ABSTRACT

Studies on Deoxynucleoside Triphosphate Pools during the Mammalian Cell Cycle

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The Deoxynucleoside triphosphate pools in both asynchronous and synchronous cultures of mammalian cells were examined.

The pools were examined in asynchronous cultures of HeLa, Chinese hamster and mouse L-cells, and it was found that the absolute amounts of the four triphosphates varied between the cell lines, and that the ratios of the amounts of the four triphosphates varied within each cell line.

In a highly synchronous population of HeLa cells it was found that the pools of all four deoxynucleoside triphosphates fluctuated in a similar, cyclic manner. The nature of these fluctuations was determined, and it was shown that in all probability the deoxynucleoside triphosphate pools do not significantly increase in size just prior to the onset of DNA synthesis.

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Bray - Mammalian Deoxynucleoside Triphosphate Pools

STUDIES ON DEOXYNUCLEOSIDE TRIPHOSPHATE POOLS DURING THE MAMMALIAN CELL CYCLE

by

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
TdR	Thymidine
C(d)R	(Deoxy)cytidine
AdR	Deoxyadenosine
GdR	Deoxyguanosine
UdR	Deoxyuridine
dTMP	Deoxythymidine 5'-monophosphate
dTDP	Deoxythymidine 5'-diphosphate
dTTP	Deoxythymidine 5'-triphosphate
(d)CMP	(Deoxy)cytidine 5'-monophosphate
(d)CDP	(Deoxy)cytidine 5'-diphosphate
(d)CTP	(Deoxy)cytidine 5'-triphosphate
(d)AMP	(Deoxy)adenosine 5'-monophosphate
(d)ADP	(Deoxy)adenosine 5'-diphosphate
(d)ATP	(Deoxy)adenosine 5'-triphosphate
(d)GMP	(Deoxy)guanosine 5'-monophosphate
(d)GDP	(Deoxy)guanosine 5'-diphosphate
(d)GTP	(Deoxy)guanosine 5'-triphosphate
(d)UMP	(Deoxy)uridine 5'-monophosphate
(d)UDP	(Deoxy)uridine 5'-diphosphate
(d)UTP	(Deoxy)uridine 5'-triphosphate
araC	Cytosine arabinoside
FUDR	Fluorodeoxyuridine
^{3}H TdR	Tritiated Thymidine
(d)RDP	(Deoxy)ribonucleoside diphosphate (base unspecified)
(d)RTP	(Deoxy)ribonucleoside triphosphate (base unspecified)
XdR Kinase	Deoxynucleoside Phosphokinase
RNR	Ribonucleotide Reductase
dCMP deaminase	Deoxycytidylate Deaminase
dTMP synthetase	Deoxythymidylate Synthetase

dTMP kinase	Deoxythymidylate Phosphokinase
poly d(A-T)	An alternating co-polymer of deoxyadenylate and deoxythymidylate
PCA	Perchloric acid
TCA	Trichloroacetic acid
Pi	Inorganic Phosphate
R_f	Distance moved by a spot on a chromatogram, as compared to the solvent front
TLC	Thin layer chromatography

INTRODUCTION

The replication of DNA is an essential part of the life cycle of any dividing cell. It is considered that a knowledge of the regulation of DNA synthesis, particularly its initiation, is of prime importance in the understanding of cellular growth. A knowledge of the control of initiation being particularly relevant to the study of cancer, since it is possible that a lack of normal controls might be responsible for the uncontrolled growth characteristic of neoplastic tissues.

1. The Regulation of DNA Synthesis

For DNA synthesis to occur the cell requires three components of the polymerization reaction, a suitable template to replicate, the proper polymerase enzyme to perform this, and precursors to use for this replication. Thus the regulation of DNA synthesis may depend on the availability of these three factors. The template, which is the pre-existing DNA of the cell, is always present, and thus any regulation at this level would necessarily involve a modification(s) of the physical state of the template molecule, such that the template is in a state in which it can be replicated. Whether such a modification actually occurs, and if it does what its nature is, is at present not clear.

DNA polymerases from many sources have been studied in detail, particularly with regard to the template specificity. In this respect the overall picture obtained in liver tissue is that DNA polymerases from normal and regenerating liver prefer a native template, while those from embryonic tissue and hepatomas prefer a denatured template (1-3). In other tissues these primer preferences are also shown (4-7), Ehrlich ascites and calf thymus polymerase preferring denatured or single stranded DNA, while that from KB cells show no preference. It is possible that the differences in template preference could be due to differences in the method of extraction used and/or possible nuclease contamination, although

at least in the liver enzymes studied by Laszlo's group (1) this is unlikely since they have separated two different enzymes on Sephadex G-200 from hepatoma tissue. The two polymerases show different template preferences and are found distributed as described above in the four states of liver examined (normal, regenerating, embryonic and neoplastic). On the basis of these results they postulated that in normal adult liver, the synthesis of the enzyme which prefers denatured DNA is repressed, derepression accompanying neoplastic transformation.

All the enzymes thus far examined show a need for all four deoxyribonucleoside triphosphates (the precursors), magnesium ion and in those examined for DNA-primer requirements 3'-hydroxyl groups on the primer, in order for synthesis to occur (8).

The level of activity of DNA polymerase in normal liver is low, and increases at the same time as DNA synthesis occurs during regeneration (9). A similar picture is seen in lymphocytes undergoing transformation, the initiation of DNA synthesis being co-incident with, and dependent on, induction of DNA polymerase activity (10). It was found that when DNA polymerase production was inhibited no DNA synthesis occurred and it was thus deduced that DNA synthesis was dependent on induction of DNA polymerase activity.

Lieberman and his co-workers studied primary rabbit kidney cultures which, when cultured in serum free medium show DNA synthesis 36-40 hours after culturing (11). During this lag period RNA and protein synthesis occur, which until 22 hours is essential for subsequent DNA synthesis; after 22 hours, if RNA and protein synthesis are blocked, DNA synthesis will occur (12). DNA polymerase activity appears at the end of the lag period, the rise in polymerase activity being independent of the simultaneous formation of DNA (13) and of the normal increase in deoxynucleotide pools (14).

~~In view of~~ The fact that the level of DNA polymerase is roughly constant throughout the mitotic cycle in rapidly growing cells (15-17), while DNA synthesis is a periodic function, indicates that factors other than enzyme level control the extent and timing of DNA synthesis

in these cells. The lack of polymerase activity in stationary phase cells would probably represent an additional control factor superimposed on those that operate in rapidly multiplying cells. There is also preliminary evidence that in the intact organism hormones may play some part in regulating DNA polymerase production (18). It can thus be seen that the picture pertaining to regulation on this level of DNA synthesis is far from clear.

The third possible level of DNA synthetic control is that of the DNA precursors, it has been assumed that the immediate precursors of DNA synthesis are the deoxynucleoside triphosphates (dRTP), although recently some work by Werner (19) has thrown doubt on this assumption. Werner found that thymine is apparently a more direct precursor of replicative DNA synthesis (as indicated by formation of Okazaki fragments) than is thymidine; thymidine appearing to be incorporated into DNA by a repair type synthesis. He postulates that replicative and repair type synthesis draw their precursors from separate pools, although the mechanism involved in the differentiation between thymine and thymidine is unclear. Whether these pools contain chemically different precursors or are physically separated also remains to be elucidated. Some of his data appear to suggest that DNA synthesis uses precursors different from dRTP. What metabolism the thymine undergoes prior to incorporation is unknown; it can be assumed that, unless the DNA backbone is a preformed entity onto which the bases are subsequently attached, the thymine must have deoxyribose and at least one phosphate group attached prior to incorporation. A possible mechanism for attachment of the deoxyribosyl moiety is by reversal of the nucleoside phosphorylase reaction:

$$\underset{\text{deoxyribose-1-phosphate}}{\text{dR-1-P}} + \underset{\text{thymine}}{\text{T}} \rightleftharpoons \underset{\text{thymidine}}{\text{TdR}} + \text{Pi}$$

or by a transdeoxyribosylation reaction:

$$\underset{\text{nucleoside}}{\text{YdR}} + \underset{\text{base}}{\text{X}} \rightleftharpoons \text{XdR} + \text{Y}$$

If the immediate precursors of DNA synthesis are the deoxynucleoside triphosphates then it is essential to have a thorough knowledge of both the metabolism of these and of their behaviour in the cell cycle. Many studies on the enzymatic synthesis of the deoxynucleotides have been performed and many of the enzymes have been shown to be finely regulated by various activators and inhibitors, and to show cyclic fluctuations in the cell cycle; although the nature of the interrelationships between these enzymes leading to the correct amount of deoxynucleotides for DNA synthesis has yet to be elucidated. As yet very few studies on the behaviour of the deoxynucleotides in the cell, particularly during the cell cycle, have been performed. In view of the fact that all the studies thus far performed on the enzymes leading to synthesis of deoxyribonucleoside triphosphates, and on the regulation of these pathways, assume the importance of the final precursor levels and of their balanced production; it would seem that direct information as to the nature of these levels, and on their cyclic fluctuations during the cell cycle, would be of importance in confirming such assumptions, speculations and deductions. The purpose of this thesis is, therefore, to directly assay quantitatively the deoxynucleoside triphosphate pools during the mammalian cell cycle.

2. The Biosynthesis of DNA Precursors and Its Regulation

The biosynthesis of deoxyribonucleotides in mammalian cells has been shown to occur via two different routes; the de novo pathway in which the purine and pyrimidine ribodiphosphonucleosides are synthesised from smaller components of the cell by a complex series of reaction; and the salvage pathway in which pre-existing deoxyribonucleosides, derive mainly from degradation of DNA, are phosphorylated by appropriate kinases.

A. The de novo Pathway

Following their de novo synthesis the ribonucleoside diphosphates (RDP) are either further phosphorylated to form ribonucleoside triphosphates (RTP) for subsequent use in RNA synthesis or are reduced to form deoxyribonucleoside diphosphates (dRDP) which in turn are further phosphorylated to form deoxyribonucleoside triphosphates (dRTP), and so become the direct precursors of DNA. Thus the DNA and RNA precursors are co-ordinately regulated during the majority of their synthesis. This regulation mainly being feedback inhibition of enzymes early in the sequence (or at branch points) by end products, and is thus mainly dependent on the rate of utilization of both RTP and dRTP.

As far as dRTP synthesis is concerned the key enzyme in the de novo pathway is ribonucleotide reductase (RNR), it being responsible for the reduction of the hydroxyl group on the 2-C position of the ribose moiety. The same enzyme is responsible for the reduction of ADP, GDP, CDP and UDP, its activity and specificity being modulated by both RTP and dRTP, as is shown in Table 1. Thus far in mammalian systems only the gross effects of these regulators are known (20-22). Moore and Hurlbert (21) show that the activators (except for the effect of ATP on GDP reduction) clearly exert their effect by modifying the affinity of the enzyme for its substrates and can thus be classified as positive effectors. Their experiments also indicated that the inhibitors compete with the activators to some degree. In addition the inhibition of CDP reduction by dATP is independent of substrate concentration; dATP thus appears as a negative effector of CDP reduction. They are, however, unable to produce any proof regarding the relative location of binding sites, nor do they have any information on the effects of activators and/or inhibitors on the conformation of the enzyme (or on subunits thereof). In this regard the activation and inhibition of mammalian RNR cannot categorically be labelled as allosteric, as has been established in bacterial systems (23, 24).

TABLE 1

Regulation of Ribonucleotide Reductase Activity in Mammalian Cells

	SUBSTRATE	ACTIVATOR	INHIBITOR
PYRIMIDINES	CDP	ATP - absolute requirement †dCTP - strong (-ATP), weak (+ATP) †dATP - weak	†dTTP, †dATP } strength of effect dependent on ATP concentration dTTP, dUTP, dGTP, dATP } *
	UDP	ATP - absolute requirement †dATP - weak †dCTP - weak	dTTP, dUTP, dGTP, dATP } *
PURINES	ADP	†GTP - strong °dGTP - †strong, dTTP - †weak } *activation by low concentration essential †UTP - weak †dUTP - weak	*° dATP
	GDP	dTTP - †strong *low concentration essential ATP - †weak UTP, dGTP } †weak *in combination with dTTP gives greater stimulation	*° dGTP °dATP - †in presence of dTTP

† Leukaemic mouse spleen only (20)

* Novikoff hepatoma only (21)

o Murine ascites cells only (22)

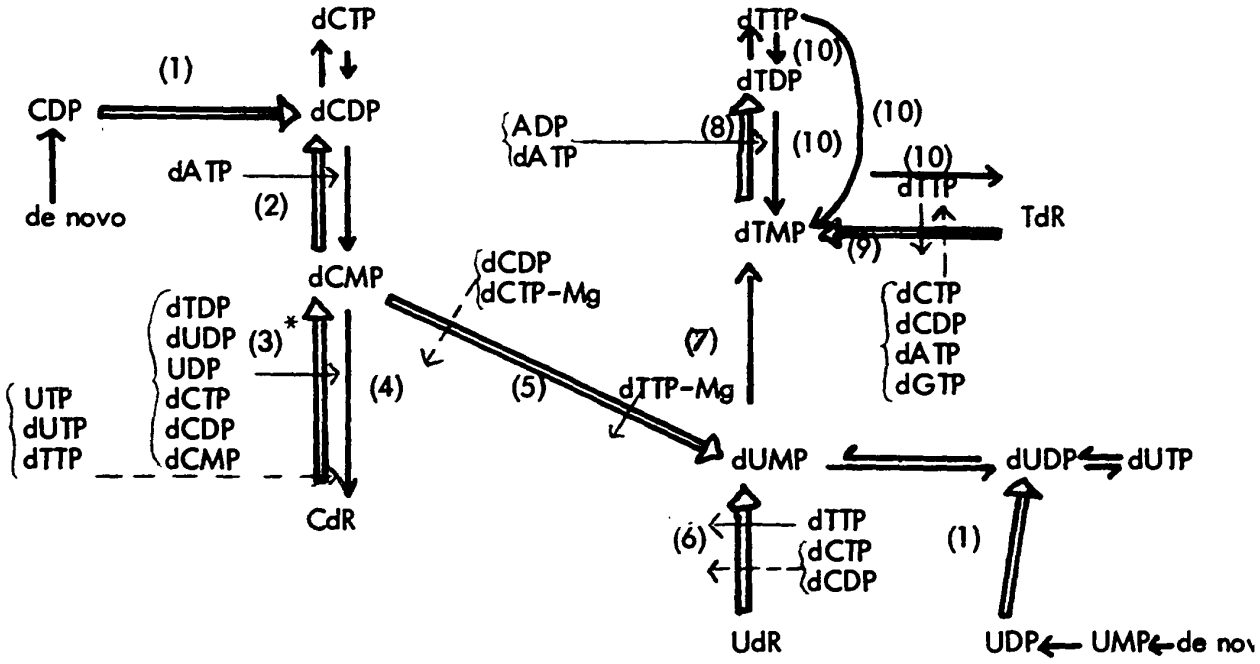
Elford et al. (25) found a very close correlation between RNR activity and growth rate in a series of rat hepatomas indicating that RNR may well be a rate limiting step in DNA synthesis and hence cell division. Thymidine kinase, deoxycytidylate deaminase, deoxythymidylate synthetase and DNA polymerase were also elevated in several tumors, but the degree of elevation did not correlate as well with growth rate as RNR activity. Elford et al. speculate that the markedly different levels of enzyme activity are due to different rates of enzyme synthesis and degradation in response to the cellular requirement for DNA synthesis, as opposed to an activation $\rightleftharpoons$ inactivation mechanism. Any allosteric effectors altering the specificity of the enzyme, and thus acting as a fine control will produce a balanced supply of deoxynucleotides, as has indeed been found in Novikoff hepatoma, murine ascites and leukaemic mouse spleen cells (see Table 1) (21-23).

Moore and Hurlbert (21) propose a scheme based on the regulatory properties observed, whereby in cells with a good energy balance, and thus a high ATP content, CDP and UDP reduction would be stimulated by the ATP. dTTP formation would thus be enhanced, this in turn would stimulate GDP reduction, producing dGTP. The dGTP will then activate the ADP reductase activity to produce dATP. Should the cell not be actively synthesising DNA, the dATP so produced will in turn inhibit all four reductase activities, prevent further reduction and thus conserve cellular energy.

B. The Salvage Pathway

Deoxythymidine (TdR) is a base unique to DNA, and it is only derived by enzymatic interconversions of other pyrimidines in the cell. The reactions leading to production of TdR and its phosphorylated derivatives have been shown to be very closely regulated by other deoxynucleotides, thus ensuring a balanced supply of precursors for

Figure 1: Pyrimidine Deoxyribonucleoside and Deoxynucleotide Interconversion and their Regulation (26-46).



: reactions under feedback control

: inhibition

: stimulation

* CdR kinase exhibits complex co-operative effects with respect to both dCTP and dTTP depending on both substrate and phosphate donor concentration.

Enzyme	Pathway
(1) Ribonucleotide reductase	de novo
(2) dCMP kinase	salvage
(3) CdR kinase	salvage
(4) dCMP phosphatase	degradative /de novo
(5) dCMP deaminase	de novo
(6) UdR kinase	salvage
(7) dTMP synthetase	de novo
(8) dTMP kinase	salvage
(9) TdR kinase	salvage
(10) Thymidine phosphatases	degradative

DNA synthesis. It is thus considered that this pathway and its regulation could be of significance in control of DNA synthesis. The interconversions of the pyrimidines and their regulation are shown in Figure 1.

It has been shown (47-49) that deoxyadenosine (AdR) and deoxyguanosine (GdR) kinases are probably the same enzyme. Krygier (48,49) showed that AdR kinase also phosphorylated GdR and CR, GdR acting as a competitive inhibitor. The enzyme is inhibited by adenine, guanine and cytosine deoxynucleoside 5' mono-di- and triphosphates as well as by the phosphorylated derivatives of cytosine arabinoside, which seem to act competitively with the phosphate donor (ATP).

Another set of salvage enzymes are the nucleoside monophosphokinases, which are responsible for the phosphorylation of ribo- and deoxyribonucleoside monophosphates to the appropriate diphosphates. Sugino and his co-workers (45) have shown that in mammalian systems there are four enzymes, which display base specificity. The CMP/dCMP enzyme also apparently phosphorylates UMP (but not dUMP), similarly the dTMP enzyme phosphorylates dUMP.

Lastly, an enzyme which partakes in both the salvage and de novo pathways is nucleoside diphosphokinase. This enzyme is totally non-specific with respect to base and sugar content, and is found at a very high level of activity in all tissues (45).

C. Nucleotide Phosphorylases

The phosphorylations previously described are not irreversible. Enzymes which dephosphorylate nucleotides have been shown to be present in a variety of tissues. In Chang liver cells there are apparently different enzymes for each phosphate (42) and the rate of release of ortho phosphates from mono-di- and triphosphates of thymidine is dependent on the rate of growth of cells at the time of harvest (43). Sugino and co-workers

isolated a "nucleoside polyphosphorylase" from calf thymus which dephosphorylates all kinds of ribo- and deoxyribonucleoside di- and triphosphates at almost equal rates to the corresponding monophosphates and inorganic phosphate (45). In regenerating liver Beltz (40) found a depression of 30-38% in monophosphatase(s) activity, as compared to normal liver, dCMP being the most susceptible to degradation. Similarly Maley and Maley (41) found normal liver extracts dephosphorylate 50% of dCMP present as compared to less than 10% in regenerating liver. Behki and Morgan (44) found that in regenerating rat liver, and thus presumably more so in normal liver, dTMP phosphatase is stimulated by UdR, which itself is produced by dUMP phosphatase, thus dTTP production is reduced in both the de novo and salvage pathways. It therefore appears that there are both specific and non-specific phosphatases present, although Eker did not test substrates other than thymidine phosphates with his enzyme (43), Behki and Morgan's data indicate specificity. Possibly the di- and triphosphatases are non-specific while the monophosphatases are base-specific. These enzymes can be considered to be both anabolic and catabolic as for example, dCDP phosphatase can be used in the synthesis of dTTP and is thus anabolic; they could play a very important role in the balancing of triphosphate pools, although no information on their cyclical fluctuations is available.

D. Differences in the Activity of the de Novo and Salvage Pathways in Rapidly Growing Tissues as Compared to Non-Proliferating Tissues

Prior to examining the differences in the activity of the de novo and salvage pathways in rapidly growing and non-proliferating tissues one must first define the two pathways in terms of enzymatic steps. A strict definition is that the salvage enzymes are only those that catalyze the phosphorylation of nucleosides, and the nucleoside monophosphates so formed to the appropriate nucleoside diphosphates. Thus the de novo enzymes are ribonucleotide reductase, deoxycytidylate deaminase and thymidylate synthetase.

Sneider et al. (50) examined the enzymes of dTTP synthesis in various Morris hepatomas, Novikoff hepatoma and Ehrlich ascites cells. They found that thymidine (TdR) kinase was consistently elevated in all these tissues (as compared to normal liver); thymidylate (dTMP) kinase was also elevated, although to a lesser extent. Deoxycytidylate (dCMP) deaminase showed normal levels in the minimal deviation hepatomas while Novikoff hepatoma and Ehrlich ascites cells showed extreme elevations. dTMP synthetase was markedly elevated in all the tissues studied. An elevated level of ribonucleotide reductase in neoplastic tissue has also been demonstrated (51). These same enzymes are high in embryonic and newborn rat liver, but decrease in activity with increasing age, such that in normal, adult rat liver they are barely detectable (50). Sneider et al. also found that the ability to degrade thymidine is lowered or completely lacking in the tissues they examined, which suggests that the salvage pathway may play an increased role in the recycling of DNA degradative products in rapidly growing tissues (50).

