

Biosynthesis of Nucleotides and Nucleic Acids in Tumours

THE BIOSYNTHESIS OF NUCLEOTIDES AND NUCLEIC ACIDS IN
EHRlich ASCITES TUMOUR CELLS AND THE EFFECTS OF COL-
CHICINE THEREON

by

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LIST OF ABBREVIATIONS

dA	deoxyadenosine
dAMP	deoxyadenosine-5'-monophosphate
dADP	deoxyadenosine-5'-diphosphate
dATP	deoxyadenosine-5'-triphosphate
dG	deoxyguanosine
dGMP	deoxyguanosine-5'-monophosphate
dGDP	deoxyguanosine-5'-diphosphate
dGTP	deoxyguanosine-5'-triphosphate
dC	deoxycytidine
dCMP	deoxycytidine-5'-monophosphate
dCDP	deoxycytidine-5'-diphosphate
dCTP	deoxycytidine-5'-triphosphate
dU	deoxyuridine
dUMP	deoxyuridine-5'-monophosphate
dUDP	deoxyuridine-5'-diphosphate
UMP	uridine-5'-monophosphate
uDP	uridine-5'-diphosphate
TdR	thymidine
TMP	thymidine-5'-monophosphate
TDP	thymidine-5'-diphosphate
TTP	thymidine-5'-triphosphate
PRPP	5-phosphoribosyl-1-pyrophosphate
ATP	adenosine triphosphate
P _i	inorganic phosphate
dR-1-P	deoxyribose-1-phosphate
R-1-P	ribose-1-phosphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
s.e.m.	standard error of mean
P	probability

CHAPTER 1

INTRODUCTION

Todd (1) has stated in a review on cancer research that "everything comes back finally to nucleic acids". This aspect of tumour cell metabolism has formed the major part of this thesis.

Several theories have been advanced to account for the initiation of neoplastic growth in cells. The two main hypotheses are that cancer may be caused by

- 1) somatic mutation
- 2) virus effects

Either factor would account for the lesions found in tumour cells. Both could result in the irrepressible growth and division characteristics of cancerous tissue.

Szent-Gyorgyi (2) has suggested that "contact inhibition" (3) is lacking in tumour cells. He has searched for, and has found, two substances from extracts of all tissues: a growth promoter, "promine", and "retine" which retards growth. It is the delicate balance of these two substances which, he feels, restore and guard the quiescent state. The establishment of a low retine/promine quotient in neoplastic tissue, relative to normal cells, would be an encouraging step in elucidating the problems of oncogenesis.

The work of Jacob and Monod (4), whose hypotheses have clarified the relation of external environmental control to genetic determination of the cell's activity, have given rise to several theories on cancer.

Pitot (5) has suggested that enzyme synthesis occurs on templates

which have been prepared at some time earlier in the life of the cell. These are maintained in a stable form to be used when the environmental situation calls for it. He has postulated that in tumours, "super stable" templates for DNA synthesizing enzymes would be advantageous for growth, and could thus give rise to neoplasms. It is cited in this paper that the lifetime of the thymidine kinase template of hepatoma is longer than in normal tissue.

Potter (6, 7) has evolved a "deletion hypothesis" which encompasses the theoretical considerations of Jacob and Monod, as well as the bulk of experimental results on the biochemistry of tumour cells. It involves successive mutations resulting in the loss of all systems responsible for negative feedback (and thus environmental control) on the specific enzyme-forming systems required for cell division. This inherently includes DNA synthesis, as well as the formation of proteins for mitotic mechanisms. This would involve, for example, deletion of the catabolic enzymes involved in DNA metabolism.

In relating cell division to the occurrence of DNA replication, one can consider the mechanism of DNA production by tumour cells as equally important for tumour growth and cell division.

Before going into the aspects of DNA most closely related to the work presented, it is necessary to discuss briefly the developments over recent years which have led to a reasonably clear understanding of the general requirements for DNA synthesis.

1.1 Requirements for DNA Synthesis

Grunberg-Manago, Ortiz and Ochoa (8) have isolated and partially

purified an enzyme from Azobacter vinlandii which catalyses reversibly the synthesis of highly purified ribonucleic acid-like polynucleotides from the 5'-nucleoside diphosphates. This enzyme has been found to require magnesium ions.

Kornberg et al (9) have purified a DNA polymerase from E. coli and have shown an absolute requirement for magnesium ions, primer DNA (from a variety of sources) and the 5'-triphosphates of the deoxyribonucleosides (dA, dG, dC, dT).

