ultraviolet fluorescence and radioactivity was determined by scintillation counting.

RESULTS

Preparations of DNA were isolated from rats which had been subjected to partial hepatectomy, injected with [^{3}H]dThd 23 h later and sacrificed at short intervals thereafter. Acid precipitation of fractions recovered after stepwise elution of DNA samples from BD-cellulose revealed a progressive decrease in the proportion of radioactivity in the caffeine-eluted fractions as the "labelling time" increased from 10 min (Fig. 1). These DNA preparations also contained acid-soluble radioactivity which, after BD-cellulose chromatography, was wholly recovered in the first four fractions, that is prior to elution of both the

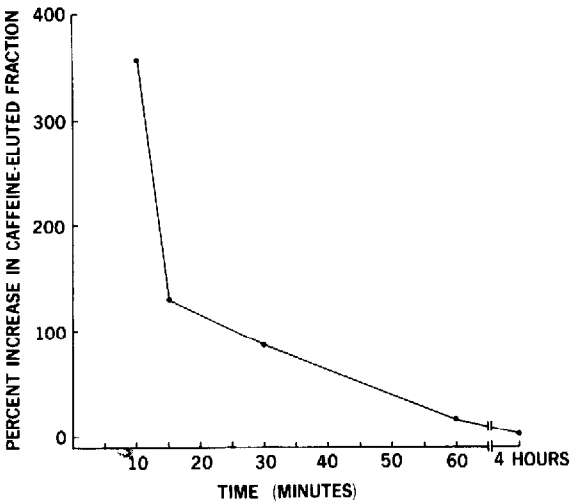

Fig. 1. Variation in the proportion of radio-labelled regenerating rat liver DNA exhibiting single-stranded character following administration of [^{3}H]dThd. DNA was isolated from pairs of animals killed at intervals up to 4 h after administration of the radioisotope and, following shearing, was subject to stepwise elution from BD-cellulose Acid-precipitable radioactivity in collected fractions was determined and the proportion of caffeine-eluted DNA expressed relative to that in preparations isolated from a large number of animals allowed to survive for at least two weeks after administration of [^{3}H]dThd. In this and all other figures, each point represents the mean from at least two experiments.

double- and single-stranded DNA fractions. When expressed as a proportion of the radioactivity recovered from the respective columns, the proportion recovered in the first four fractions also fell as labelling time increased (Fig. 2). It was also noted that dTTP, when applied to BD-cellulose in 0.3 *M* sodium chloride, was not bound and thus recovered in the initial fractions collected. The inference from these observations, that dThd incorporation into DNA might be characterized in terms of precursor, intermediate and final stages,

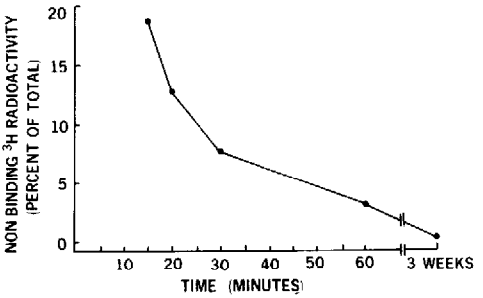

Fig. 2. Progressive decrease in acid-soluble radioactivity contained in preparations of rat liver DNA radiolabelled as described in Fig. 1. Following BD-cellulose chromatography, total and acid-soluble radioactivity in collected fractions were determined. Acid-soluble radioactivity was wholly recovered in the first four fractions, which were eluted with 10 m*M* Tris—HCl—1 m*M* EDTA containing 0.3 *M* sodium chloride, and, for each preparation, was expressed as a percentage of the total radioactivity recovered from the column.