There is also some evidence for an elevation in thymidine phosphorylation activity in LM-cells following vaccinia virus infection (52), the enhancement being greater in monolayers which have been cultured over a period of several days, non-infected cultures having no detectable activity. Similarly McAuslan and Joklik (53) using HeLa S_3 infected with pox virus showed a marked increase in TdR kinase 4-6 hours after infection, reaching a plateau, approximately 9 hours after infection, representing a ten to fifteen fold increase in activity. DNA polymerase activity was also shown to be elevated, although by no more than 50%. They were unable to detect any increase in dTMP, dTDP, dAMP and dCMP kinases. Protein synthesis was found to be essential for the increase in TdR kinase activity to take place, and induction of enzyme activity was only caused by particles which could be "uncoated" in the cells. This evidence indicates that perhaps this enhancement of activity is due to the synthesis of a viral enzyme.

It can be seen from the foregoing account that both salvage and de novo enzymes are elevated in rapidly growing tissues, as compared to normal tissues, as would be expected in view of the fact that DNA synthesis in normal tissues is minimal, and so there is a minimal need for precursors. The lack of thymidine degradation found suggests a more important role for the thymidine salvage pathway. However, in view of the lack of information concerning the other salvage enzymes, it is impossible to draw any conclusion as to whether one or the other pathway plays a greater role in the synthesis of deoxyribonucleoside triphosphates in rapidly growing tissues.

3. The Relationship of Precursor Biosynthesis to DNA Replication

A. The Cell Cycle

One way to study the involvement of precursor regulation in relation to DNA synthesis is to look at the sequence of events occurring in the cell cycle. Cells multiplying in culture and in the body pass through successive cycles of growth, one cycle being defined as "that interval between the completion of mitosis in a cell and completion of the subsequent mitosis in one or both daughter cells" (54). The cell cycle is divided into four phases which are a) G_1, the interval between completion of mitosis and the onset of DNA synthesis; b) S, the period during which the DNA of the cell undergoes replication; c) G_2, the period between completion of DNA synthesis and cell division and d) M, the four stages of mitosis (prophase to telophase).

In the intact organism the majority of cells are quiescent, but can be induced to divide by appropriate stimuli; these cells are considered to be in a static phase (G_o) which can probably be regarded as a prolonged early G_1 state. Cells in tissue culture which do not exhibit contact inhibition, whether in monolayer or suspension culture, are continuously

dividing and thus continually passing through the cell cycle. Those which exhibit contact inhibition in a monolayer culture divide continuously until they reach confluence whereupon they enter, and remain in G_o (54).

B. Cell Synchrony

From the foregoing account it can be seen that DNA synthesis is a discontinuous function of cell growth. Cells in culture are approximately randomly distributed throughout the cell cycle and the cell population is said to be asynchronous. As a rough approximation it can be said that the fraction of cells in any one stage is proportional to the duration of that stage relative to the total generation time. Hence any sample taken from such a culture is useless for studying processes occurring at various specific stages of the cell cycle, it representing an average of the levels of that particular function in all four stages.

The ideal system to study is a single cell, however this is, in the majority of cases, impractical because of the minute size of a cell, and because most biochemical techniques require destruction of the cell and are not sensitive enough to examine a single cell. One must therefore approximate to the ideal by using a culture in which the majority of the cells are in the same stage of the cycle; such a culture is termed synchronous. Regardless of the method used to obtain a synchronous culture, it is found that it is not possible, with mammalian cells, to maintain synchrony for more than two cycles. This loss of synchrony is due to variation in cycle length of individual cells.

There are many methods of obtaining synchrony which can be divided into two groups. The first involves synchronization by the application of exogenous blocking agents, which cause the cells to accumulate at some stage of the cell cycle, on removal of the blocking agent the cells proceed synchronously through the cycle. The second is based on selecting a fraction of the total asynchronous cell population in which all of the

selected cells are at the same stage of the cell cycle (55).

(i) Synchronization by Blocking Agents

Most of the methods of producing synchrony by blocking agents utilize a block at some specific stage in the cycle, in particular those agents that inhibit DNA synthesis and those that produce metaphase arrest. There are, however, two relatively non-specific blocking methods, temperature shock and nutritional manipulation (55).

Temperature shock, or cyclical changes of temperature between optimal and sub-optimal (56) seems to slow down faster growing cells and/or speed up slower cells resulting in a partially synchronous population; it is only of limited use as mammalian cells display a complex and variable lag of each phase of the cell cycle on change of temperature. Nutritional manipulation is of very limited use in mammalian cells as relatively few mammalian cells are very sensitive to manipulation of the nutritional content of the growth medium; however, transfer of cells from low to high serum medium will produce a parasynchronous wave of growth (57).

Excess deoxythymidine (58) or the double thymidine block method (59) prevent synthesis of other DNA precursors by feedback inhibition, and so cause arrest at the beginning of S. High concentrations of exogenous deoxyadenosine or deoxyguanosine can be used in place of deoxythymidine (60). Other inhibitors of DNA synthesis are fluorodeoxyuridine (FUdR) (61), amethopterin (62), actinomycin D (63) and cytosine arabinoside (araC) (64). The effects of the first two are reversed with thymidine. FUdR and amethopterin seem to act by preventing entry into S; actinomycin D produces a reversible block in G_1, apparently as a result of inhibition of ribosome synthesis. AraC is lethal to cells in S, deoxycytidine added on removal of the drug can rescue only those cells which were in G_1 at the time of exposure to araC. Hydroxyurea (65) has been used to

some extent to inhibit DNA synthesis, however it has a tendency to produce cytotoxic effects, the degree of cytotoxicity varying between different cell lines.

The main agents producing non-lethal metaphase arrest are colcemid (66,67) and low concentrations of vinblastine sulphate (68). Colcemid appears to act by preventing the centrioles from organizing the microtubules which are necessary for their migration to the poles; on removal of colcemid normal mitosis proceeds within five minutes and it appears that the cells are unaffected. The mode of action of vinblastine is unknown, but the end result is a reversible arrest of metaphase.

(ii) Synchronization by Selective Methods

All the methods of obtaining synchrony thus far described have the disadvantage that the cells obtained have been exposed to unphysiological conditions, and it is considered that they are liable to show metabolic disturbances; thus the validity of data obtained with these cells is questionable.

There are, however, two methods of producing synchronous cells which do not have this disadvantage. Both select for cells at a certain stage in the cell cycle. The first is a sedimentation velocity through a gradient (69,70) which is based on the observation that cells just prior to M are much larger (theoretically twice as large) than cells in very early G_1, with the cells in the intervening stages of the cycle showing a gradation of sizes intermediat between these two extremes. An exponentially growing population of cells in suspension is layered onto a buffered sucrose gradient; by gentle centrifugation (69) or separation at unit gravity (70) these cells separate such that the oldest sediment to the bottom and the youngest remain near the top of the gradient. One can therefore obtain fractions from the gradient, representing all stages of the cell cycle, at the same time, thus removing the neec for sampling throughout the cycle. One disadvantage is that one does not know the tempora

relationship between fractions for relating observations to precise location in the cycle.

The second method is selective mitotic detachment (71) which makes use of the observation that in a monolayer culture, cells in mitosis round up and become more loosely attached to the substratum, and are thus easily dislodged by agitation of the culture. Both of these methods do, however, have the disadvantage that relatively small numbers of cells are obtained. For the second method this disadvantage is overcome by collecting cells from a large area of monolayer and/or repeated harvests where cells are chilled to 0°C immediately after harvesting, it having been shown (72) that cells remain in mitosis for up to three hours at 0°C and will resume normal, synchronous growth on rapid rewarming to 37°C.

The aforementioned methods of obtaining synchrony produce variable degrees of synchrony e.g. transfer from low to high serum medium produces approximately 50% synchrony (57) while with mitotic selection up to 95% synchrony can be obtained (O. Bernard, personal communication). The degree of synchrony obtained will effect the resolution of fluctuations in the cycle, the better the synchrony, the better the resolution. Thus to study any transient changes occurring in the cycle it is necessary to have a high degree of synchrony.

Once a suitable synchronous culture has been obtained one can study discontinuous functions in the cell cycle. Most work done thus far relates to events concerned with DNA synthesis.

C. Periodicity of DNA Synthetic Enzymes in the Cell Cycle

One element of the control process for the initiation and termination of DNA replication in mammalian cells may be the level of enzymes associated with the synthesis and polymerization of deoxyribonucleoside triphosphates. Thus many studies have been performed on the changes in activity of these enzymes occurring in synchronous cell populations. The enzymes in question being ribonucleotide reductase, deoxycytidylate

deaminase, the deoxynucleoside phosphokinases, deoxythymidylate phosphokinase, deoxythymidylate synthetase and DNA polymerase.

(i) Ribonucleotide Reductase

Turner, Abrams and Lieberman (62) using L-cells partially synchronized with amethopterin and thymidine found a strict parallelism between the measured enzyme levels of ribonucleotide reductase (RNR) and the fraction of the population forming DNA. The peak of enzyme activity was found at, or just before, mid S, the activity fell to less than one third of that value in G_2, rising again with the onset of mitosis. They showed that the enzyme had a half life of less than two hours in vivo, and that the loss of activity is associated with termination of DNA synthesis and not with the subsequent division. In contrast Nordenskjold et al. (57) using cultured mouse embryo cells, stimulated to a wave of DNA synthesis by transfer from low to high serum medium, found that the peak of RNR activity came about 17 hours after serum stimulation, this being between 2 and 3 hours after the peak of DNA synthesis. Whether this is a real difference or can be attributed to experimental error is not clear.

Whether the difference between these two mouse cell lines is a tissue difference or a normal/neoplastic difference, or is due to different experimental systems, is unknown. Either way it is clear that RNR activity does not peak before DNA synthesis.

(ii) Deoxycytidylate Deaminase

Deoxycytidylate (dCMP) deaminase activity is elevated in cells and tissues undergoing rapid proliferation (73,74). In a population of HeLa S_3 cells synchronized by mitotic selection, Gelbard, Kim and Perez (74) showed that dCMP deaminase activity was present in mitotic cells, decreased after cell division, increased during S to reach a

peak towards the end of G_2, at which time activity is three to five fold higher than in early G_1. Following the peak, activity declined reaching the same low level in the subsequent G_1. The peak of activity was approximately 4 hours after the peak of DNA synthesis. Their results suggest that dUMP may be made in the cell cycle immediately preceding its actual use (74). They also showed, by use of inhibitors that the fluctuations of activity seen represented changes in the rate of synthesis and degradation, and that DNA synthesis is not essential for the increase in dCMP deaminase activity. In addition the lower levels of activity seen in G_1 are not the result of the presence of competing enzyme systems or inhibitors.

Mittermayer et al. (75), using L-cells synchronized by mitotic detachment found an essentially similar pattern, with a peak, representing a five fold increase in activity, occurring at 16 hours after mitosis (6 hours after the peak of S) followed by a rapid fall in activity co-inciding with the second mitosis. In addition, by use of puromycin and actinomycin D, they showed that the increase in enzyme activity was probably due to de novo synthesis.

(iii) The Deoxynucleoside Phospholkinases

Thymidine (TdR) kinase has been shown by several groups of workers (47,66,57, 75-78) (using HeLa, L-cells, Chinese hamster fibroblasts and mouse embryo cells, synchronized by mitotic selection, various metabolic blocking agents and serum stimulation) to show an essentially similar pattern to dCMP deaminase; that is, a low level during G_1, rising at the same time as, or slightly before, the onset of S to reach a peak towards the end of G_2, followed by a rapid decline during M, reaching a low level in the subsequent G_1. The peak level is from two to nine fold higher than the basal level of activity. It has also been shown (47,66,75,78) that the observed rise in TdR kinase is directly due to

the enzyme's biosynthesis.

Deoxycytidine (CdR) kinase in HeLa was found to display a temporally similar fluctuation to that found for TdR kinase (47), although the overall level for this enzyme is about twenty fold lower than that of TdR kinase. Brent also found evidence that deoxyguanosine (GdR) and deoxyadenosine (AdR) kinases are the same enzyme, as did Krygier with purified calf thymus enzyme (48,49), and that they showed a constant high activity, no clear periodicity being observed (47).

(iv) Deoxythymidylate Phosphokinase

Brent et al. (76) using HeLa cells synchronized by mitotic detachment found that this enzyme shows a similar, if lesser, response to TdR kinase. It has also been observed that deoxythymidylate (dTMP) kinase levels are elevated in a series of hepatomas (50), whereas it is barely detectable in normal liver.

(v) Deoxythymidylate Synthetase

There seems to be very little information on the rhythmicity, if any, of deoxythymidylate (dTMP) synthetase. It is known that the level in normal liver is very low, rising after hepatectomy (79). It has also been shown (50) that it is elevated, in a manner parallel with growth rate, in a series of normal karyotype Morris hepatomas. It thus seems possible that, if the periodicity of this enzyme is studied in synchronous cultures, it too will be found to fluctuate in a manner similar to that displayed by the enzymes previously described.

(vi) DNA Polymerase

Very few studies on the periodicity of DNA polymerase activity have been carried out. Littlefield et al. (15) and Gold and Helleiner (16) using L-cells synchronized with FUdR, and Friedman (17) using HeLa cells synchronized with amethopterin found that during the S phase there was a decrease in DNA polymerase activity in the cell supernatant, with a corresponding increase in activity in the particulate fraction; the total activity remained approximately constant throughout the cell cycle. It thus appears that for this enzyme cellular location and changes thereof play a greater role than overall fluctuations of activity in any regulatory role DNA polymerase might have.

From the foregoing account it can be seen that most of the enzymes concerned with precursor biosynthesis, particularly those involved in pyrimidine production, do undergo periodic fluctuations during the cell cycle. It is thus of interest to see whether this observed periodicity produces similar periodicity in the end products of these pathways, the deoxyribonucleoside triphosphates(dRTP).

4. Previous Studies on Precursor Pool Sizes

Despite the amount of interest in, and the number of investigations on, the synthesis of dRTP, very few studies on the size and characteristics of the dRTP pools have been made. It would seem that the main reason for this omission has, in the past, been the lack of a suitably sensitive method for the estimation of these pool sizes.

A. Methods used in the Determination of Deoxynucleoside and Deoxynucleotide Pool Size

The methods used can be divided into four groups, although various combinations, particularly of the first three are frequently used. The first is a microbiological assay for

deoxyribosidic compounds; the second chromatographic separation, and identification, of the four deoxyribonucleosides and their different phosphate derivatives; the third, usually used in conjunction with the second, is spectrophotometric identification and estimation of the different bases; the fourth which is the most recent and most sensitive method is based on the in vitro enzymatic synthesis of DNA.

Prior to using any of these four methods a suitable cell or tissue extract must be prepared. This is usually made in 0.5-2.0 N perchloric acid (PCA), although trichloroacetic acid (TCA) (80,81) or methanol have also been used (80). PCA extracts must be neutralized to stabilize the deoxynucleotides, this is usually done with KOH followed by removal of the $KClO_4$ precipitate so formed. TCA extracts can be neutralized by extracting the TCA with ether.

(i) Microbiological Assay

This assay makes use of a microorganism which requires deoxyribose for growth. There are two such bacteria which are used for this purpose, Lactobacillus Acidophilus and L. Leichmannii, although L. Leichmannii can by-pass this defeciency by using vitamin B_{12} for growth, thus necessitating a step to destroy any possible vitamin B_{12} in the extract (82). This assay was originally developed by Hoff-Jørgensen (83) and has subsequently been modified by several workers (81,82,84,85). The growth requirement is specific for deoxynucleosides and deoxynucleoside monophosphates; thus prior to being assayed the di- and triphosphates must be dephosphorylated, which can be done either by incubation of the extract with snake venom (82) or potato apyrase (86,87). Autoclaving of extracts also degrades dRTP and dRDP (88).

If the assay of specific deoxynucleosides or deoxynucleotides is required, prior separation by ion exchange or paper chromatography has been used (86,88-90). It is also

possible to separate purine deoxynucleosides from pyrimidine deoxynucleosides by acid hydrolysis (89). Nucleosides or nucleotides present in the extract have been shown not to interfere with the assay and thus there is no need to remove them before assaying (82, 89). The minimum sensitivity of this method is about 0.5 μmoles (82).

(ii) Chromatographic Assay

Using either ion exchange, paper or thin layer chromatography, either separately or in conjunction with each other, it is possible to separate all the different deoxynucleosides, dRMP, dRDP and dRTP. This separation is purely qualitative and thus this method is used in conjunction with methods (i) and (iii). Substances are identified by comparison of Rf values of the sample and appropriate standards. Localization of peaks and spots being achieved either by monitoring OD_{260} of column effluent or by scanning paper and TLC chromatograms with an ultraviolet light. Chromatography has been widely used (86-92) in determining which deoxynucleosides and deoxynucleotides appear to be present in various tissues.

Once an appropriate separation of deoxynucleosides and deoxynucleotides has been made chromatographically, it is possible to assay the eluates (in the case of ion-exchange columns) or eluted spots (from paper and TLC) either microbiologically (81,87-91) or spectrophotometrically (86,90,92,93). For use in chromatographic separation it is necessary to have between 10 and 20 μmoles of the sample to be assayed; if less is present localization of the peaks and spots is not possible.

(iii) Spectrophotometric Assay

This method makes use of the fact that the purine and pyrimidine bases absorb light in the ultra-violet region of the spectrum, each base having a characteristic spectrum

which can thus be used for positive identification (86,90,93). It is also possible to determine the absolute concentration of base present from the molar extinction co-efficient, concentration being equal to the optical density of the solution divided by the extinction co-efficient. The minimum concentration that can be determined by this method is about 20 μmolar. Before using this assay, prior chromatographic separation is necessary.

(iv) Enzymatic Assay

Following the isolation and purification of DNA polymerase, Kornberg and his co-workers, in studying the synthetic requirements of this enzyme, observed that the presence of all four of the deoxyribonucleoside triphosphates is necessary for enzymatic synthesis of DNA (8). Solter and Handschumacher (94) utilized this observation to develop a very sensitive assay which is specific for the dRTP. If in the polymerization reaction DNA primer, Mg^{2+} and three of the triphosphates are present in excess, and if the fourth triphosphate is supplied in limiting quantities, then the reaction will proceed until essentially all of the limiting dRTP is incorporated. The amount of DNA formed should then be proportional to the amount of the limiting dRTP present. If one of the excess dRTP is labelled then it is possible to determine the amount of limiting triphosphate present by precipitating the acid-insoluble polymer and assaying for radioactivity. With this method it is possible to determine as little as 50 picomoles of dRTP.

Lindberg and Skoog (95) modified this method and increased the sensitivity by using poly d(A-T) as the primer and by increasing the specific activity of the labelled triphosphate present. With this method they were able to measure 0.5 picomoles of dATP or dTTP. The reliability of this modification depends on the state of the poly d(A-T) primer, a fairly large and uniform size being required; it also has the disadvantage of not being able

to assay dCTP and dGTP.

In addition to the four previously described methods some estimates of pool size have been made using isotope-dilution techniques (96), these have mainly been applied to thymidine nucleotides following administration of ^{3}HTdR. With this method exact quantitation is not possible, since it is probable that addition of external deoxynucleoside alters the equilibrium pool size present in vivo.

The microbiological, chromatographic and spectrophotometric assays have the disadvantage that they are not very sensitive and that, if quantitative determination of a specific deoxynucleoside or deoxynucleotide is required, it is necessary to purify the particular deoxynucleoside or deoxynucleotide from the acid-soluble cell extract. Solter and Handschumacher's assay method does not need prior separation of the dRTP from the extract, it is specific for each of the four dRTPs and is much more sensitive than the other three. It is therefore a very valuable tool for use in determinations of dRTP pool sizes.

B. Size and Localization of Deoxynucleoside and Deoxynucleotide Pools in Mammalian Tissues

Using the microbiological, chromatographic and spectrophotometric assays previously described investigations on various tissues showed the presence of pyrimidine deoxynucleosides and deoxynucleotides, but in only one instance (93) was a purine, dATP, demonstrated. The probable reason for this is not that the purines are lacking in tissues but rather that they are present at a concentration too low to be detected. Lark (88) estimated that there could be approximately 20,000 molecules of deoxyadenosine and deoxyguanosine, and their phosphate derivatives,in the cell which would not be detected by the microbiological assay. Cleaver (97) has estimated that the total pool size of the deoxythmidine phosphates is between 4×10^{-18} and 8×10^{-17} moles per cell; this was calculated from data obtained by other

workers (86,90,98). Cleaver also estimated that dTTP accounts for more than 70% of this pool. The dTTP pool in regenerating rat liver was found to be 3-4 nm/mg DNA (92) which is estimated to be 2-3 times that in normal liver. The deoxycytidine pool in normal rat liver is approximately the same size as the deoxythymidine pool (10 μg/g tissue), while in regenerating liver it is less elevated than the deoxythymidine pool (TdR: dNS + dNT = 30 μg/g tissue; CdR: dNS + dNT = 20-25 μg/g tissue (91, 99)).