Bollum and Potter (10) have found a similar enzyme in the high speed fraction of homogenates from mammalian tissues (regenerating rat liver). The unusual location of the enzyme in the cytoplasmic rather than the nuclear portion of the cells, has led them to postulate that the enzyme system may be of nuclear origin, but it is liberated into the cytoplasm when the nuclear membrane disintegrates during mitosis. Using the incorporation of tritiated thymidine as a measure of DNA synthesis, they have shown similarity in the characteristics of the mammalian system to those of the bacterial systems.

Mantsavinos and Canellakis (11) have demonstrated requirements for DNA synthesis in mouse leukemic cells similar to those of normal tissue. A net synthesis of DNA in Ehrlich ascites tumour cells has been shown by Davidson and co-workers (12).

All this work has indicated the common denominator in requirements for DNA synthesis in most tissues to be:

- 1) the component deoxynucleoside triphosphates
- 2) DNA primer serving as a template
- 3) ATP or an ATP generating system
- 4) magnesium ions

- 5) purified or partially purified extracts containing the DNA polymerase

1.2 The Breakdown and Synthesis of DNA Precursors

In keeping with the experimental work presented in this thesis, the aspect of DNA biosynthesis most extensively discussed will be the metabolism of the deoxynucleoside triphosphates - their breakdown and synthesis.

The results of several investigations have suggested that the availability of these deoxynucleotide precursors is the key aspect of DNA synthesis. Deoxyribosides and their phosphorylated derivatives are compounds whose syntheses are apparently specifically directed for the manufacture of DNA. The rates of their production would thus have a controlling effect on DNA formation.

Lark (13) has been able to demonstrate the disruption of periodic DNA synthesis in synchronous cultures of Alcaligenes fecalis by the addition of the four deoxynucleosides during DNA synthesis. He has suggested that during DNA replication, these deoxynucleosides pair with the corresponding bases on the uncoiled strands of DNA and this leads to additional DNA synthesis.

Stern (14) has shown a periodic accumulation of deoxyribosides in developing lily anthers. This is preceded by an increase in DNase activity which may be the source of the accumulated deoxyribosides. He suggests that their presence results in the morphogenetic development of the pollen mother cells.

It has been postulated that the proliferative rates of tumours are related to the ability of the tissue to furnish the deoxynucleoside

triphosphate precursors. This can be related to the balance between synthesis and degradation of these precursors (15).

In many aspects of deoxynucleoside triphosphate biosynthesis, there exist more than one metabolic route. Cells furnish their nucleotides by means of stepwise 'de novo' biosynthesis (16, 17) from glycine, glutamine and 5-phosphoribosyl-pyrophosphate (PRPP) in the cases of purines, and aspartic acid, carbon dioxide, and ammonium ions in the case of pyrimidines. The end products in these reaction sequences are the purine and pyrimidine nucleoside 5-monophosphates. The purine and pyrimidine bases and their nucleosides and deoxynucleosides are not intermediates in these sequences. However, alternate routes, (referred to by Kornberg (18) as "salvage pathways"), do exist, whereby tissues can utilize preformed precursors (bases, nucleosides, deoxynucleosides) for the synthesis of the phosphorylated nucleotides required for RNA and DNA synthesis. These preformed precursors can be either exogenously supplied, or obtained by the enzymatic cleavage of RNA and DNA followed by dephosphorylation of the nucleotides. It has been frequently suggested that an abundance of these salvage mechanisms leads to conditions amenable to DNA synthesis, and that they exist to a much greater extent in rapidly proliferating tissues than in normal cells.

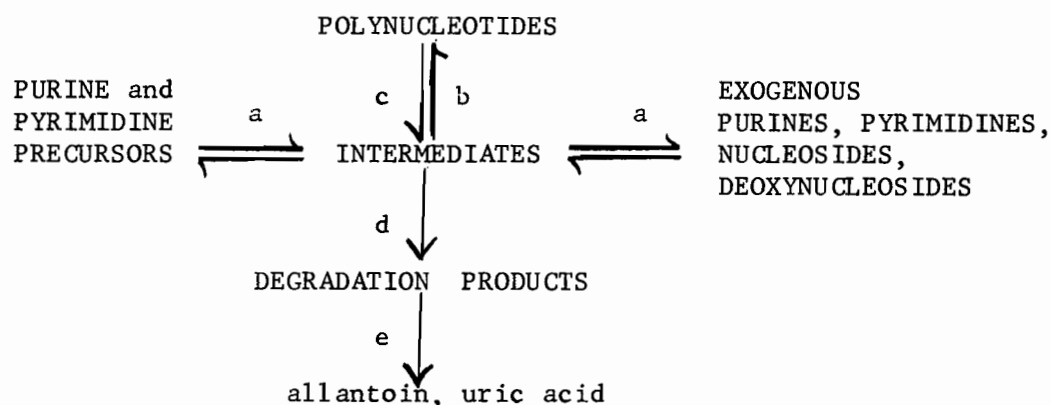
The problem of the breakdown and synthesis of precursors by nucleic acids is a key one. In a rapidly dividing cell in which there would obviously be an increase in the synthesis of DNA, it would be expected that there would be either:

- a) increase in the anabolic reactions which produce DNA precursors, or
- b) a decrease in the catabolic reactions which degrade DNA precursors, or

- c) increased utilization and reutilization of preformed precursors, or
- d) all of the above.

Bennet et al (19) have put forth a scheme which illustrates the relationships of the various aspects of nucleic acid synthesis and breakdown.

The following is a slightly modified reproduction of the scheme:



a, b... areas of possible increased activity or capacity in rapidly proliferating cells

c, d, e... areas of possible deficiency associated with rapid cell division

Bennet et al (20), in seeking biochemical distinctions between normal and tumour cells, have observed lower incorporation of formate -C¹⁴ into the nucleic acids of liver tissue than into the nucleic acids of tumour or intestinal tissues. Similarly, tumour cells utilize 2, 6-diaminopurine as a source of polynucleotide guanine to a much greater extent than do liver or intestinal cells. Skold (21), in studying uracil metabolism in various tissues, has presented evidence that the anabolic enzymes responsible for uracil incorporation increase in concentration during liver regeneration.

There have also been some reports on decreased catabolism in

proliferating tissue. De Lamirande et al (22) have found decreased activities of 5-nucleotidase, nucleoside phosphorylase, guanase, and adenase in hepatoma compared with those in normal liver. They have also reported that xanthine oxidase and uricase activities are non-existent in this tumour. This has led to the conclusion that the catabolism of purine and pyrimidine derivatives is not only impaired but completely blocked at the stage of xanthine and hypoxanthine. Wheeler and Alexander (23) have also reported decreased xanthine oxidase activity in sonicates of tumour cells.

On the basis of experimental results, Canellakis (24) has suggested, that tissues which can incorporate uracil into RNA (e.g. intestinal mucosa) have a decreased capacity to degrade it. He has proposed that there is an inverse relationship between the capacity to degrade uracil and to incorporate it into RNA. This may be part of a homeostatic mechanism which regulates the rate of RNA synthesis.

There have been a great many reports on the utilization and re-utilization of preformed precursors in actively growing tissues. The utilization of uracil-2-C¹⁴ by preneoplastic rat liver and rat hepatoma, in contrast to the relatively small incorporation in normal rat tissues, has been demonstrated (25). Much greater utilization of uracil for acid-soluble nucleotides, RNA and DNA in Flexner-Jobling carcinoma and intestinal cells than in liver has been reported (26). This is also attributed to decreased catabolism of uracil.

Recently, a study has been made of ribose and deoxyribose distribution in nucleic acids. With glucose-6-C¹⁴ as the label, as much as a 3-fold increase in purine-bound sugars as in pyrimidine-bound sugars has been found. This would imply, in the case of the purines, a certain

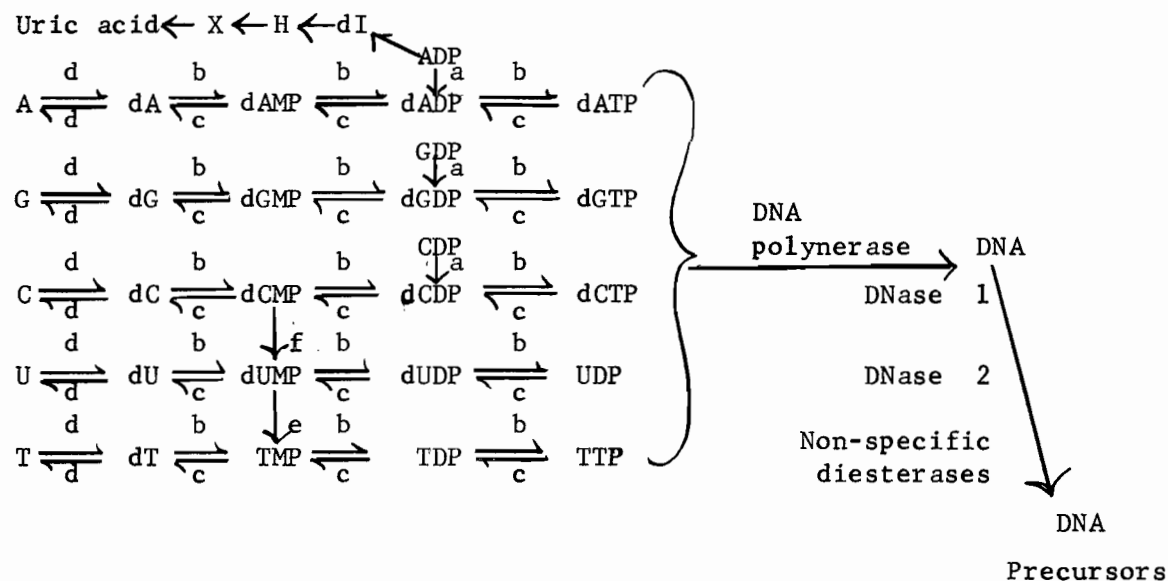
amount of breakdown and reutilization (27, 28).