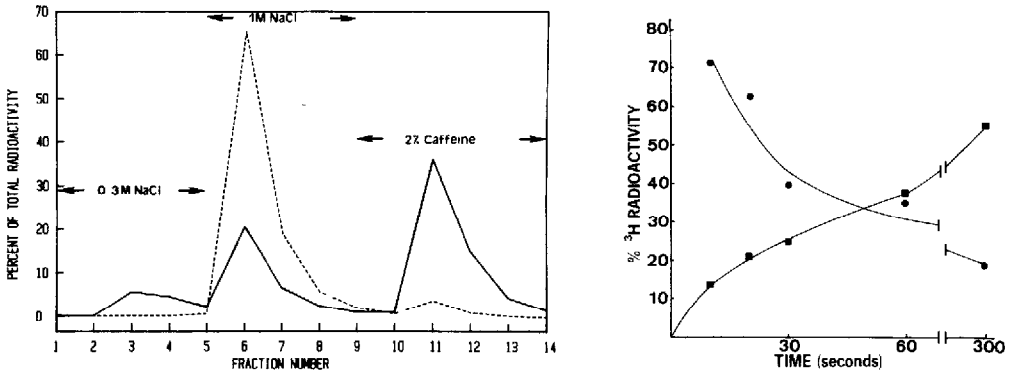

Fig. 3 Typical chromatogram generated by elution of DNA from CCRF-CEM (human leukaemia) cells. Cells labelled 24 h previously with [^{14}C]dThd (broken line), were exposed to [^{3}H]dThd (continuous line) for 2 min and incubated in fresh medium for a further 2 min. DNA was isolated and, after shearing, loaded onto a BD-cellulose column. Elution was continued with the loading solution, 0.3 *M* sodium chloride in TE buffer, and then, after fraction 4, with 1.0 *M* sodium chloride in TE buffer (double-stranded DNA) followed after fraction 9 by 2.0% caffeine—1.0 *M* sodium chloride in TE buffer (double-stranded DNA with single-stranded regions). Total radioactivity in each fraction expressed in terms of the total of the respective isotope contained in all fractions

Fig. 4. Relationship between ^{3}H-radioactivity recovered in the 0.3 *M* (•) and 1.0 *M* (▪) sodium chloride-eluted fractions following BD-cellulose chromatography of DNA preparations isolated from human leukaemia cells incubated with [^{3}H]dThd for periods up to 5 min. Stepwise chromatography was conducted as described in Fig. 3 and for each chromatogram, the amount of radioactivity recovered in the designated fractions is expressed as a percentage of the total in all fractions.

was assessed using DNA isolated from cells in culture. The latter permitted more precise manipulation of the various parameters.

In double-labelled preparations of DNA from leukaemic cell lines, stepwise elution from BD-cellulose invariably generated three radioactive peaks. Radioactivity in the 0.3 *M* sodium chloride-eluted peak was restricted to tritium, all ^{14}C-activity being bound to the column and mostly recovered in the 1.0 *M* sodium chloride (double-stranded DNA) peak (Fig. 3). From certain preparations, the first four fractions from BD-cellulose columns were pooled and subjected to thin-layer chromatography. Under conditions which permitted separation of thymidine mono-, di- and triphosphate (typical R_F values being 0.91, 0.68 and 0.55, respectively), greater than 90% of radioactivity co-chromatographed with the triphosphate, the remainder being associated with the other two standards. The expected precursor—product relationship between dTTP and DNA, as quantified by BD-cellulose chromatography, could be demonstrated by utilization of short incorporation times. Approximately half the analysed ^{3}H-radioactivity was recovered in double-stranded DNA if incubation of the cells were continued for 60 s (Fig. 4).

To express the dynamics of DNA synthesis in terms of an intermediate stage, a pulse—chase protocol was required. Removal of medium containing [^{3}H]-dThd and continuing the incubation with fresh medium established the intermediate status of caffeine-eluted radioactivity. Radioactivity thus associated with replicating DNA by virtue of its single-stranded character increased to a maximum proportion within 2 min of the "chase" period after which it progressively decreased (Fig. 5).

In preliminary studies, the effect of inhibitors of DNA synthesis could be expressed in terms of the relative sensitivities of early and late stages of replication. Sensitivity of early stage replication to inhibition was assayed by addition of the inhibitor at the same time as labelled dThd. By comparison with the appropriate control, both aphidicolin and hydroxyurea retarded incorporation

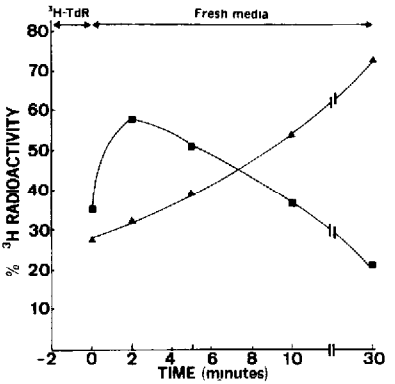

Fig. 5. Relationship between radioactivity recovered in the 1.0 *M* sodium chloride-eluted (▲) and caffeine-eluted (■) fractions following pulse—chase labelling of human leukaemic cells with [^{3}H]dThd. Cells were incubated in the presence of the labelled precursor for 2 min, and after a change of media, the incubation was continued for the periods of time indicated. At the conclusion of each experiment, DNA was isolated and subjected to stepwise elution from BD-cellulose, radioactivity in respective fractions being expressed as described in Fig. 4.

TABLE I

EFFECT OF INHIBITORS ON DISTRIBUTION OF RADIOACTIVITY IN BD-CELLULOSE-DERIVED FRACTIONS AFTER PULSE LABELLING OF CCRF-CEM CELL DNA

Inhibitors were either added together with [^{3}H]dThd (no chase period) or with the fresh medium in experiments involving a chase period.