It has been shown that in regenerating liver the relative concentration of phosphorylated deoxynucleosides increases with respect to the deoxynucleosides (87,99, 100); in normal liver more than 90% of the deoxyribosidic compounds found are in the nucleoside form whereas in regenerating liver approximately 33% are in the nucleotide form (99). This relative increase is also seen in hepatoma tissue (100) and would thus seem to be of importance in rapidly growing tissues as deoxynucleotides are needed for DNA synthesis.

The one available estimate of purine pool size (93) assayed microbiologically, is 3-4 mμmoles dATP/g tissue (Flexner-Jobling carcinoma). This can be seen to be of the same order of magnitude as the dTTP pool (1.45 mμmoles/g rat spleen; 32.3 mμmoles/g rat thymus) (90). The absolute levels cannot be compared as it is inevitable that different tissues and cell systems, having different growth rates,will have different pool sizes (101).

The acid soluble pool of TdR derivatives has been localized, by autoradiography, and by nuclear isolation in a non-aqueous medium, in the nucleus (100,102,103). This is what one might expect to find as it seems probable that the nucleus is the site of DNA replication.

Nordenskjöld et al. (57) using Lindberg and Skoog's modification (95) of Solter and Handschumacher's (94) assay, studied the dATP and dTTP pools, in relation to DNA synthesis, in cultured mouse embryo cells. The cells were induced to a parasynchronous

wave of DNA synthesis by transfer from low to high serum culture medium or by infection of low serum cells with polyoma virus. They found that during low serum cultivation the levels of dATP and dTTP were about 0.5 picomoles/μg DNA. Following induction of DNA synthesis the pools started to increase at about the same time as DNA synthesis began, reaching a peak either simultaneously with, or just after the peak of DNA synthesis. The peak pool sizes being a 4-5 fold increase over the low-serum levels, in all cases the dTTP pool was larger than the dATP pool.

C. The Possible Significance of the Precursor Pool Sizes in the Regulation of DNA Synthesis

In order to synthesise DNA, the cell must produce the necessary precursor deoxyribonucleotides, and it appears that these are in the triphosphate form. To what extent, if any, does such precursor production regulate or control DNA biosynthesis during the division cycle of the normal cell?

In a synchronous bacterial culture (Alcaligenes fecalis L.B) Lark (80,88) showed that deoxypyrimidine nucleotide synthesis was a periodic function, an increase in deoxynucleotide pool size occurring 10 minutes prior to onset of DNA synthesis (10 minutes being equivalent to 1/7th of the cell cycle). However, if DNA synthesis was made continuous, by supplying deoxynucleosides to the medium during a previous cycle of synthesis, the pattern of deoxynucleotide synthesis is unaltered and would thus appear to indicate that the production of deoxynucleotides is closely coupled to DNA synthesis, but is neither dependent upon, nor responsible for, the onset of DNA synthesis.

Another system which has been studied in connection with a possible role for deoxynucleotides in DNA synthetic regulation is microspore development in the lily and trillium anther (104-106). In these systems microspore production is synchronized with

respect to time, the length of the flower bud enclosing the anthers being correlated with the synchronous activities of the microspore. Another aspect of microspore growth which is useful is that the interphase in a few weeks long andthus sequential events in the life cycle are sufficiently separated in time to provide easy resolution. Hotta and Stern (104-105) chose to study the appearance and disappearance of TdR kinase activity, the activity appears on approximately the twentieth day of interphase and persists for only 24 hours. They found that immediately prior to TdR kinase activity appearing (which is immediately prior to DNA synthesis) a pool of deoxynucleosides appears in the fluid surrounding the microspores; this pool disappears abruptly as DNA is made. The pool contains all four deoxynucleosides and originates from the destruction of a polydeoxyribotide pool in extra-sporangeous tissue, these deoxynucleosides are rapidly converted into dRTP by the microspores or the microspore walls. These dRTP are not incorporated into microspore DNA, as if they are labelled, the label is not incorporated into microspore DNA (107). Hotta and Stern have postulated that their function might be to induce TdR kinase, which they have shown to be synthesized de novo (104). Takats (107) suggests that the deoxynucleotide pool might serve to induce the formation of a second deoxynucleoside or deoxynucleotide pool which is the one used to supply the necessary precursors for microspore DNA synthesis.

The appearance of the phosphorylating enzyme may, in certain instances, make DNA synthesis possible, but it does not make it mandatory. It would thus appear that in this system deoxynucleosides and deoxynucleotides may play an indirect role in the initiation of DNA synthesis. However, in regenerating liver (86,87,99) and embryonic mouse cells (57) the increase in deoxynucleotide pool sizes occurs at the same time as DNA synthesis occurs suggesting that deoxynucleotides are supplied for DNA synthesis on demand, neither accumulating to any great extent before DNA synthesis nor diminishing greatly during synthesis.

From data available, it would appear that the precursor pools do not play a direct role in the initiation of DNA synthesis. The increase in pool size seen concomitant with DNA synthesis probably occurring as a result of demand, their synthesis being possible because of the elevated synthetic enzyme activities seen at this time.

The work reported in this thesis was undertaken to examine the pool sizes of all four deoxyribonucleoside triphosphates in a highly synchronous culture of mammalian cells (HeLa), in order to determine whether these pools fluctuate, the nature of such fluctuations and their relationship to DNA replication

MATERIALS AND METHODS

1. Materials

A. Deoxynucleoside Triphosphates

Tritium labelled dTTP, dATP and TdR were obtained from New England Nuclear, specific activity 13-18 Ci/m mole, 5.74 Ci/ m mole and 18.5 Ci/m mole respectively.

Unlabelled deoxynucleoside triphosphates were purchased from Sigma or P-L Biochemicals.

The purity of both labelled and unlabelled triphosphates was checked by descending paper chromatography for 16 hours on Whatman #1 paper strips in Isobutyric acid: .880 NH_4OH : 0.1 M EDTA : H_2O, 66 : 1 : 1 : 32, final pH 4.6 (108). Spots were located under an ultra-violet lamp. Radioactivity was localized by scanning the strips in a Packard model 7201 Radiochromatogram Scanner. When any spots other than the triphosphates were present on the chromatogram, they were purified by chromatography through a DEAE-cellulose column; it was normally found that the unlabelled triphosphates had more than one spot, whereas the labelled ones appeared pure.

Column chromatography was by the method used by Brown and Reichard (24). Elution was monitored by following OD_{260} of the fractions collected. Following flash evaporation the purified triphosphates were redissolved in double distilled water to a final concentration of 4 mM; concentrations were determined both spectrophotometrically and by phosphate content, good agreement between the two methods was found; from this stock appropriate dilutions were made prior to use.

B. DNA

Undenatured calf thymus DNA was purchased from Worthington. For use in the assay it was made up to a concentration of 1.2 mg/ml in 1 M NaCl. The concentration was determined by OD_{260} (1 mg/ml = 22 OD_{260}).

Calf thymus DNA, activated by the method of Loeb (109) at a concentration of 1.2 mg/ml in 0.01 M KCl was a gift from Dr. J. Slater (Institute for Cancer Research, Fox Chase, Philadelphia, Pa.).

C. DNA Polymerase

E. coli DNA polymerase purified to fraction 7 according to Jovin et al. (110) and then subjected to hydroxylapatite chromatography, was also a gift from Dr. J. Slater. The activity was 16,500 Kornberg units/ml (using an activated DNA template, one unit being that amount that will incorporate 10 n moles of deoxynucleoside triphosphate into an acid-insoluble product per 30 minutes), and it was at a concentration of 0.28 mg protein/ml. For use in assays it was diluted 80 fold in a solution of 0.055 M Tris-HCl, 0.01 M 2-Mercaptoethanol, 0.1 M $(NH_4)_2SO_4$, 1 mg/ml bovine serum albumin, pH 8.5 (enzyme diluent) (94), and stored at -80°C.

D. Cell Culture

(i) HeLa Cells

HeLa cells were routinely maintained in suspension culture at 37°C in Eagle's minimum essential spinner medium (F_{14} from GIBCO), supplemented with 5% aseptic calf serum (A.C.S.) (Tissue Culture Services, Slough, Bucks, England). Stock cultures were maintained as monolayer culture in 250 ml Falcon flasks in Eagle's minimum essential

medium for monolayers (F_{11} from GIBCO) containing 5% A.C.S. These cells originated in 1963 from Dr. H. Harris (John Innes Institute, England) and have been maintained since then at the Chester Beatty Research Institute, Pollards Wood Station, Chalfont-St-Giles, Bucks, England, and in our laboratory.

(ii) Chinese Hamster Cells

Chinese hamster cells, strain V79-753-B3 1-F39, were a gift from Miss K. Mann (McGill University Biology Department). This strain is a clonal derivative of strain V and was originally obtained from Dr. M. M. Elkind at N.I.H. Strain V was obtained from male Chinese hamster lung tissue. These cells were routinely maintained in monolayer culture in Eagle's MEM supplemented with 0.003% trypsin, 0.04% NCTC 135 and 15% foetal calf serum (BBL, a division of Beckton Dickinson and Co, Clarkson, Ontario). At least three hours prior to harvest, monolayer cultures approaching confluence were trypsinised and placed in suspension culture at a concentration of approximately 2×10^5 cells/ml.

(iii) Mouse Fibroblast Cells

Mouse fibroblasts cells, strain L929, were a gift from the laboratory of Dr. A. F. Graham (McGill University, Biochemistry Department). These cells were maintained in suspension culture in Eagle's minimum essential spinner medium (F_{14}) supplemented with 5% foetal calf serum (Tissue Culture Services, England).

For the production of large areas of monolayer culture, roller bottles and apparatus from New Brunswick Scientific were used.

E. General

Toluene scintillation fluid was prepared by dissolving 10 mg POP and 200 mg POPOP in 2l toluene.

All other chemicals used were purchased locally from Fischer or Canlab.

2. Methods

A. Deoxynucleoside Triphosphate Assay

The method used was a modification of the method of Solter and Handschumacher (94).

Optimal assay conditions were determined (see Results, section 1) and were as follows: Glycine-NaOH buffer (pH 9.2), 20 μmoles; 2-mercaptoethanol, 0.3 μmoles; $MgCl_2$, 2.04 μmoles; DNA template, 12 μg undenatured calf thymus DNA; excess triphosphates, 1 nmole; labelled triphosphate, 1 μC (1 nmole); limiting triphosphate, 0.005-0.1 nm; in a final volume of 0.1 ml. 0.1 ml water, standard or cell extract was added. 0.9 ml enzyme diluent was added to the reaction mixture while in an ice-water bath, incubation tubes were then warmed to 37°C and the reaction started by the addition of 2.06 Kornberg units of enzyme. Incubation was carried out at 37°C for 45 minutes. The reaction was terminated by the addition of 3.0 ml of ice-cold 5% trichloroacetic acid (TCA) containing 0.01 M tetrasodium pyrophosphate ($Na_4P_2O_7$). The reaction tubes were then placed in an ice-water bath (0°C) and allowed to stand for 10 minutes, to allow precipitation. The acid-insoluble material was then collected by filtration through Whatman GF/C glass fibre filter discs in Gelman filter holders. The tubes were washed twice with ice-cold 5% TCA plus 0.01 M $Na_4P_2O_7$ and once with distilled water; the filters were rinsed once with distilled water and then dried at 210°C. Radioactivity

was measured by placing the dried filters in vials containing 5.0 ml of toluene scintillation fluid and counting in a Packard Tri-carb liquid scintillation spectrometer, model 3375, for 10 minutes or until 20,000 counts were accumulated.

Incorporation of label was calculated as the percentage of the total added label recovered in the acid-insoluble fraction. This percentage could be converted to picomoles of the limiting deoxynucleoside triphosphate incorporated by reference to a standard curve, which was empirically determined during each assay. The pool size of dRTP was expressed as p moles/μg DNA or p moles/cell, and using Terasima and Tolmach's estimation of HeLa cell volume (71), as absolute concentration in the cell.

B. Preparation of Cell Extracts

Approximately 2×10^7 cells were used per extract. It was found that when more than 10^8 cells were used per extract, a substance present in the extract inhibited the DNA polymerase reaction (see Results, section 1 A (iv)).

Cells in suspension at 37° were rapidly chilled to 4° in a dry ice-ethanol bath (by swirling in an Ehrlenmeyer flask, rate of cooling: 100 ml from 37° to 4° in 1 minute). All subsequent operations were carried out at 4°C. The cells were then centrifuged for four minutes at 120 g in a Sorvall GLC-1 centrifuge, resuspended in 50 ml phosphate buffered saline (P.B.S. = 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2 . 6H_2O$, pH 7.4), and re-centrifuged. The resulting pellet was transferred in PBS to a 2.0 ml nitrocellulose tube (Beckman), centrifuged for 4 minutes at setting 7 of a bench-top clinical centrifuge (I.E.C.) and resuspended with a glass rod into 0.2 ml 0.5 N perchloric acid (PCA). The extracted cells were centrifuged for 15 minutes at 39,000 rpm (105,000 g) in a Beckman model L preparative ultracentrifuge, using an AL40 rotor. The supernatant was removed and neutralized by addition of 15 μl 1 M potassium

phosphate buffer, pH 7.4, plus 25 µl 4 N KOH. Neutrality was judged with 'neutralit' pH papers, any value between pH 6.0 and pH 8.5 being accepted. The resulting $KClO_4$ was spun down and the neutralized extract was removed and frozen at -20°C until use . It has been found that the triphosphates in such an extract are stable for several months at -20°C (94,95). The extracted pellet was retained at -20°C for subsequent DNA assay. It was found that up to 0.1 ml $KClO_4$ (saturated at 4°C) did not interfere with the deoxynucleoside triphosphate assay (see Results, section 1 A (iii)).

C. Production of Synchronous Cells

Synchronous HeLa cells were obtained by selective detachment of mitotic cells from a monolayer. Monolayer cultures were grown in roller bottles. These were prepared for use by gassing with 95% CO_2 - 5% air mixture, adding 100 ml F_{11}, (10% A.C.S.), and rolling for 2 days. For innoculation 2×10^8 cells in suspension culture, at about 10^6 cells/ml, were added to each roller bottle, about 300 ml of fresh F_{11} (10% A.C.S.) was also added. Ten to 15 roller bottles were used per experiment, and following innoculation were allowed to roll for 24 hours prior to harvesting so that the cells could become established in a monolayer. Before harvesting monolayers were washed three times at half-hourly intervals with 50 ml F_{14} (5% A.C.S.) by vigorous agitation and discarding of the cells so detached, this was to remove loosely attached but non-mitotic cells. Mitotic cells were harvested by agitation of the roller bottles and collection into an Ehrlenmeyer flask. Where more than one harvest was necessary to obtain enough cells, harvested cells were chilled rapidly by swirling in an ice-water bath, centrifuged for 10 minutes at 4° at 1000 rpm in an I.E.C. centrifuge. The cells were resuspended in a total volume of 100-200 ml of ice-cold F_{14} (5% A.C.S.) and maintained in a spinner flask in an ice-water bath until sufficient cells had been obtained for use. Usually it was necessary to perform two or three harvests

at half-hourly intervals; cells at 4°C were rewarmed to 37°C in a water bath at 45-50°C such that they were at 37°C at a time co-incident with the completion of the last harvest. All the cells were then combined and placed either in a large spinner flask or in a Vibromix (Chemap, Germany) depending on the final volume present, this being taken as time zero for the cell-cycle experiment.

Cell counts were performed, using a haemocytometer, on cell harvests and on the final suspension throughout the cell cycle. The degree of synchrony obtained was determined by estimating the mitotic index of the harvested cells (see Methods, section D). The rate of DNA synthesis was followed during subsequent progression through the cell cycle by incorporation of ^{3}H-TdR (see Methods, section E).

In HeLa cells the cell cycle is 18-20 hours long which is divided thus: G_1, 6-8 hours; S, 8 hours; G_2, 3 hours; M, 1 hour. Samples were therefore taken at intervals of 1,2 or 3 hours for a minimum of 18 hours, and in some cases up to 28 hours, in order to examine changes during the cell cycle.

D. Determination of Mitotic Index

A 1 or 2 ml sample of harvested cells was taken immediately after harvesting. The cells were chilled rapidly by pipetting into ice cold PBS. The resulting suspension was centrifuged for 4 minutes at setting 5 on a clinical centrifuge at 4°C. The resulting pellet was resuspended by dropwise addition of 5 ml ice cold 0.1 M sucrose with constant agitation. The suspension was allowed to stand at 4°C for 3 minutes to allow the cells to swell. Following centrifugation the cells were slowly resuspended in 5 ml fixative (Absolute ethanol, glacial acetic acid, 3.1) and allowed to stand at 4°C for 10 minutes. The cells were again centrifuged and resuspended into 0.1 ml fixative; the resulting suspension was applied dropwise to a microscope slide, air dried, stained for 10-15 minutes in

giemsa stain, rinsed and examined under the microscope. The mitotic index was determined by scoring that percentage of the cells observed that were in mitosis. Normally 90-95% of the cells were in mitosis.

E. Monitoring of DNA Synthesis

Throughout the cell cycle samples were taken at 1 or 2 hourly intervals to follow the course of DNA synthetic activity of the cells. In order to measure DNA synthesis 0.5 ml of cell suspension was added to 1.0 ml F_{14} (5% A C S), 1 μC TdR/ml and was incubated in a sealed tube at 37°C for 30 minutes. The reaction was stopped by addition of excess ice cold P B S. The samples were then filtered through Millipore HAWP 025 00 filters. The tubes and filters were rinsed twice with ice cold P B S and the filters washed twice with ice-cold 5% TCA, once with distilled water and dried at 210°C. 3H-TdR incorporation was determined by counting the dried filters in 5.0 ml toluene scintillation fluid.

It was usually found that DNA synthesis began at about 5 hours after mitosis, reached a peak at 12-14 hours, declining to a minimal activity at 20-22 hours after mitosis. When the beginning of the second cycle was also studied, the second rise in synthetic activity began at about 24 hours.

F. DNA Assay

DNA was assayed according to the method of Burton (111) using diphenylamine. Calf thymus DNA was used as standard. Cell pellets, the residue remaining following acid extraction of the cell, were hydrolyzed twice with 2.5 ml 0.6 N PCA at 70-80° for 15 minutes. The two extracts were pooled and 0.5 and 1.0 ml samples were assayed.

G. Phosphate Assay

Phosphate, both inorganic and total, was assayed by the method of King (112) using KH_2PO_4 as a standard.

RESULTS

1. Deoxynucleoside Triphosphate Assay

A. The Determination of Optimal Deoxynucleoside Triphosphate Assay Conditions

(i) Assay According to Solter and Handschumacher

Initially Solter and Handschumacher's assay (94) was followed almost exactly. This assay makes use of the observation that all four deoxyribonucleoside triphosphates (dRTP) are necessary for in vitro synthesis of DNA to occur. If a DNA templete, DNA polymerase and three of the dRTP are present in excess, if one of the excess dRTP is labelled, and if the fourth dRTP is present in a limiting amount, then the amount of synthesis, as determined by incorporation of label into acid insoluble material, will be proportional to the amount of the limiting dRTP. Solter and Handschumacher used 10 μg undenatured calf thymus DNA as template, 14.1 units of DNA polymerase and 10 n moles of excess dRTP, the labelled dRTP was at a specific activity of 0.1 μC/n mole, and the limiting dRTP at a concentration of 0.1 to 1.0 n moles. This assay method does not measure rate of incorporation, but, rather, measures the amount of incorporation after complete consumption of the limiting dRTP. Thus, it is necessary to ensure that the experimental conditions allow for total utilization of the dRTP that is in a limiting concentration.

Since the amount of label incorporated should depend on the amount of limiting dRTP present, then using different amounts of enzyme should merely change the time required to reach the plateau of incorporation; it was therefore decided to determine whether, with the enzyme preparation being used, the same amount of enzyme as Solter and Handschumacher used was necessary to obtain the requisite incorporation within a reasonable time

period. To test this 2.85, 5.7 and 11.4 units of enzyme were used in the assay system, with all four triphosphates present in equal amounts (10 n mole), for incubation times ranging from 0 to 90 minutes. Since the limiting triphosphate was to be used at a maximum of 1.0 n mole, which is 10% of the amount of the triphosphate used in this experiment, it was desireable to have sufficient enzyme present to produce more than 10% incorporation in a reasonable incubation time. Table 2 shows that with 2.85 units of enzyme 10% incorporation is reached after between 30 and 60 minutes of incubation, with 5.7 units it occurs between 15 and thirty minutes, while with 11.4 units 10% incorporation occurs before 15 minutes. It can be seen that different amounts of enzyme do change the time required to reach 10% incorporation, and that 5.7 units of enzyme enable this level of incorporation to be reached in a reasonable time. Therefore 5.7 units of enzyme were used in all subsequent experiments.

In order to verify that this amount of enzyme was correct, and to determine what time of incubation should be used, a time course of incorporation was examined with one dRTP present at a limiting concentration (1 n mole). Figure 2 shows that a plateau of incorporation was obtained after 30 minutes of incubation, which is in good agreement with a similar experiment done by Solter and Handschumacher. Therefore it was decided to use a 60 minute incubation time in conjunction with 5.7 units of enzyme in subsequent experiments.

A standard curve of dCTP concentration between 0.01 and 1.3 n moles was made to determine the range of concentration over which the assay is applicable. Figure 3 shows that incorporation of ^{3}H-dTTP is linear over a range from 0.1 to 1.3 n mole, and it is apparent that this deoxynucleoside triphosphate assay can be used to determine the concentrations of dRTP in cell extracts with a maximum sensitivity of about 0.1 n mole (100 p mole). At this stage, standard curves for the other three dRTP were not obtained, as it was decided that while optimal assay conditions were being determined it was not necessary to

TABLE 2

Time Course of Incorporation of ^{3}H-dTTP in the DNA Polymerizing Reaction with Varying Amounts of DNA Polymerase

Enzyme (units)	Time of Incubation (minutes)				
	0	15	30	60	90
2.85	0	5.29 (15.03)	7.53 (3.98)	13.39 (3.36)	17.82 (4.85)
5.7	0	8.63 (2.20)	14.31 (8.70)	23.73 (2.40)	31.09 (3.14)
11.4	0	13.56 (1.22)	20.96 (2.17)	32.84 (1.93)	39.96 (2.66)

The results represent the percentage conversion of the total radioactivity present into an acid-insoluble product.

The figures in parenthesis represent the percentage error between duplicates.

$$\% \text{ Error} = \frac{x-y}{\frac{(x+y)}{2}}$$: where x and y are the values of the duplicate samples.

The reaction mixture contained 10 n mole of each dRTP, the labelled dRTP was at a specific activity of 0.1 μc/n mole, 10 μg undenatured DNA was used as template and incubation was at 37° for the indicated times with the indicated amounts of enzyme.

Figure 2: Time course of ^{3}H-dTTP incorporation in the presence of a limiting concentration of dCTP.

The incubation mixture contained 10 nmole dATP, dGTP and ^{3}H-dTTP (specific activity 0.1 μC/nmole), dCTP was at a concentration of 1 nmole. 5.7 units of DNA Polymerase were used. Other components of the incubation mixture were as described under Methods. Incubation was at 37°C for the indicated times.

In this and all subsequent figures, the bars represent the range between duplicate samples, where no bar is present the variation was within the limits of the point.

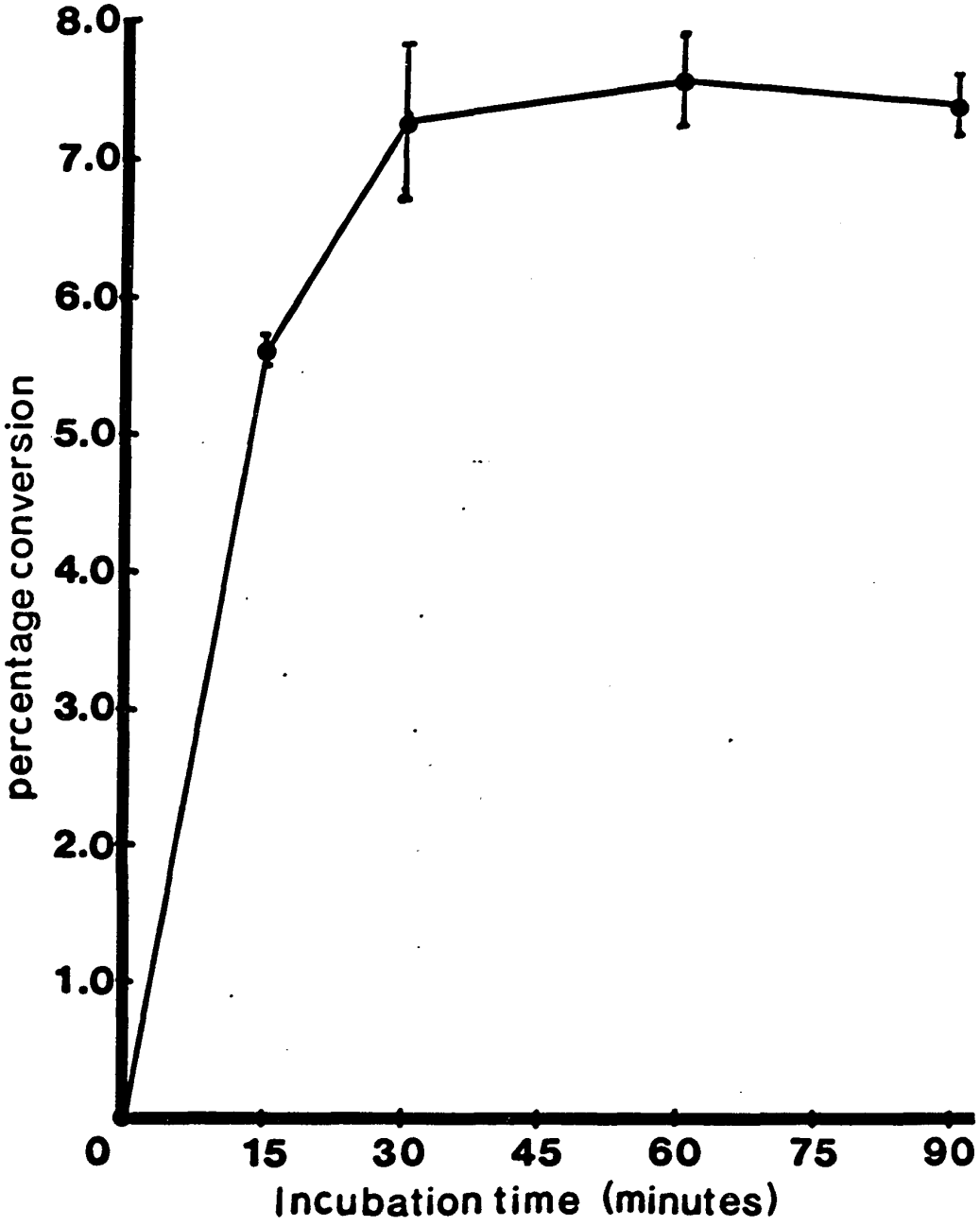
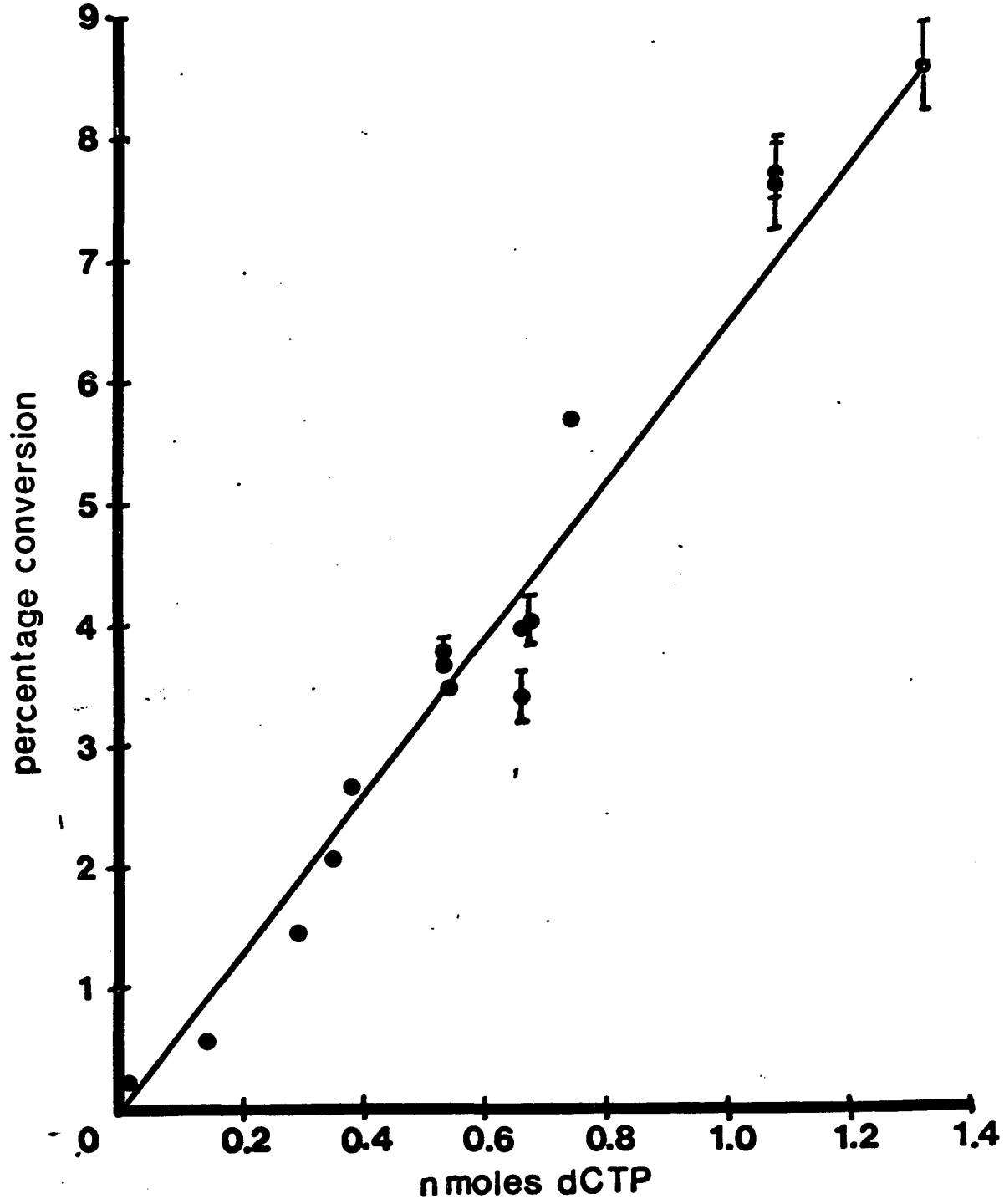

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
percentage conversion
0
15
30
45
60
75
90
Incubation time (minutes)

Figure 3: dCTP standard curve (compiled from several different experiments)

The incubation mixture was as described for Figure 2 with the exception that a range of concentrations of dCTP from 0.01 to 1.3 nmole was used with a 60 minute incubation period.


9
8
7
6
5
4
3
2
1
0
percentage conversion
0.2
0.4
0.6
0.8
1.0
1.2
1.4
n moles dCTP

work with more than one dRTP at a limiting concentration.

(ii) Determination of the Procedure for Extracting Deoxynucleoside Triphosphates from Cells

The most commonly used procedure for obtaining a preparation of the nucleotide pool is extraction with perchloric acid (PCA) followed by neutralization with KOH. However in view of the extremely low concentration of deoxynucleoside triphosphates in the cell (approximately 0.9 p mole/μg DNA (57), or 13 p mole/10^6 cells), and because the minimum sensitivity of the triphosphate assay was 100 p mole (thus necessitating the use of at least 8×10^6 cells/assay), minimal dilution of cellular material was desired.

It was therefore decided to disrupt the cells by sonication followed by boiling to denature enzymes and nucleic acids and centrifugation, to prepare a supernatent containing the nucleotide pool. Forty seconds sonication at 40% power setting with a microprobe (Sonic Dismembrator, Artek Systems Corp.) produced complete cell breakage, as judged by microscopic observations. Approximately 1×10^8 asynchronous HeLa cells per extract were harvested, washed and collected in nitrocellulose tubes as described under Methods. An equal volume of water was added to the final cell pellet and the slurry so formed was sonicated, boiled for one minute and centrifuged for 45 minutes at 39,000 rpm (105,000 g) at 4°C in a Beckman model L preparative centrifuge using an AL40 angle head. Four extracts were prepared, and to check the effect of boiling on the stability of deoxynucleoside triphosphates, a range of standard amounts of triphosphate were added to each of three extracts prior to boiling, the fourth extract contained no exogenous dRTP. These extracts were assayed for dRTP. However it was found that no proportionality existed between the amount of exogenous triphosphates and the amount of incorporation into DNA. This result indicates that this method results in considerable loss of dRTP and the extraction procedure by sonication and boiling was therefore discarded.

It was therefore decided to use an acid extraction procedure with minimal volume increase. Approximately 2×10^8 asychronous HeLa cells per sample were extracted as described in the Methods section, the packed cell volume was approximately 0.2 ml and an equal volume of 0.5 N PCA was added. Two extracts were prepared, standard being added to one prior to addition of acid. 0.1 ml of each extract was assayed, together with a standard. Table 3 shows that the addition of internal standards to the cell extract gave additive values. This indicates that the acid extraction procedure results in no apparent loss of triphosphate.

From the value for extract alone in Table 3 it was calculated that there were approximately 4 p mole dCTP in 10^6 asynchronous cells.

The acid extraction procedure was used in all subsequent experiments.

(iii) The Effect of $KClO_4$ on the Deoxynucleoside Triphosphate Assay

The use of the PCA extraction procedure introduces a saturated $KCLO_4$ solution into the dRTP assay mixture when the extract to be assayed is added, it is possible that this might affect the polymerization reaction. This possibility was examined by adding $KClO_4$, saturated at 4^oC, to reaction tubes containing a standard amount of a limiting triphosphate. Table 4 shows that the incorporation of labelled precursor is not significanly affected by the addition of up to 0.1 ml saturated $KClO_4$.

(iv) Demonstration of the Presence of an Inhibitor of the Deoxynucleoside Triphosphate Assay in the Extract

Up to this point all assays had been performed on the dCTP content of the extracts. Examination of the proportionality of incorporation of ^3H-dTTP with increasing concentrations of extract showed a lack of proportionality, and in addition, when the level of dTTP in extracts was assayed, none could be detected. This result suggests either that

TABLE 3

The Effect of the Extraction Procedure on Added Internal Standards

Sample	% conversion (% error in duplication)
0.1 ml extract	0.87 (5.75)
0.1 ml extract + 0.25 nmole dCTP	2.26 (3.56)
0.25 nmole dCTP	1.40 (5.88)

The incubation mixture contained 10 nmole dATP, dGTP and ^{3}H-dTTP (specific activity 0.1 μC/nmole) and 5.7 units of DNA polymerase. All other components of the reaction were as described under Methods. Incubation was at 37°C for 60 minutes. dCTP standard was added to the second extract prior to addition of PCA during the extraction procedure.

TABLE 4

The Effect of $KClO_4$ on the
Deoxynucleoside Triphosphate Assay

ml $KClO_4$	% conversion (% error in duplication)	
	0.538 nmole dCTP	0.039 nmole dGTP*
0	3.49 (0.57)	1.41 (3.19)
0.005	-	1.50 (3.00)
0.01	-	1.43 (3.49)
0.02	-	1.45 (0.69)
0.03	3.63 (1.65)	-
0.05	4.16 (0.0)	1.38 (2.90)
0.07	3.34 (2.39)	-
0.10	3.72 (2.96)	-

* This test was performed following further modification of the assay.

The reaction mixture contained 10 nmole (*1 nmole) of dATP, dGTP (*dCTP) and ^{3}H-dTTP (specific activity 0.1 μC/nmole (*1 μC/nmole))and 5.7 (*2.06) units of DNA polymerase. Incubation was at 37° for 60 (*45)minutes.

the dTTP is acid-labile or that there is an inhibitor of the dRTP assay present in the extract.

The first possibility was checked by incubating standard amounts of dTTP with 0.5 N PCA at 4°C for 15 minutes followed by neutralization and subsequent assay for dTTP. Table 5 shows that the dTTP was stable under these conditions.

The second possibility was examined by assaying a constant amount of dTTP and, separately, dCTP with varying amounts of extract. In both cases a decrease in incorporation of ^{3}H label was observed with increasing extract volume in the reaction mixture (see Figure 4). It was thus evident that more than 30 µl of extract produced an inhibition of the polymerase reaction. 30 µl of extract is equivalent to approximately 0.15 nmole of dTTP (0.5% conversion), which is close to the lower limits of sensitivity of the reaction. It was therefore decided that, rather than attempt to identify and remove the inhibitor, and as extract amounts of less than 30 µl contain too little dRTP to assay, the results obtained necessitated making the assay more sensitive.

B. Increasing the Sensitivity of Solter and Handschumacher's Deoxynucleoside Triphosphate Assay

Lindberg and Skoog's (95) modification of Solter and Handschumacher's assay, which resulted in a 200 fold increase in sensitivity, made use of a poly d(A-T) template combined with a higher specific activity of the labelled triphosphate. The use of poly d(A-T) as a template has the disadvantage that it is only possible to assay for dATP and dTTP. In the present study it was desired to assay for all four deoxynucleoside triphosphates and, as it has been reported (113) that activated DNA is a better template for purified DNA polymerase than undenatured DNA (because of the increased number of 3'-OH groups available for the enzyme to utilize) it was decided to use an activated DNA template. In addition it was desired to decrease the total triphosphate concentration in the assay such

TABLE 5

The Acid Stability of dTTP

Sample (nmole dTTP)	% conversion (% error in duplication)	n mole present from a standard curve
0	0	used in the derivation of the standard curve
0.195	0.47 (6.80)	
0.790	2.51 (1.63)	
0.41 acidified and 0.20 neutralized	1.46 (0.68) 0.76 (1.32)	0.47 0.24

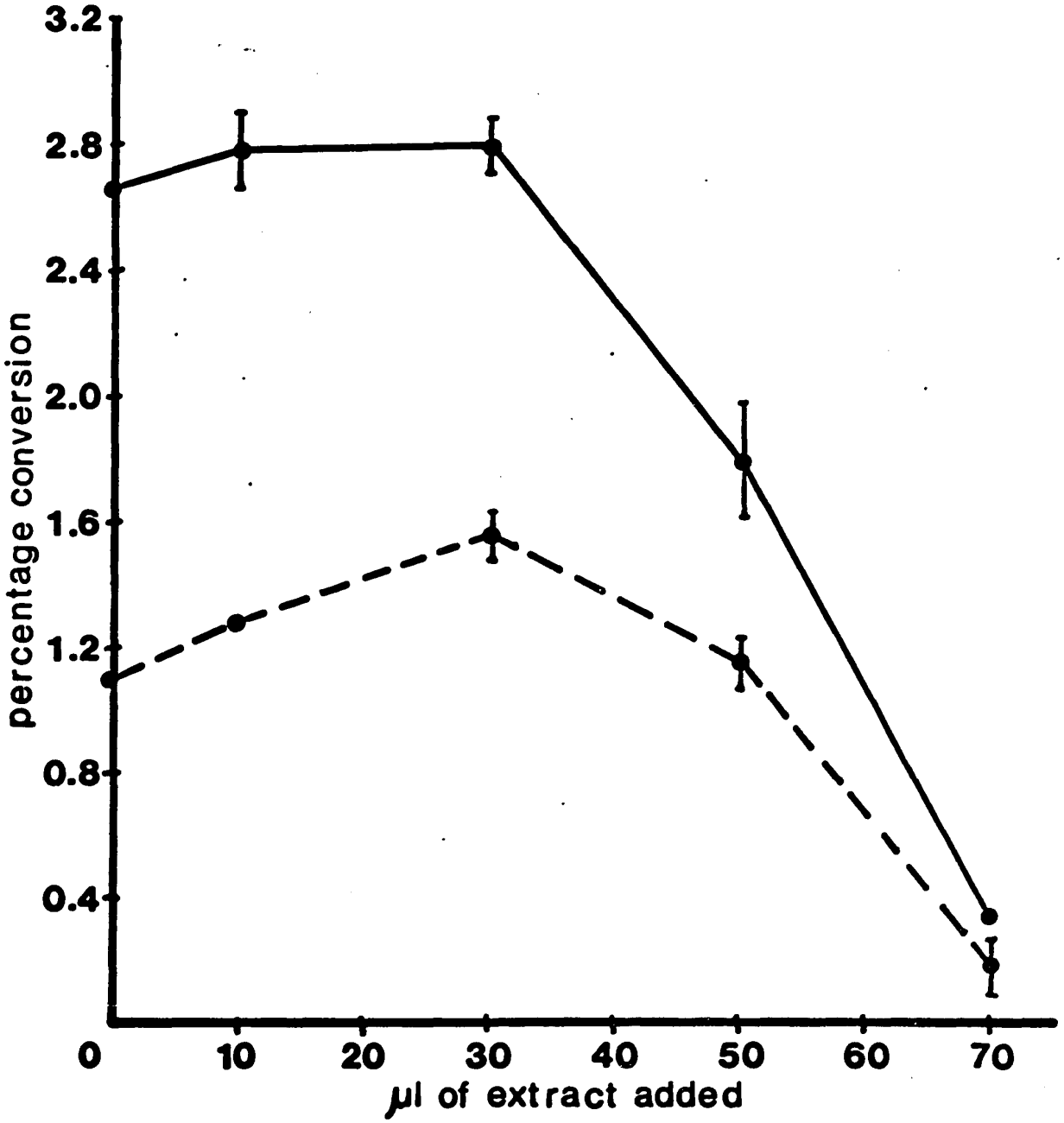
The incubation mixture contained 10 nmole dATP, dGTP and ^{3}H-dCTP (specific acitivity 0.05 μC/nmole). 5.7 units of DNA polymerase were used with an incubation time of 60 minutes at 37°C. The 0.41 and 0.20 nmole standards were incubated with 0.5 N PCA at 4°C for 15 minutes and then neutralized prior to assay.