Inhibitor	Labelling time (min)		Distribution of radioactivity (%)		
	Pulse	Chase	TTP	Double-stranded	Single-stranded
None	2	—	37.2	27.4	35.4
	2	2	10.2	32.4	57.4
Aphidicolin (2.9 μM)	2	—	67	27	5.2
	2	2	38.1	24.6	37.3
Hydroxyurea (2 mM)	2	—	58.3	23.1	18.6
	2	2	34.5	24.15	41.5
3-Aminobenzamide (5 mM)	2	—	27.2	25.2	47 6
	2	2	31.0	21.6	47.4

of radioactivity into caffeine-eluted DNA, radioactivity persisting in the precursor dTTP fraction (Table I). Of these inhibitors, aphidicolin was most effective in the pulse—chase protocol, reducing the proportion of radioactivity in the double-stranded fraction by one third, whilst also inhibiting the fall in dTTP-associated radioactivity. In contrast, 3-aminobenzamide could be distinguished as most effectively causing accumulation of radioactivity in the single-stranded (caffeine-eluted) DNA.

DISCUSSION

Gillam et al. [1] described separation of the four common ribonucleoside 5′-phosphates on BD-cellulose using a sodium chloride gradient. However, their study principally concerned separation of tRNA of various types on different preparations of derivatized DEAE-cellulose. The DNA-binding properties of such resins were not characterized until some years later. Despite the similar binding characteristics of double-stranded DNA and its precursors to BD-cellulose in particular, there appears to have been no specific attempt to examine this material in detail.

Recovery of labelled dTTP in 0.3 M sodium chloride (Fig. 3) is consistent with the results of Gillam et al. [1], and permits ready separation of this nucleotide from double-stranded DNA which is not eluted until 0.6 M sodium chloride [11]. Habener et al. [14] noted that dTTP was not removed by exhaustive dialysis, consistent with our findings that the labelled triphosphate was not lost from DNA preparations despite overnight dialysis. That dThd should have been metabolised to dTTP is consistent with numerous observations that thymidylate kinases are not rate-limiting during S phase: analyses of the acid-soluble pool from eukaryotic cells have revealed predominantly dTTP [15—17]. Thus, determination of the specific activity of the dTTP pool is the principal problem in measuring DNA synthesis on the basis of thymidine incorporation [18].

Replicating DNA of eukaryotic cells has been found by numerous investi-

gators to exhibit single-stranded character [19—21]. In labelling studies, lengthening of the pulse time results in a progressive decrease in the relative labelling of the single-stranded fraction [4, 22] suggesting its close association with the replicating fork. During DNA replication at least some, if not most, dTTP is initially incorporated into Okazaki fragments [23]. Such fragments are implicitly associated with the replicating fork and hence with single-stranded regions. Larger intermediates, of approximately 10 kilobases, have been described prior to the maturation of chromosomal DNA [24]. In the present studies, depletion of radioactivity associated with dTTP is clearly associated with DNA synthesis (Figs. 2 and 4). It is equally clear that initially, radioactivity accumulates in the caffeine-eluted fraction (Fig. 1). The intermediate status of this fraction is readily evidenced by the pulse-chase phenomena characterized in Fig. 5. Incorporation of thymidine into caffeine-eluted DNA may correspond closely with initiation of polymer synthesis, whilst migration of radioactivity to the double-stranded fraction may mark a later stage than that characterised by chain elongation [23].

Use of inhibitors, such as aphidicolin, in conjunction with BND-cellulose chromatography to characterize DNA replication intermediates has been recently described by Burhans et al. [25]. In the present studies, aphidicolin, a specific inhibitor of DNA polymerase alpha [26], severely inhibited incorporation of dTTP into caffeine-eluted DNA. Hydroxyurea was almost as effective, an observation compatible with effects of this compound which are not simply explained in terms of inhibited ribonucleotide reductase [27]. In contrast to hydroxyurea and aphidicolin, 3-aminobenzamide did not have a marked effect on incorporation of TTP into single-stranded DNA, but nonetheless reduced relative labelling of double-stranded DNA after pulse—chase incorporation studies. 3-Aminobenzamide has implicated a role for poly(ADP)-ribosylation in the late, possibly ligation stage of DNA repair [28] and our data suggest an effect of this inhibitor on the later stages of DNA replication. Whilst these effects require confirmation in more detailed studies, it would appear that the simple separation of dTTP from replication intermediates and double-stranded DNA provides an improved basis for determination of the rate of DNA synthesis and for more mechanistic analysis of DNA replication.

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