Figure 4: The effect of increasing volumes of cell extract on the DNA polymerase assay for dCTP and dTTP.

The reaction mixture contained 10 nmole of the three excess dRTP (specific activity of ^{3}H-dTTP and ^{3}H-dCTP, 0.1 μC/nmole and 0.05 μC/nmole respectively) together with indicated amounts of the limiting triphosphate and extract. Incubation was at 37°C for 60 minutes using 5.7 units of enzyme.

●——●: 0.384 nmole dCTP

●— —●: 0.33 nmole dTTP


3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
percentage conversion
0
10
20
30
40
50
60
70
µl of extract added

that the limiting level of triphosphate producing 10% conversion of the total radioactivity present was 0.1 nmole instead of 1 nmole; the levels of the excess triphosphates were also reduced, from 10 nmoles to 1 nmole, and the specific activity of the labelled triphosphate was increased 10 fold such that adequate levels of counts were obtained with the lower levels of limiting triphosphate.

Using 5.7 units of enzyme and 12 μg activated DNA with the reduced triphosphate concentration, it was found that incorporation was maximal at 15 minutes followed by a steep decline to 60 minutes. The peak of incorporation obtained corresponded to the calculated theoretical maximum incorporation, and was found to be five fold higher than the maximum incorporation obtained in a similar experiment where undenatured DNA was the template. The decrease in incorporation observed after 15 minutes incubation is probably due to the exonuclease activity of the E. coli polymerase. It therefore seemed that activated DNA was a better template than undenatured DNA, but was only useful when used with a short incubation time.

However, when further studies on this system were performed, particularly with a view to determining the amount of enzyme needed and the optimal incubation time, it was found that although more rapid incorporation was obtained and an elevated plateau level of incorporation was observed, there was increased degradation with longer incubation times and the observed incorporation was about four times higher than the calculated theoretical maximum. When an incubation was performed omitting one triphosphate it was found that over a 90 minute incubation period up to 24% conversion was obtained (see Table 6), thus indicating the presence of terminal addition activity in the enzyme preparation. When this terminal addition activity was subtracted from the incorporation obtained in the presence of 0.1 nmole dCTP it was found that the values obtained were still two fold higher than the theoretical maximum incorporation (see Table 6).

TABLE 6

Terminal Addition with an Activated DNA Template

Time of incubation (min)	% conversion (% error in duplication)		
	0.1 nmole dCTP	0 nmole dCTP	Difference between 0.1 and 0 nmole*
0	0	0	0
15	25.22 (5.50)	10.58 (0.90)	14.64
30	28.83 (2.53)	17.67 (13.38)	11.16
60	36.42 (0.16)	19.94 (3.68)	16.48
90	34.18 (0.85)	24.33 (1.99)	9.85

* Theoretical maximum incorporation = 8.49% conversion.

The reaction mixture contained 1 nmole of the three excess triphosphates including ^{3}H-dTTP at a specific activity of 1 μC/nmole, indicated amounts of dCTP and 18 μg activated DNA as template. 4.13 units of DNA polymerase were used for incubation at 37°C for the indicated times.

When low concentrations of the limiting triphosphate are used the extent of incorporation will approach the background level, therefore it is important to have the lowest background possible. It was therefore decided to determine whether the use of an undenatured template would reduce or eliminate this background caused by terminal addition. An experiment identical to that shown in Table 6 was therefore conducted using 19 µg undenatured DNA template both in the presence of 0.1 nmole limiting triphosphate and in the absence of one triphosphate. Table 7 shows that in the presence of 0.1 nmole dCTP a plateau of incorporation at approximately 9.5% conversion was obtained after 15 minutes of incubation, and that in the absence of dCTP, up to 1% incorporation was obtained after 60 minutes. The difference between these two values gives a plateau of about 8.4% conversion which is in good agreement with the calcaulated theoretical maximum of 8.49% conversion.

Further evidence that the high background seen with activated DNA is due to non base-specific terminal addition was obtained by carrying out an assay in the presence of only one triphosphate. With undenatured DNA incorporation was negligible, whereas with activated DNA incorporation increased in a linear fashion with time up to 60 minutes (see Figure 5).

It was therefore decided to use undenatured DNA as the template in subsequent assays, as it was thought that reduction of the terminal addition activity was more important to the reliability of the dRTP assay than the slight loss of sensitivity incurred with the use of this template.

(i) Determination of the Optimal Amount of Undenatured DNA Template

For the deoxyribonucleoside triphosphate assay to be useful it is necessary for the DNA template to be present in excess, therefore, a range of concentrations of undenatured

TABLE 7

Terminal Addition with an Undenatured DNA Template

Time of incubation (min)	% conversion (% error in duplication)		
	0.1 nmole dCTP	0 nmole dCTP	Difference between 0.1 and 0 nmole*
0	0	0	0
15	10.08 (6.47)	0.83 (8.43)	9.25
30	9.47 (7.67)	0.93 (2.15)	8.54
60	9.49 (11.16)	1.15 (2.61)	8.34
90	9.44 (1.37)	1.08 (7.41)	8.36

* Theoretical maximum incorporation = 8.49% conversion.

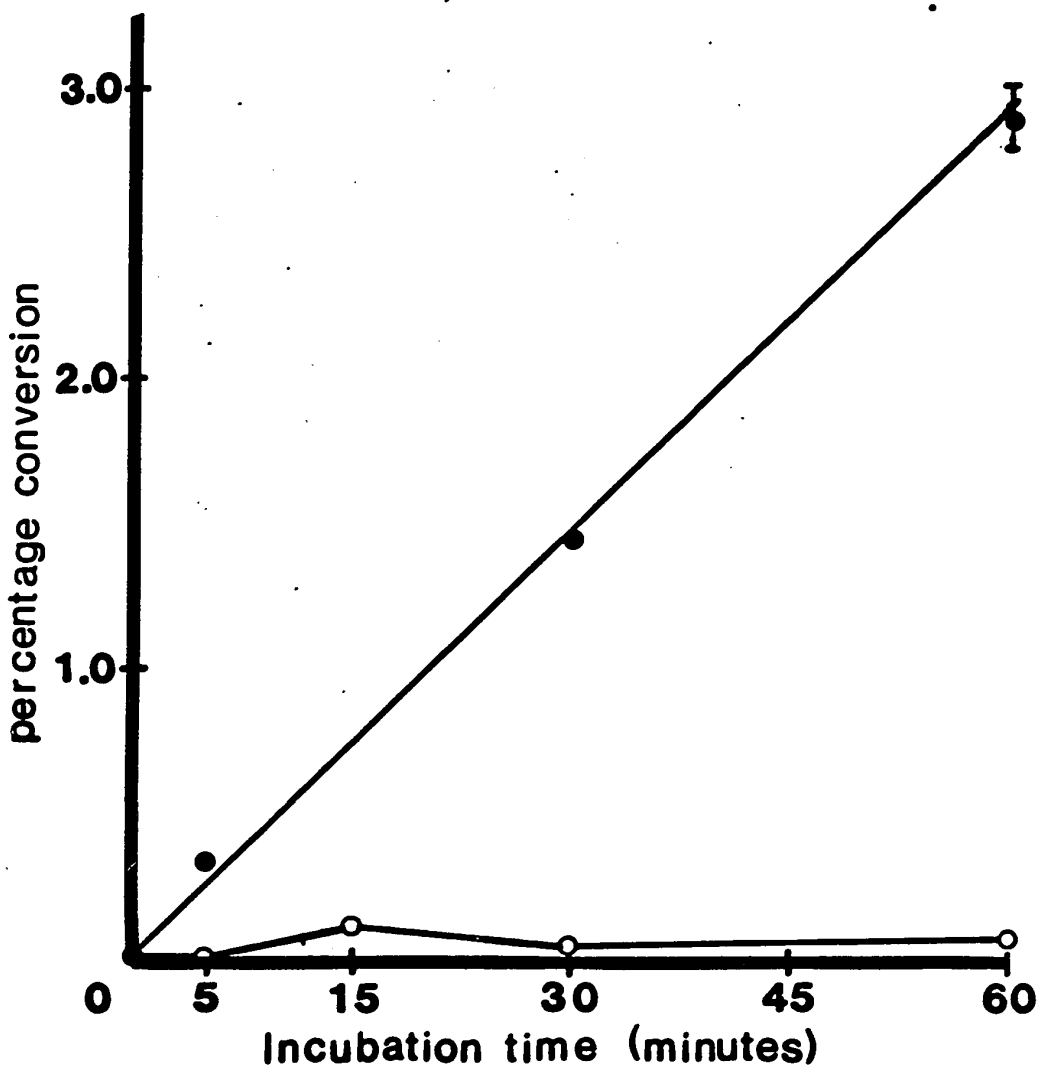
Assay conditions were as in Table 6 with the exception that 19 μg undenatured DNA was the template.

Figure 5: Time course of incorporation of a solitary triphosphate into an acid insoluble product with activated or undenatured DNA.

The reaction mixture contained 1 nmole ^{3}H-dATP (specific activity 1 μC/ nmole), and 12 μg of either activated or undenatured DNA as indicated.

●——● : Activated DNA

○——○ : Undenatured DNA


3.0
2.0
1.0
percentage conversion
0
5
15
30
45
60
Incubation time (minutes)

DNA were assayed using zero and 0.1 nmole of limiting triphosphate with 4.13 units of enzyme and an incubation time of 60 minutes, because this was on the plateau of incorporation in the experiment shown in Table 7. Table 8 shows that in the presence of 0.1 nmole dCTP a linear increase in incorporation was observed with increasing DNA content up to 12.8 μg per incubation, which gave 10% conversion. With more than 12.8 μg (19.3 μg) a slight decrease in incorporation was observed. Terminal addition also increased with increasing DNA content. It was therefore decided that in order to obtain a balance between that amount of DNA allowing 10% incorporation and minimal terminal addition activity, 12.8 μg of undenatured DNA would be used in future experiments.

(ii) Determination of the Amount of Enzyme and the Time of Incubation Required

When the concentration of the limiting triphosphate is reduced, the use of that amount of enzyme found to produce a plateau of incorporation in a reasonable time of incubation with 1 nmole of limiting triphosphate (see Table 2 and Figure 2), will give quicker total consumption of a lower level of limiting triphosphate. It is therefore possible to use less enzyme with 0.1 nmole of limiting triphosphate. To determine the amount of enzyme needed a time course of incorporation was studied using 12.8 μg of undenatured DNA, 0.1 nmole of limiting triphosphate and different amounts of enzyme. Figure 6 shows that with 1.38 units of enzyme a plateau of incorporation was reached after 60 minutes of incubation; with 4.13 units an identical plateau of incorporation was reached in fifteen minutes. To ensure that samples were taken during the plateau period of the time course and to obviate lengthy incubation periods, which would be required by the use of 1.38 units of enzyme, it was decided that an amount of enzyme intermediate between these two would be desireable. The assay was therefore repeated using 2.06 units of enzyme with 0.1 nmole and 0.042 nmole of limiting triphosphate. Figure 7 shows that, after correcting for terminal

Table 8
Determination of the Optimal Amount of Undenatured
DNA Template

µg DNA per assay	% conversion (% error in duplication)		
	0.1 nmole dCTP	0 nmole dCTP	Difference between 0.1* and 0 nmole
0	0	0	0
2.57	3.51 (11.39)	0.15 (13.3)	3.36
6.44	6.06 (5.44)	0.43 (4.65)	5.63
12.87	10.90 (0.09)	1.02 (3.92)	9.88
19.3	9.49 (1.37)	1.30 (3.00)	7.14

* Theoretical maximum incorporation = 8.49% conversion.

The incubation mixture contained 1 nmole dATP, dGTP and ^{3}H-dTTP (specific activity 1 µC/nmole) and indicated amounts of dCTP and undenatured DNA template. Incubation was at 37°C for 60 minutes with 4.13 units of DNA polymerase.

Figure 6: Time course of ^{3}H-dTTP incorporation in the presence different amounts of enzyme.

The incubation mixture contained 1 nmole dATP, dGTP and ^{3}H-dTTP (specific activity 1 μC/ nmole), 0.1 nmole dCTP and 12.8 μg of undenatured DNA template. Incubation was at 37°C for the indicated times.

●——● : 1.38 units of enzyme

●– – –● : 4.13 units of enzyme

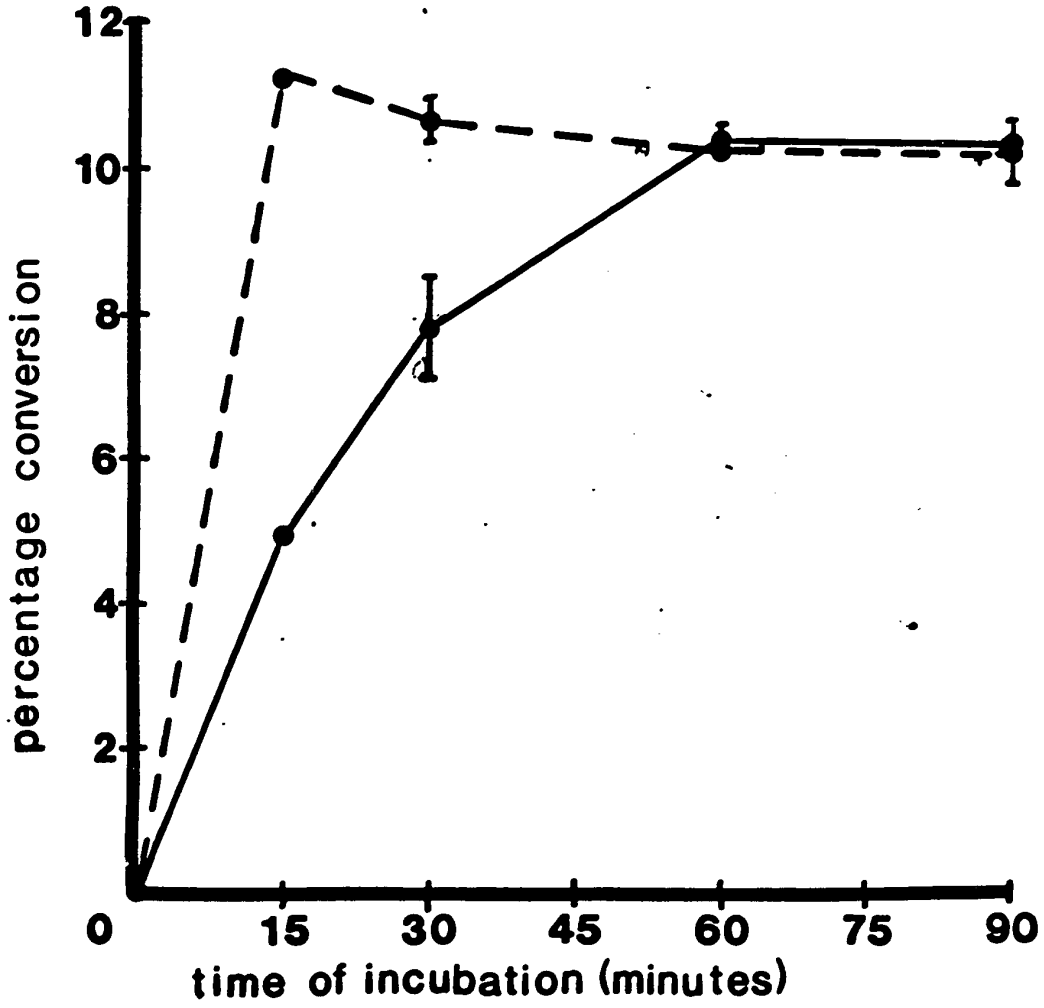
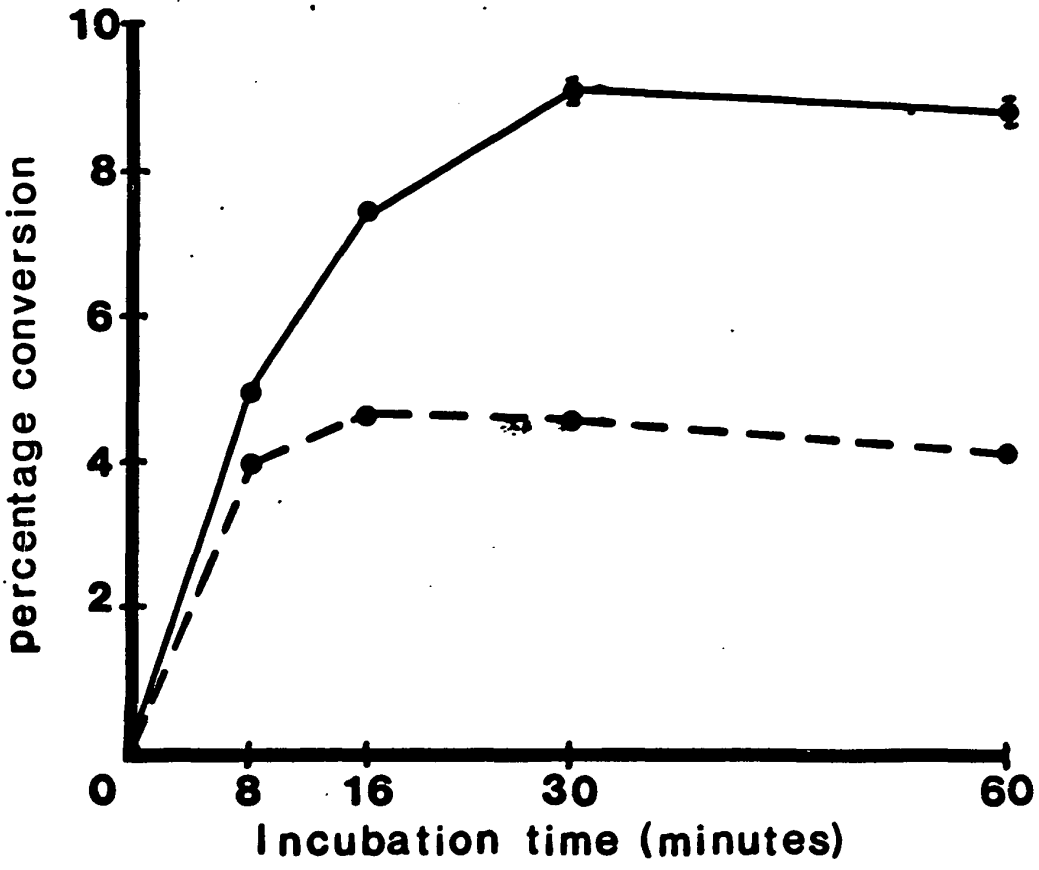

12
10
8
6
4
2
0
percentage conversion
15
30
45
60
75
90
time of incubation (minutes)

Figure 7: Time course of ^{3}H-dTTP, in corportion in the presence of two different limiting concentrations of dCTP.

The incubation mixture was as described for Figure 5 with the exception that the amounts of the limiting triphosphate (dCTP) were as indicated. 2.06 units of DNA polymerase were used. A control lacking one triphosphate was used to correct for terminal addition activity.

●——● : 0.107 nmole dCTP

●- - -● : 0.042 nmole dCTP


10
8
6
4
2
0
8
16
30
60
percentage conversion
Incubation time (minutes)

addition, a plateau of incorporation was obtained after 16 minutes of incubation with the lower concentration, whereas with the higher concentration, a plateau, proportionately higher, was obtained after 30 minutes.

It was therefore decided that this amount of enzyme (2.06 units) was appropriate when combined with a 45 minute incubation period.

From Figure 7 it can be seen that 0.1 nmole (100 pmole) of limiting triphosphate produces approximately 9% conversion of the total radioactivity present into an acid-insoluble product, thus 10 pmole would give 0.9% conversion, which is equivalent to approximately 3500 cpm; this is about 1000 cpm above the incorporation observed from the terminal addition reaction, and is thus just above the lower limits of sensitivity of this reaction (between 5 and 10 pmole, see Figure 8).

2. Standard Curves for the Four Deoxynucleoside Triphosphates

Following modification and sensitisation of Solter and Handschumacher's assay, as described above, standard curves of incorporation were determined for each of the four dRTP to obtain the range of concentrations over which incorporation was linear. ^{3}H-dTTP was used for the dATP, dGTP and dCTP assays and ^{3}H-dATP was used for the dTTP assay. To check that incorporation with the different labelled precursors was equivalent, a standard curve for dCTP was also made using ^{3}H-dATP.

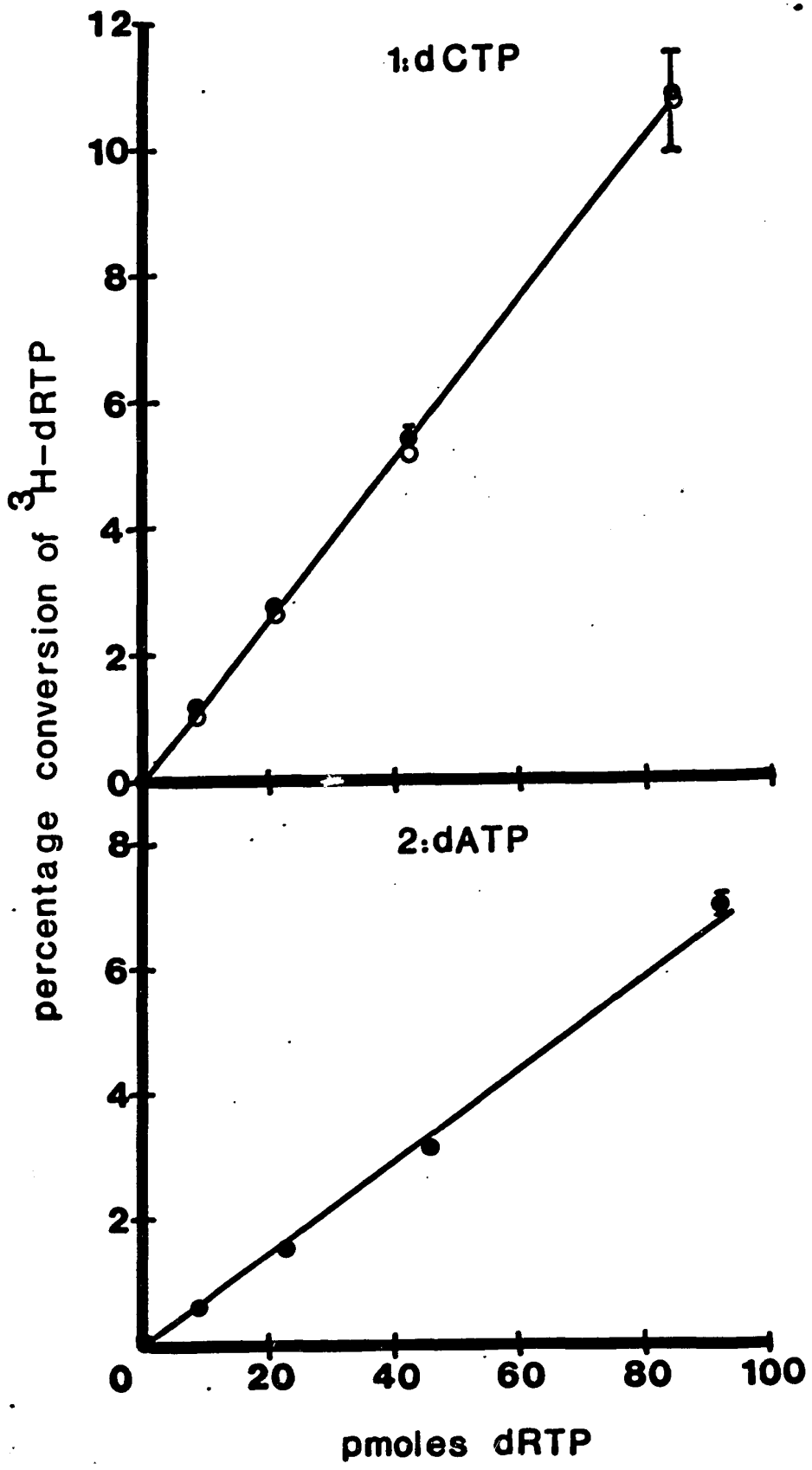
Figure 8 shows that for all four dRTP's incorporation is linear over the range of concentrations tested, from about 5 pmoles to 80 or 90 pmoles. It can also be seen that there is no significant difference in ^{3}H incorporation with either ^{3}H-dTTP or ^{3}H-dATP as the labelled precursor in the dCTP standard curve. It should also be noted that the percentage conversion obtained for a given number of picomoles of triphosphate varies between the four triphosphates, and is markedly higher for dCTP. This variation was observed in all standard

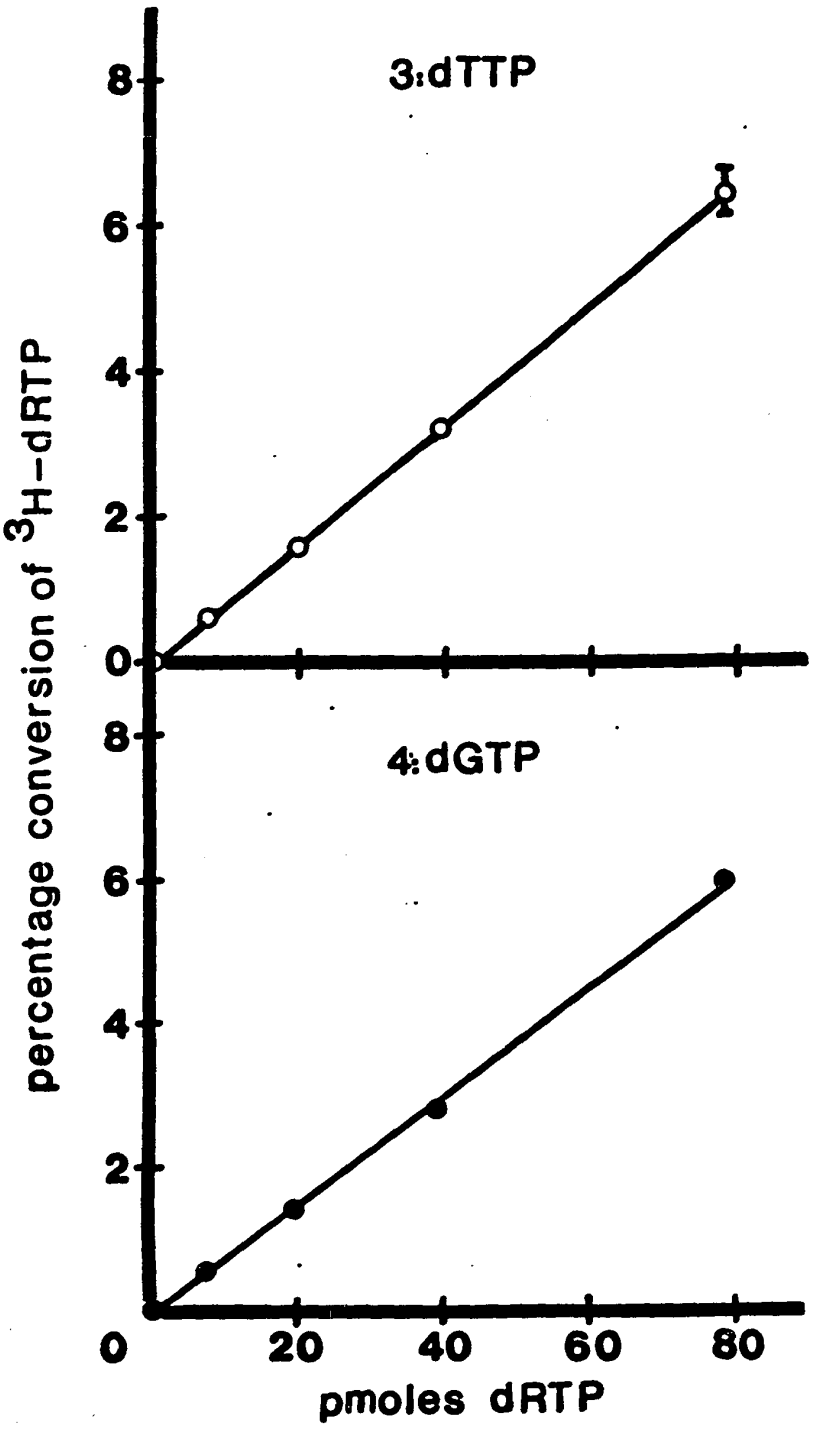
Figure 8: Standard curves of incorporation for all four deoxynucleoside triphosphates.

The deoxynucleoside triphosphate assay was as described under Methods, with the indicated amounts of limiting triphosphates.

●——● : ^{3}H-dTTP precursor

○——○ : ^{3}H-dATP precursor


1:dCTP
2:dATP
percentage conversion of ^{3}H-dRTP
pmoles dRTP
0
2
4
6
8
10
12
20
40
60
80
100


3:dTTP
4:dGTP
percentage conversion of ^{3}H-dRTP
0
2
4
6
8
0
20
40
60
80
pmoles dRTP

curves obtained, the reason for it is unknown. In subsequent experiments a standard curve was determined for each assay, to correct for variations in the polymerization reaction.

Following the previously described modifications to the deoxyribonucleoside triphosphate assay, a test was conducted to see whether these modifications did serve to circumvent the inhibition found previously (see Figure 4). An assay was therefore conducted using the assay conditions described under Methods with 28 pmole dTTP present in all samples together with increasing amounts of a cell extract prepared from 3×10^7 asynchronous HeLa cells. Figure 9 shows that using up to 25 µl of extract produced no inhibition of the polymerase reaction, a slight inhibition was observed between 25 and 50 µl. With this extract 25 µl was equivalent to 75 pmoles dTTP, which is in the upper range of concentrations expected in cell extracts. Therefore the modification of Solter and Handschumacher's dRTP assay does circumvent the inhibition found earlier; the assay in this form was therefore used in all subsequent experiments together with extract amounts up to 25 µl.

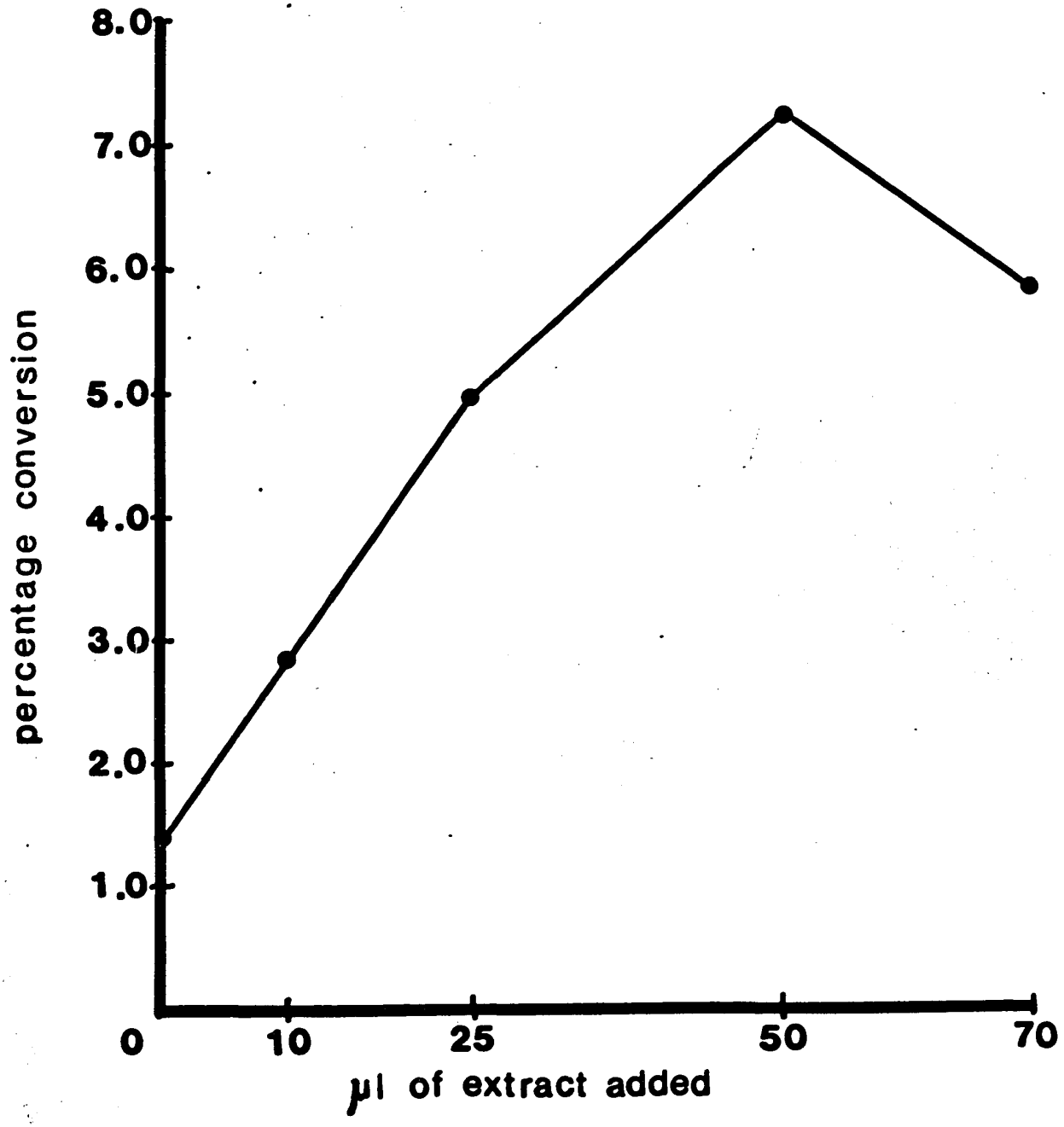
3. The Amounts of Deoxynucleoside Triphosphates Present in Asynchronous Cells

The pool sizes of deoxynucleoside triphosphates were examined in asynchronous cultures of HeLa, Chinese hamster and mouse L-cells to compare the pool sizes in the three types of cells, and to examine the relative pool sizes of the triphosphates of the four different bases.

Perchloric acid extracts of the cells were prepared as described under Methods and these extracts were subsequently assayed for the four triphosphates by the modification of Solter and Handschumacher's assay described in the Methods section. The amounts of triphosphates present were determined from appropriate standard curves derived at the same time as the samples were assayed. The DNA content of the cell pellets remaining after

Figure 9: The effect of increasing volumes of cell extract on the assay for dTTP.

The reaction mixture contained 1 nmole dCTP, dGTP and ^{3}H-dATP (specific activity 1 μC/nmole) and 28 pmole of dTTP with indicated amounts of a cell extract. Incubation was for 45 minutes at 37°C with 2.06 units of DNA polymerase.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
percentage conversion
0
10
25
50
70
µl of extract added

extraction of the cold PCA soluble fraction was determined as described under Methods.

Table 9A shows that there is considerable variation in the pool size distribution of the four triphosphates both within and between the different cell lines. In HeLa cells the dTTP pool is the largest, followed in size by the dCTP pool with the dGTP and dATP pools being the smallest and similar in size. In Chinese hamster cells the dCTP pool is largest, the dTTP pool is second largest, both being considerably larger than either purine pool, which are again similar in size. In the mouse L-cells essentially the same pattern was found as in the Chinese hamster cells, except that the dTTP pool was nearer in size to the purine pools. Table 9B shows more clearly the relative pool sizes, expressed as a percentage of the dCTP pool. The variation in the absolute levels of each triphosphate pool observed in the different determinations for each cell line could be due to different growth states of the different cultures. This variability makes inter cell line comparisons difficult, but has not significantly changed the base ratios of the triphosphates within a single cell line.

4. Changes in the Deoxynucleoside Triphosphate Pool Sizes in a Synchronous Population of HeLa Cells During the Cell Cycle

A synchronous population of HeLa cells was obtained as described in the Methods section; two or three harvests were necessary to provide a sufficient number of cells for the experiment. The mitotic index, determined at the time of harvest, was always found to be between 90 and 95% indicating that a highly synchronous population had been obtained.

The growth of mitotic cells after harvesting was followed by counting aliquots of the cell suspension in a haemocytometer at intervals throughout the experiment. It was found that after an initial rise, caused by cells in mitosis at the time of harvest completing that mitosis and dividing, there was no increase in cell number until 16 hours after harvesting. The cell number then doubled over a period of 8 hours following which it was again constant

TABLE 9

Deoxynucleoside Triphosphate Pool Sizes in Asynchronous Cultures of HeLa, Chinese Hamster and Mouse L-Cells

A

Cell Line	picomoles/μg DNA (% error in duplication)			
	dCTP	dATP	dGTP	dTTP
HeLa	1.80 (12.77) 3.66 (3.55)	1.44 (6.25) 2.47 (14.17)	1.39 (8.63) 3.04 (9.53)	2.68 (7.08) 6.69 (13.00)
Chinese Hamster	4.98 (1.61) 9.30 (3.01)	0.82 (4.88) 1.97 (4.57)	0.94 (24.46) 2.64 (3.03)	3.25 (3.07) 4.84 (10.12)
Mouse L-Cells	0.94 (6.38) 1.59 (1.89)	0.41 (2.44) 0.53 (20.75)	0.41 (24.39) 0.38 (15.79)	0.40 (10.00) 0.74 (17.56)

B

Cell Line	Relative Pool Size Expressed as a Percentage of the dCTP Pool			
	dCTP	dATP	dGTP	dTTP
HeLa	100 100	80.0 67.5	77.2 83.1	148.9 182.8
Chinese Hamster	100 100	16.5 21.2	18.9 28.4	65.3 52.0
Mouse L-Cells	100 100	43.6 33.3	43.6 23.9	42.5 46.5

Cell extracts were prepared, and between 10 and 25 μl were assayed, as described in the Methods section. Two experiments are shown for each cell line.

(see Figure 10). The length of the cell cycle has been taken as extending from the time of harvest to the time when a 50% increase in cell number had occurred. In the cells being used this was found to be approximately 20 hours.

The rate of DNA synthesis during the cell cycle was estimated by pulse labelling with ^{3}H-thymidine at intervals throughout the cycle, and assaying for ^{3}H incorporation into the acid-insoluble fraction of the cell, as described in the Methods section. Figure 10 shows a typical ^{3}H-thymidine incorporation rate curve during 28 hours after mitosis. It can be seen that DNA synthesis occupies a discrete portion of the cycle. The length of the various phases of the cell cycle has been determined from the curve for the rate of DNA synthesis as follows: G_1 is the time from mitosis to the half maximum rise in the rate of DNA synthesis; S is the time from the half maximum rise in the rate of synthesis to the half maximum point in the following fall in DNA synthetic rate; G_2 is the difference between the cell cycle length, determined from the cell number plot as described above, and the sum of G_1 and S. For example, in the experiment shown in Figure 10 these values are G_1 : 8 hours, S : 7 hours, G_2 : 4 hours.

Samples of the culture for the determination of the deoxyribonucleoside triphosphate pool sizes were taken at one, two or three hourly intervals throughout the cell cycle. Extracts were prepared and assayed in the same manner as for the asynchronous pool sizes.

The amounts of deoxynucleoside triphosphates found in the cell extracts can be expressed in a variety of ways, e.g. against the DNA content of the cells extracted or against the cell number, in a synchronous culture these references change in a discontinuous fashion.

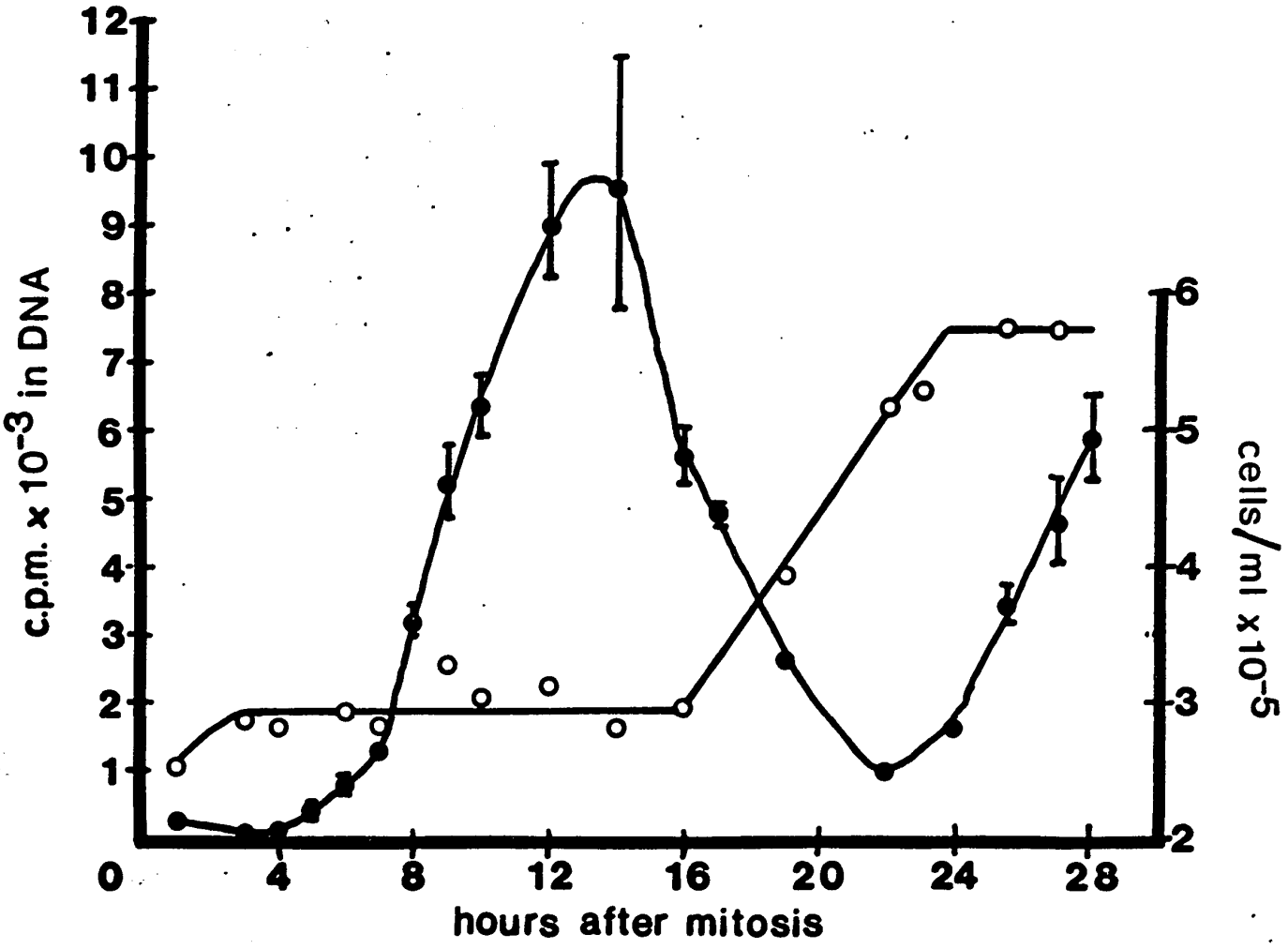
In Figures 11 and 12 the triphosphate pools from a typical experiment are shown expressed as pmole/μg DNA and pmole/cell x 10^{-5} respectively.

Figure 10: Cell growth and DNA synthesis in a synchronous culture of HeLa cells.

A synchronous culture of HeLa cells was prepared as described in the Methods section. Cell counts were performed using a haemocytometer. DNA synthesis rate was followed by pulse labelling samples of the culture with ^{3}H-TdR for 30 minutes and assaying for ^{3}H incorporation into acid-insoluble material.

●——● : DNA synthesis

○——○ : Cell number


c.p.m. x 10⁻³ in DNA
cells/ml x 10⁻⁵
hours after mitosis
0
4
8
12
16
20
24
28
1
2
3
4
5
6
7
8
9
10
11
12
2
3
4
5
6

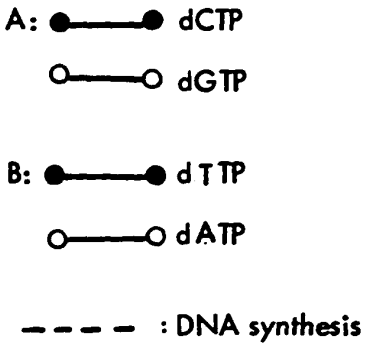
In Figure 11 it can be seen, by comparison with Table 9, that the observed fluctuations largely fall within the range of pool sizes found in the asynchronous HeLa populations; this is expected as the asynchronous pool sizes represent an average value of all the fluctuations present in the cell cycle. It can be seen from Figure 11 that the pyrimidine pools show a rapid increase in size at the same time as the onset of DNA synthesis, and show a slight decline, or plateau, when DNA synthetic activity is maximal. The dTTP pool then has a second peak late in G_2 followed by a rapid decline during the second M period and into the beginning of the subsequent G_1. The decline seen in the second M and the rise following it are equivalent to the decline seen between one and three hours after the first mitosis and the subsequent rise between 5 and 7 hours, showing that the variations in dTTP pool size are cyclical in nature. Similarly the dCTP pool size declines slowly during G_2 and M and begins to rise again in the second G_1, which also parallels the rise seen between 1 and 3 hours in the first cycle; thus this pool also exhibits cyclical fluctuations in size. After an initial slight decrease both purine pools show a small increase, concomitant with the rapid increase in pyrimidine pool size. A slight plateau is seen at a time corresponding to the rapid increase in the rate of DNA synthesis. The dATP pool then exhibits a pattern similar to the dTTP pool, while the dGTP pool shows a small increase when DNA synthesis starts to decline and then decreases in a pattern similar to that seen for the dCTP pool.

It can therefore be seen that all four triphosphates do exhibit cyclical fluctuations in their pool sizes.

In Figure 12 the deoxynucleoside triphosphate pools shown in Figure 11 are expressed in terms of p mole/cell x 10^{-5}; in this Figure the peak of pyrimidine pools seen at 7 hours after mitosis in Figure 11 is eliminated, and all four triphosphate pools show a more gradual increase in size throughout S. The dTTP pool shows a peak at about 18 hours after mitosis (late G_2), followed by a decline, of greater magnitude than than seen in Figure 11, reaching a minimum at 25 hours with a subsequent increase, as in Figure 11. The dCTP

Figure 11: Deoxynucleoside triphosphate pools during the cell cycle of a synchronous culture of HeLa cells; expressed as p mole/μg DNA.

A synchronous culture of HeLa cells was prepared as described under Methods. Extracts were prepared at the indicated times. The rate of DNA synthesis (from Figure 10) is plotted as a reference.



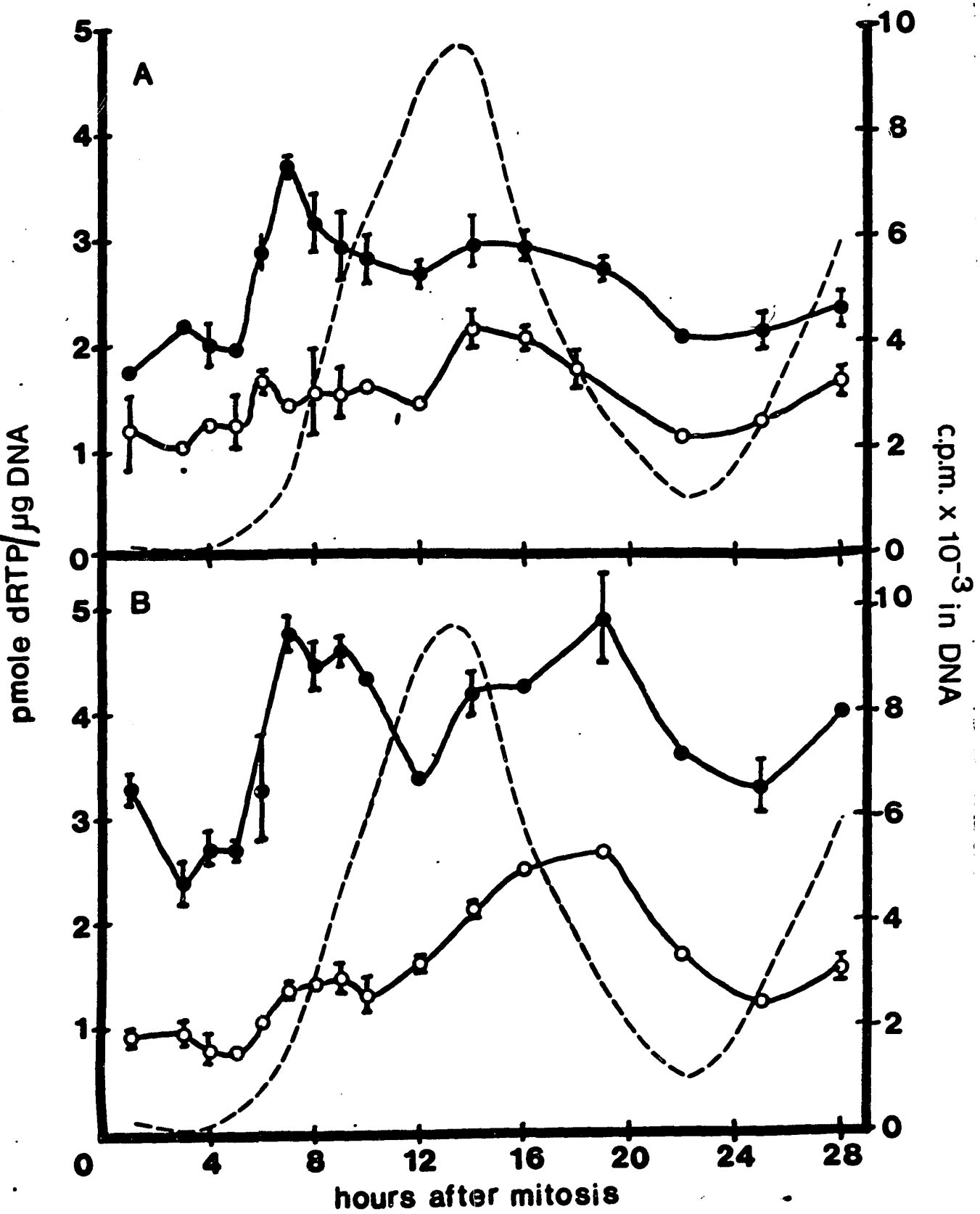
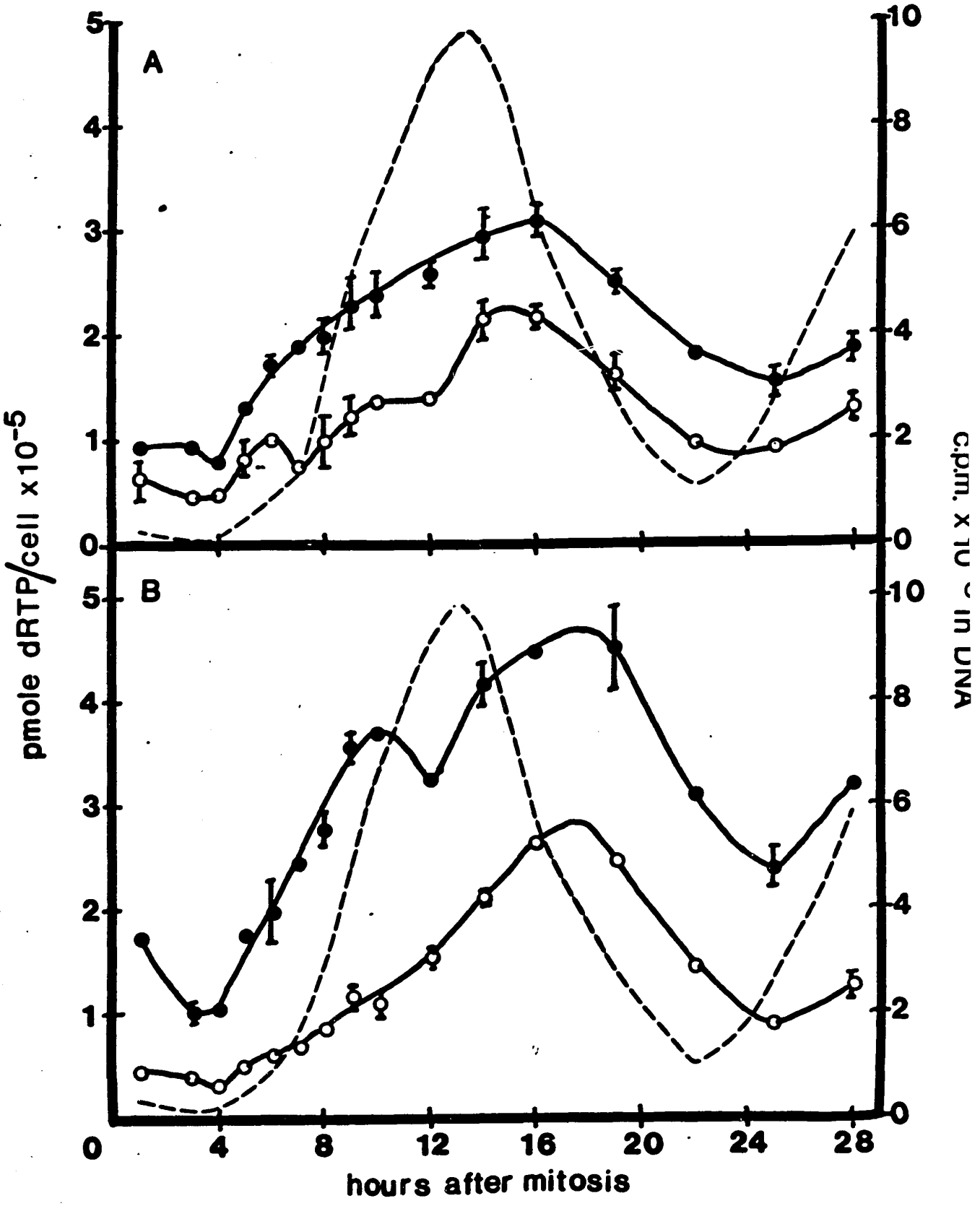

A
B
pmole dRTP/μg DNA
c.p.m. x 10⁻³ in DNA
hours after mitosis
0
4
8
12
16
20
24
28
1
2
3
4
5
6
8
10

Figure 12: Deoxynucleoside triphosphate pools during the cell cycle of a synchronous culture of HeLa cells; expressed as pmole/cell x 10^{-5}.

Legend as in Figure 11.


A
B
pmole dRTP/cell x10⁻⁵
c.p.m. x 10⁻³ in DNA
hours after mitosis
0
4
8
12
16
20
24
28
1
2
3
4
5
6
8
10

pool reaches a shallow peak at 16 hours after mitosis which is followed by a decline to 24 hours, again accompanied by a slight increase to 28 hours; the decrease seen is also of greater magnitude than than seen in Figure 11. The dATP and dGTP pools show a steady increase in size, reaching peaks at 17 and 15 hours respectively. Following the peak both show a decline to about 24 hours and a subsequent increase in pool size.

Figure 13 shows the experiment shown in Figures 11 and 12 with the pools plotted as the absolute cellular concentration, this is the most meaningful way of calculating the pool sizes, particularly for reference to the K_m and K_i's for the enzymes involved in the biosynthesis of the triphosphates; in addition the problem of having discontinuous changes in the reference is removed. To obtain this curve certain assumptions were made, namely that cellular volume increases in a linear fashion between mitoses, and that the pools are evenly distributed throughout the cell. Terasima and Tolmach's data on average cellular volume (71) during the HeLa cell cycle was used to provide the minimum and maximum cell volumes, and the Figures were adjusted to a 20 hour cycle. The assumption was made that the degree of synchrony obtained by Terasima and Tolmach and in the present experiment was similar, therefore the average cell volume at the end of one cycle would be the same. Terasima and Tolmach only gave the volumes for one cell cycle, therefore an extrapolation was made into the second cycle on the basis of the rate of volume increase in the first cycle. Table 10 shows the cellular volumes used in calculating the points for Figure 13.

This Figure shows that the dTTP concentration increases in size sharply from 4 to 9 hours after mitosis, declines slightly during the peak of DNA synthesis and the initial rate of increase is resumed between 16 and 19 hours to give a second peak at the end of G_2. This peak is followed by a sharp decline in size up to approximately 24 hours, followed again by a rise. The dCTP concentration increases rapidly from 4 to 6 hours after mitosis when a plateau concentration is reached, and maintained for the next 10 hours while DNA is being synthesised, after which a small peak is observed late in G_2. This is followed by a decline

TABLE 10

HeLa Cell Volumes During a Synthronized Cycle of Growth

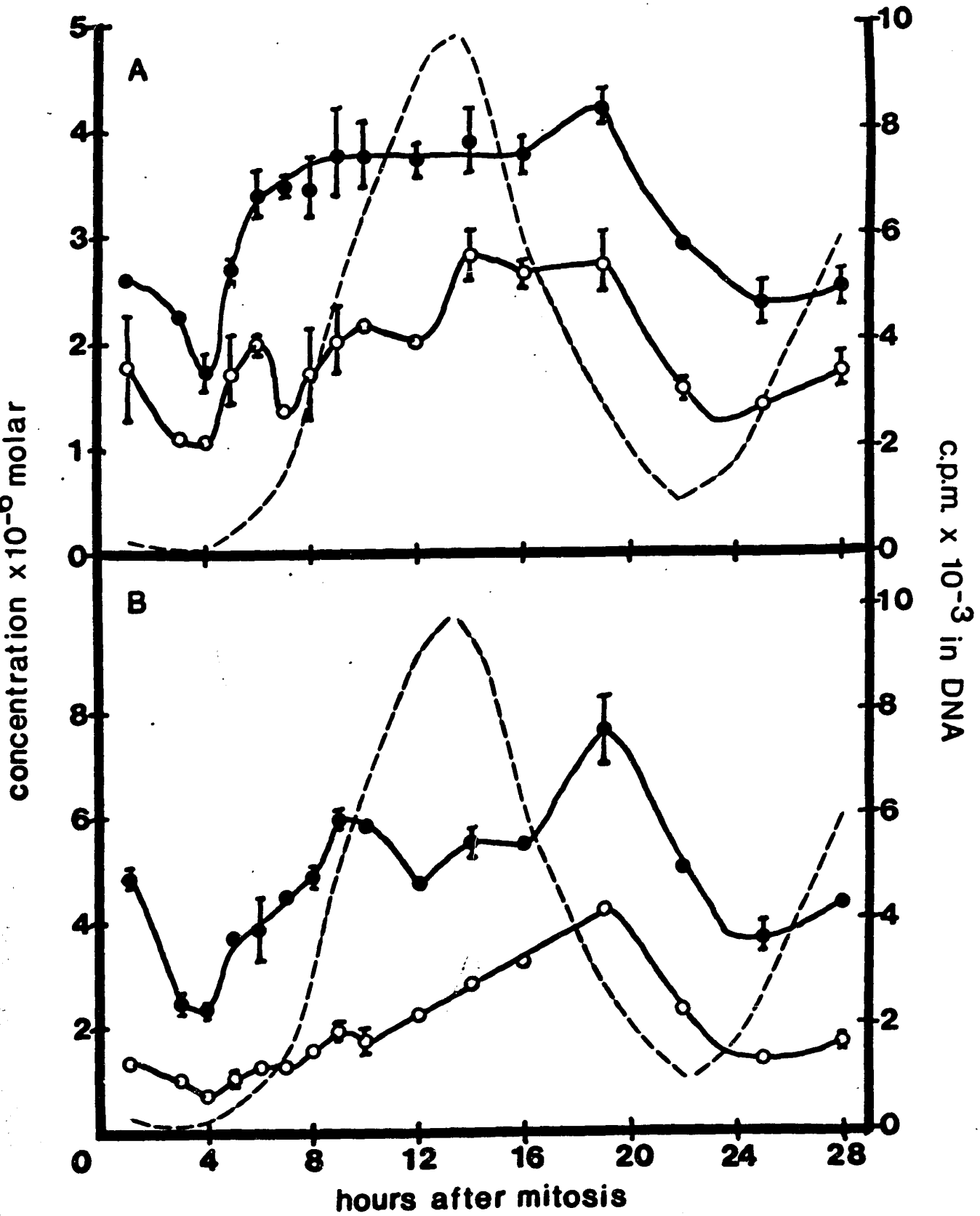
Time after Mitosis (hours)	Cell Volume μ^3
1.4 *	3650
3	4180
6	5100
9	6020
13	7260
16*	8280
20*	4960
24	6200
28	7440

The figures given in this Table were calculated from the data of Terasima and Tolmach (71), who obtained the volume measurements from photomicrographs of synchronous cells.

* Values taken from Terasima and Tolmach's data.

Figure 13: Deoxynucleoside triphosphate pools during the cell cycle of a synchronous culture of HeLa cells; expressed as concentration x 10^{-6} molar.

Legend as in Figure 11.


A
B
concentration x10-6 molar
c.p.m. x 10-3 in DNA
hours after mitosis
0
4
8
12
16
20
24
28

and subsequent rise in concentration. The dGTP concentration shows an initial small peak at 6 hours after mitosis, as was evident to a lesser extent in Figures 11 and 12, followed by a fairly steady rise in concentration to a plateau between 14 and 19 hours after mitosis (late S and G_2), after which the concentration declines up to 23 hours and then begins to increase. The dATP pool exhibits a steady increase in concentration from 4 to 19 hours after mitosis, after which it shows a decline to 24 hours and subsequent increase.

All four pools show an initial decline in concentration up to 4 hours after mitosis followed by an increase, which is repeated during the subsequent mitosis, again indicating the cyclical nature of the fluctuations in the deoxyribonucleoside triphosphate pool sizes.

DISCUSSION

1. Deoxynucleoside Triphosphate Assay

The assay method developed by Solter and Handschumacher is clearly an advance over all the previously used methods for assaying for the deoxynucleoside triphosphates (dRTP) in that it is 100 fold more sensitive than the microbiological assay, and prior separation of the four dRTPs is not necessary. It has an additional advantage in that the time necessary to perform an assay is very short. This assay was shown to have a lower limit of sensitivity of approximately 0.1 nmole (Figure 3), however, as was shown in Figure 4, an extract prepared from sufficient cells to enable detection of triphosphates contained an inhibitor of the polymerization reaction when more than 30 µl of extract were used. The assay method was therefore made more sensitive by increasing the specific activity of the labelled dRTP precursor, decreasing the concentration of both limiting and excess dRTP ten fold, and adjusting the amounts of DNA template and DNA polymerase present in the reaction mixture, such that they were in excess, and the only factor limiting the reaction was the triphosphate to be assayed.

Following modification of the assay the lower limit of sensitivity was found to be between 5 and 10 picomoles, which represents a 10-20 fold increase in sensitivity. Incorporation was linear over the range of concentrations tested (5 to 90 pmoles) as was shown in Figure 8.

The presence of an inhibitor of the DNA polymerizing reaction in an extract of a neoplastic tissue has not been previously reported; it is possible that either it is a normal component of the cell or is produced by the action of PCA on some cell component. Neither Solter and Handschumacher (94) nor Lindberg and Skoog (95), using leukaemic mouse cells and mouse embryo cells respectively, reported any inhibition of their reactions

in the tissue extracts assayed; it is therefore possible that the existence of the inhibitor is a property unique to HeLa cells. In the determinations of the asynchronous pool sizes shown in Table 9, different amounts of Chinese hamster and L-cell extracts were assayed and, within the range used, good reproducibility was found. It is possible that if an inhibitor is present in these tissues it was not detected at the low concentration of extract used. Further studies similar to the experiments shown in Figures 4 and 9 with other cell lines would indicate whether the inhibitor is unique to HeLa cells or not.

From Figures 4 and 9 it should be noted that significant inhibition of the polymerase reaction occurs only with extracts containing more than 150 pmoles of dTTP (30 µl of extract Figure 4, 50 µl Figure 9). This corresponds to between 5.2 and 6.6 x 10^6 cells, calculated from the number of cells used to prepare the extract assayed in Figure 9, and from an average of the values for pmole dTTP/cell x 10^{-5} during the first 20 hours (1 cell cycle) after mitosis from Figure 12.

The DNA polymerase preparation used in these studies appeared as one band on polyacrilamide gel electrophoresis, although the sample used for electrophoresis was dilute, and therefore the presence of contaminating enzymes, at concentrations below the level of resolution of the gel, cannot be ruled out (Dr. J. Slater, personal communication). It therefore seems probable that the terminal addition activity seen was an activity of the DNA polymerase itself rather than of a separate enzyme. Kornberg and his co-workers in studying the multiple functions of the E. coli DNA polymerase (114) found no evidence of terminal addition activity; however, it is known that E. coli DNA polymerase, when incubated with one or more dRTP and Mg^{2+}, in the absence or presence of a primer, will catalyse de novo incorporation into a double stranded polynucleotide of defined repeating sequence (115). It is possible that the terminal addition activity seen is this de novo synthesis. The finding of a higher level of terminal addition with activated DNA than with undenatured DNA in a given time of incubation (Tables 6 and 7) can be attributed to the increased

number of priming (3'-OH) ends present on activated DNA than on the same amount of undenatured DNA. The results shown in Figure 5, when only one triphosphate was present, support the possibility that the incorporation seen is due to de novo synthesis, in this case of poly dA.

2. The Pool Sizes of Deoxynucleoside Triphosphates in Asynchronous Cells

The pool sizes of all four deoxynucleoside triphosphates varied in distribution both within and between the three asynchronous cell lines examined (Table 9).

The values for pool sizes reported in the Introduction (section 4 B) are obtained from a variety of tissues, as opposed to cultured cells, and can, therefore, be considered to be asynchronous, with the exception of regenerating liver, in which the tissue grows in a parasynchronous fashion following partial hepatectomy. If the values cited are converted to absolute concentration (taking the upper limit for the volume of 1 gram of tissue to be 1 ml) they all fall into the μmolar range, which is in the same range of concentrations as the asynchronous pool sizes found in this study. However, it must be remembered when comparing these figures, that the majority of the pool sizes cited in the Introduction are the pools of the total deoxynucleotide and/or deoxynucleoside of the particular base, rather than the triphosphates, which were studied here.

In cultured mouse embryo cells, Nordenskjöld et al. (57), using Linberg and Skoog's modification of Solter and Handschumacher's triphosphate assay, found asynchronous pool sizes to be about 1.6 pmole/μg DNA for dTTP and 0.8 pmole/μg DNA for dATP, which is also in the same range of concentrations as the values obtained in all three cell lines examined here, although in the mouse cell line examined (L-cells) the dATP and dTTP pools were of a very similar size (Table 9).

The variations in the absolute concentration of triphosphates observed between the different cell types cited in the Introduction, are to be expected in view of the fact that different tissues in the body have different growth rates, and will therefore be synthesising DNA at different time intervals. They will therefore have different precursor requirements and hence different pool sizes. It is also possible that the differences seen might be species differences as well as tissue differences.

It should also be noted that in the three cell lines examined the pools of the triphosphates of the four different bases varied (Table 9B). It has previously been assumed, particularly in studies on the enzymes responsible for the synthesis of the triphosphates, that the triphosphates are produced in balanced amounts. The nature of this "balance" was not specified; however, it appeared that the majority of workers assumed the balance to mean equal amounts. The reason for the imbalance found is not clear, particularly as the nature of the imbalance varied in all three cell lines, although it was found that the pyrimidine pools were usually larger than the purine pools. The synthesis of the pyrimidines is more closely regulated than that of the purines, and, as shown in Figure 1, the pyrimidine phosphates play a larger role in the regulation of dCTP and dTTP production than the purine phosphates, the imbalance seen could therefore be connected with this regulation.

3. The Deoxynucleoside Triphosphate Pool Sizes in Synchronous HeLa Cells

A. Expression of Pool Sizes

In the Results section the pool sizes of the deoxyribonucleoside triphosphates were shown expressed in three different ways (Figures 11-13). The reason for doing this was that each method has certain drawbacks; therefore, by using several normalizing

procedures, and comparing the results obtained, a more accurate picture of the nature of changes in pool size occurring can be obtained.

(i) pmole/μg DNA (Figure 11)

Expressing the pools as pmole/μg DNA is a commonly used way of citing the levels; one of the primary reasons for its use here was so that a comparison of the results obtained with those of Nordenskjöld et al. could be made. This method has the advantage that the DNA reference is determined from the acid insoluble fraction of the same aliquot of cells as the acid soluble fraction for the dRTP assay. However, it has the disadvantage that during the S period of the cell cycle the DNA content is undergoing duplication, and therefore any changes in the pools occurring between about 6 and 20 hours after mitosis (Figure 10) will cause an underestimation of the dRTP pools as compared to those determined earlier in the cell cycle.

(ii) pmole/cell x 10^{-5} (Figure 12)

The second mode of expression, using the cell number as the reference parameter, enables a more accurate picture of changes during the first 16 hours after mitosis (G_1 and S) to be obtained, since the cell number is constant over this period. The values for the cell number/sample were taken from the curve in Figure 10; therefore, variations between individual determinations, possibly attributable to error in sampling and haemocytometer counting, were eliminated. However during the G_2 and M periods this reference also changes in a stepwise manner, a doubling in cell number occurring over a period of 8 hours; therefore the pool sizes during this period appear to decrease in a manner more rapid than perhaps they in fact do.

(iii) Absolute Concentration (Figure 13)

This method of expression is perhaps the best way of giving the information on pool sizes. It has the major advantage that discontinuously changing functions are not used as the reference point. This method of expressing the pool sizes has the most widespread application of the three used, in that the concentrations found can be directly related to K_m and K_i values for the enzymes involved in the synthesis of the deoxyribonucleoside triphosphates and DNA. The disadvantages of this mode of expression lie in the difficulty of calculating the concentration. As was mentioned in the Results (section 4) two assumptions were made: that the average cellular volume increases in a linear fashion throughout the cell cycle, and that the triphosphate pools are evenly distributed throughout the cell.

The former assumes that the data on average cell volume, determined by Terasima and Tolmach (71), applies to the the cells being used in this study; it is likely that this is so since the difference between the HeLa cells used here and the HeLa S_3 cells used by Terasima and Tolmach is minimal. The increase in cell volume during the cell cycle also depends on the growth state of the cells, and it is likely that cells in logarithmic growth would behave in a similar manner regardless of the cell type. Secondly, it was assumed that an equal degree of synchrony existed in Terasima and Tolmach's culture and that used in the experiment shown in Figures 10-13. The mitotic index in both cases was found to be greater than 90%, therefore this assumption is valid. Thirdly, Terasima and Tolmach only examined the volume changes occurring during one cell cycle; as was explained in the Results section, an extrapolation into the beginning of the second cycle was made. In view of the similarity between the two cell lines and in the degree of synchrony obtained, it is probable than an equal loss of synchrony would have occurred during the first cell cycle, therefore the average cell volumes at the end of the first cycle would be similar. Also the rate of increase of the cellular volume in

the second cycle is probably similar to that seen in the first cycle. Therefore providing that the minimum and maximum average cell volumes determined by Terasima and Tolmach are accurate, this first assumption is valid.

The second assumption, concerning the distribution of the pools in the cell is, however, not as likely to be valid, as there is evidence that the acid soluble pool of thymidine phosphates, and therefore presumably of the other three bases as well, is localised in the nucleus (100, 102,103). However, as no data is available on changes in nuclear volume during the cell cycle this approximation was used. If the triphosphate pools are localized in the nucleus as opposed to being evenly distributed throughout the cell, the error introduced by this assumption is that the values obtained will be underestimated.

B. Changes in Pool Sizes During the Cell Cycle

(i) Cyclical Nature of the Changes in the Pools

The nature of the fluctuations in the deoxyribonucleoside triphosphate pools during the cell cycle have been fully described in Results, section 4, and are shown in Figures 11-13. It can be seen that the three different methods of expressing the same results, as described above, do produce different patterns for the pool size fluctuations. However, a basic pattern, for all four triphosphates, common to all modes of expression emerges: namely, an initial decline (except for dCTP, Figure 11) followed by an increase starting at the same time as the onset of DNA synthesis; a peak (or plateau) in G_2, followed by a decline in late G_2, through the second mitosis and into the second G_1, with a subsequent rise concomitant with the onset of the second DNA synthetic activity. The primary conclusion that is evident is that the changes in dRTP pools are cyclical in nature.

The fluctuations in pool sizes will vary depending on three factors, namely synthesis, utilization and degradation. In the following discussion, the first two will be considered together and the third separately.

As was described in the Introduction (section 3 C), some of the enzymes involved in dRTP synthesis undergo cyclical changes in activity during the cell cycle, and these changes are of a very similar nature to the gross fluctuations seen for the dRTP pools.

In the following discussion, more reference will be made to Figure 13 than to Figures 11 and 12 since, as previously seen (Discussion, section 3A), the expression of pool sizes as absolute concentration would appear to be the most relevant mode of expression.

(ii) Purine Triphosphate Pools and Possible Mechanisms for the Fluctuations Observed

Of the enzymes involved in purine dRTP biosynthesis, ribonucleotide reductase (RNR) is the only enzyme known to exhibit cyclical changes in activity (57,62), deoxyadenosine/deoxyguanosine kinase having a constant, high activity throughout the cell cycle (47). Thus, if the changes in the purine pools are dependent on changes in activity of any enzyme, it must be associated with RNR. Turner et al. (62) showed the peak of RNR activity to be at mid S with a decline during G_2 followed by an increase coincident with the onset of mitosis; while Nordenskjöld et al. (57) found the peak of activity to be between 2 and 3 hours after the peak of DNA synthesis. Nordenskjöld et al.'s data fit the fluctuations observed for the dGTP pool in all three figures; but it appears that neither resembles the changes in the dATP pool, with its peak late in G_2; however, both Nordenskjöld et al.'s data, and that obtained in the present study is not adequately precise to draw any firm conclusions. It would thus seem that dGTP synthesis is fairly closely linked to changes in RNR activity, whereas some other factor is involved in regulating the dATP pool. In view

of the extreme similarity between the fluctuations in the dATP and dTTP pools seen between 16 and 28 hours after the first mitosis in all three figures, it is possible that their syntheses are co-ordinately regulated. As dTTP only has a weak stimulatory effect on ADP reduction (Table 1) it is unlikely that this co-ordinated regulation is mediated through RNR. On the other hand dGTP is a strong activator of ADP reduction and could thus stimulate dATP production; however, dATP is an inhibitor of its own synthesis, and thus, when the concentration reaches a critical level, dATP production will be inhibited. As the decrease in RNR activity after its peak is not very rapid (57), there would probably be enough enzyme available for this mechanism to operate.

With both dATP and dGTP, a fairly steady increase in concentration is seen throughout the S period (Figure 13). In view of the fact that DNA is being synthesised during this time, synthesis of both dATP and dGTP must be extremely rapid to account for the increase seen.

(iii) Possible Mechanisms for the Changes in dCTP and dTTP Pool Sizes

The possible explanations for the changes occurring in the dCTP and dTTP pools are much more complicated since many more enzymes are involved.

a) dCTP

The main enzymes affecting the dCTP pool size are RNR, dCMP deaminase and CdR kinase. The latter two enzymes show a low level of activity in G_1, increasing during S to reach a peak late in G_2 followed by a rapid decline during M and into the subsequent G_1 (47,74,75). An increase in dCMP deaminase activity will result in a siphoning off of dCMP from the available pool into dUMP, and subsequently to dTMP; however, an increase in CdR kinase acitvity will increase the dCMP pool, provided that

CdR is present in the cell. It is thus possible that the co-incident changes in activity of these two enzymes partially neutralise each others effect on the dCMP pool; dCTP production would then become more dependent on CDP reduction. In Figures 11 and 12 the dCTP pool does show fluctuations similar to that found for RNR activity by Nordenskjöld et al. (57); however, in Figure 13, a peak in the concentration is observed late in G_2, following a plateau concentration extending from late G_1. It seems probable that the dCTP is in fact being produced at a rapid rate throughout the time when this plateau is observed, but no increase in concentration is seen due to utilization of the pool for DNA synthesis. One cause for the peak in dCTP concentration observed in late G_2 is that DNA synthesis has effectively ceased, while dCTP synthesis evidently continues. Additional reasons can be found in the regulation of CDP reductase, dCMP deaminase and CdR kinase. Since the control of these enzymes is very complex (see Figure 1) no one component can be positively identified as acting to effect a change in the dCTP pool. It is possible that the high dTTP pool seen during S and G_2 might inhibit dCMP deaminase and stimulate CdR kinase such that the dCMP pool, and hence the dCTP pool, would increase in size. The fact that CdR kinase activity peaks late in G_2 adds credence to the above possibility. However, UTP, dUTP, UDP and dUDP also affect CdR kinase activity and no information as to their levels is available, probably for want of a suitable assay . Thus, the possible mechanism for the increase in the dCTP pool described above may well be only a small part of entire regulatory process. It must also be remembered that, as shown in Figure 1, the synthesis of dCTP and dTTP is very closely regulated, with the di- and triphosphates of both bases affecting both their own, and each other's synthesis. Therefore, the dCTP and dTTP pool sizes are closely co-ordinated to their respective levels.

b) dTTP

dTTP production is affected by dCMP deaminase, UdR kinase, dTMP synthetase,

TdR kinase, dTMP kinase and, indirectly through CDP and UDP reduction, RNR (figure 1). Gelbard et al's results suggest that dUMP is made from dCMP in the cell cycle prior to it's utilization (74). Since no information on the periodic fluctuations of UdR kinase and dTMP synthetase is available, it is not possible to speculate on the validity of Gelbard et al's suggestion. Sneider et al's finding (50) that thymidine degradation is at a low level, or completely lacking, in the various hepatomas they examined, suggests that, providing TdR is present in the cell, either from DNA degradation or from de novo synthesis, the salvage pathway, and thus TdR kinase may play an increased role in the recycling of DNA degradative products in rapidly growing tissues. HeLa cells can be considered as rapidly growing tissues, and therefore TdR kinase may well be fairly important in regulating dTTP production. Both TdR and dTMP kinases exhibit cyclic fluctuations in activity (47,57 66,75-78). The activity is low in G_1, increases at the same time as, or slightly before, the onset of S, reaching a peak late in G_2, which is followed by a rapid decline during M producing a low level in the subsequent G_1. This pattern very closely resembles that seen for the dTTP pool in figure 13. It is therefore possible that TdR and dTMP kinases are important in regulating dTTP production; however, it could be that both the changes in enzyme activity and dTTP pool size are the result of a common regulatory effector. In addition, as can be seen in figure 1, TdR kinase is stimulated by dCTP, dCDP, dATP and dGTP, all of which, especially the three triphosphates are present throughout the time that the dTTP concentration is elevated (figure 13), although whether they are present at effective concentrations is not clear. As with the dCTP pool shown in figure 13, an initial increase in concentration is observed from 4 hours after mitosis. This lasts until the beginning of S, when the concentration declines slightly and then increases, at the same time as initially, from the end of S to give a peak in late G_2 (figure 13). The slight decreasein concentration seen during S probably reflects a slightly faster utilization than synthesis. However, the rate of synthesis appears to be constant

throughout the latter part of G1, S and early G_2, since the rates of increase seen before and after the plateau are similar. It is likely that the decrease in the dTTP pool seen at 12 hours in the experiment shown in figures 11-13 is an artefact, since it was not observed in other experiments.

(iv) Similarities in the Changes of the Deoxynucleoside Triphosphate Pools

In figure 13 it can be seen that both the dCTP and dTTP pools increase in concentration at a very rapid rate between 4 and 6, and 4 and 8 hours respectively. The plateau dCTP concentration, which persists throughout the S period, is reached when DNA synthesis is only at approximately 10% of its maximum rate, similarly the plateau dTTP concentration is reached when DNA synthesis is approximately 50% maximal. The resolution of the experiment unfortunately does not permit one to determine whether the beginning of this rise occurs before the initiation of DNA synthesis or not. If it is prior to the initiation of DNA synthesis, there are implications that the pyrimidine pools may be involved in the mechanism of initiation of DNA synthesis. The dGTP pool also shows a small rise from 4 to 6 hours followed by a decline at 7 hours prior to a second steady increase (figure 13). The beginning of this initial rise may also be prior to the onset of DNA synthesis.

It should also be noted that, in figures 11 and 12, following the S period the triphosphate pools appear to fluctuate in pairs corresponding to the pairing found in DNA, dATP with dTTP and dGTP with dCTP. Whether this observation is of any significance, as DNA synthesis does not occur outside of the S period, is not known. However in figure 13 the fluctuations seen after 19 hours are very similar for all four triphosphates which, as discussed below, would seem to result from degradation of the dRTP in the absence of synthesis.

(v) Degradation of Deoxyribonucleoside Triphosphates

Another factor that should be taken into account when studying the fluctuations of the four triphosphate pools in the cell cycle is the nucleoside phosphorylases (see Introduction section 2.C.). No information as to their cyclical fluctuations is available, although if it were it might be shown to be a significant factor in the changes in the levels of the triphosphates. It is possible that a role of the nucleotide phosphorylases is in causing the decrease in concentration seen for all four triphosphates from late G_2, through M and into the subsequent G_1, when the enzymes responsible for their synthesis are declining in activity. It seems that the di- and triphosphatases are non base specific (45), which would account for the similar rates of decline of all four dRTP pools seen at the end of the cell cycle. In addition dRTP are extremely unstable at 37°C, and therefore once synthesis has ceased it is probable that non-enzymatic degradation will occur within a very short time period.

4. The Possible Significance of the Changes in Deoxynucleoside Triphosphate Pool Sizes During the Cell Cycle in the Regulation of DNA Synthesis

In the Introduction (section 4.C.) it was shown that in bacteria (80,88) deoxynucleotide production was closely coupled to DNA synthesis, but was neither dependent upon, nor responsible for, the onset of DNA synthesis. Similarly in regenerating liver (86,87,99) and embryonic mouse cells (57), the deoxynucleotide pools increase in size at the same time as DNA synthesis occurs, which suggests either that the deoxynucleotides are supplied on demand, or that DNA synthesis and dRTP synthesis is co-ordinately induced. However, Hotta and Stern's studies on lily and trillium anther development (104-106) do suggest that the deoxynucleoside pool, appearing in the fluid surrounding the microspores, does play an indirect role in the initiation of DNA synthesis

by inducing TdR kinase activity. The main evidence for this is the temporal relationship between the appearance of the deoxynucleoside pool, the synthesis of TdR kinase and the onset of DNA synthesis.

In the present study, it was shown that the dRTP pools increased at approximately the same time as the onset of DNA synthesis, which is suggestive of a common initiator for synthesis of dRTP and DNA synthesis. Before the triphosphates can be synthesised, the enzymes responsible for their synthesis must either be activated or synthesised (usually the latter (47,66,74,75,78)). This indicates that there is perhaps a master "DNA synthesis switch" which is turned on about 4 hours after mitosis, 5 hours prior to the start of the S period (as defined in Results section 4 and shown in figure 10), and activates the synthesis of the dRTP synthesising enzymes (and thus dRTP synthesis), the movement of DNA polymerase from the cytoplasm to the nucleus (15-17) and initiation of DNA synthesis. The nature of this "switch" is at present not clear.

It seems possible that the dRTP pools do not increase in size prior to DNA synthesis, and thus are probably not responsible for initiating DNA synthesis, as has occasionally been thought, particularly following the publication of Hotta and Stern's work.

SUMMARY

1. The deoxynucleoside triphosphate pools in both asynchronous and synchronous cultures of mammalian cells were examined using an enzymatic assay based on the in vitro synthesis if DNA.

2. The pools were examined in asynchronous cultures of HeLa, Chinese hamster and mouse L-cells. It was found that the absolute amounts of the four triphosphates varied between cell lines, and that the ratios of the amounts of the four triphosphates varied within each cell line. The asynchronous pool sizes were found to be in the same range of concentrations as those previously reported.

3. In a highly synchronous culture of HeLa cells it was found that the pools of all four deoxynucleoside triphosphates fluctuated in a similar, cyclic manner. The nature of these fluctuations was determined, and it was shown that in all probability the deoxynucleoside triphosphate pools do not significantly increase in size just prior to the onset of DNA synthesis.

CLAIMS TO ORIGINAL RESEARCH

1. A pre-existing assay for the four deoxynucleoside triphosphates was made 10 to 20 fold more sensitive.

2. The changes in the four deoxynucleoside triphosphate pools in a highly synchronous culture of mammalian cells (HeLa) were determined, in relation to DNA synthesis.

3. The dCTP and dGTP pools in both asynchronous and synchronous cultures of mammalian cells were estimated.

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