Williams and LePage (29) have been able to show a high rate of incorporation of guanosine and adenosine into polynucleotides of mouse ascites cells.

Thompson et al (30), in studying the incorporation of formate -C¹⁴ into DNA-thymine in mammalian cell cultures, have found that DNA thymine retains about 75% of its initial activity when grown in the absence of thymidine. When exogenous thymidine is supplied, the DNA-thymine drops to 50% of its original activity. This has been interpreted as the reutilization of the breakdown product for synthesis of new DNA-thymine, as revealed by its reduction in the presence of exogenously supplied thymidine.

1.3 The Enzymes Involved in DNA Biosynthesis

Roth (15) has constructed a scheme which illustrates the various pathways of formation of the deoxynucleoside triphosphates. The diagram represents the main anabolic and catabolic reactions that are involved in DNA synthesis. It is presented below in a somewhat modified form.



According to the scheme above, the enzymes involved are:

- a) reductase (nucleotide reductase)
- b) kinase (deoxynucleoside, mono- and dinucleotide kinase)
- c) phosphatase (tri-, di-, monodeoxynucleotide phosphatase)
- d) phosphorylase (deoxynucleoside phosphorylases)
- e) thymidylate synthetase
- f) deoxycytidine deaminase

In general, the phosphatases, deaminases and phosphorylases (if they catalyse the cleavage of the deoxynucleoside to its base plus deoxyribose-1-phosphate) are considered catabolic. The kinases, reductases, DNA polymerase and phosphorylases (if they catalyse the synthesis of deoxynucleoside from base plus deoxyribose-1-phosphate) are considered anabolic.

The two general systems of enzymes, the reductases and the phosphorylases have been shown to be alternate sources of the deoxyribose moiety of DNA. The reductases represent the usual mode of deoxyribose formation in most tissues. The deoxynucleoside phosphorylases are a means of synthesizing deoxyribosides from preformed (exogenously supplied or reutilized) precursors and, as has been discussed previously, tend to be more active in highly proliferating systems. Both enzyme systems can, and often do, operate simultaneously in a tissue.

1.4 Nucleotide Reductase

It has been shown that when mammalian tissues are incubated with ribosides labelled in the base and in the pentose, the deoxyribosides isolated from DNA also contain label both in the pentose (deoxyribose) and in the base. The ratio of $-C^{14}$ in pentose to base is identical with that

found in the RNA, indicating that no cleavage of the glycosidic bond has occurred. This would indicate that ribonucleosides or ribonucleotides can be directly reduced to the corresponding deoxyribose derivatives (31).

It has also been demonstrated (32, 33, 34) that in tumour and liver cells, labelled glucose produces very similar patterns of radioactivity in the nucleotide-linked ribose and deoxyribose.

These indications have led to investigations of the factors controlling the direct reduction of the ribose moiety of nucleotides to deoxyribose.

Reichard (35, 36) has presented evidence for the conversion of uridine monophosphate and cytidine monophosphate to the corresponding deoxy-compounds. Extracts of E. coli contain enzymes which can convert CMP to dCMP. The first reaction product is dCDP. This enzyme requires ATP, Mg^{++} and TPNH. Similar enzymes have been demonstrated in extracts of Novikoff ascites cells (37, 38).

An enzyme termed cytidine nucleotide reductase has been purified 100-fold by Moore and Reichard (39). The reduction definitely occurs at the diphosphate level, and requires ATP, Mg^{++} and reduced lipoic acid, which can be replaced by TPNH.

Bertani et al (40) have demonstrated the conversion of UDP to dUDP in the same enzyme fraction that contains cytidine nucleotide reductase, a process also requiring ATP, Mg^{++} and reduced lipoate. It is interesting that this fraction contains an active and highly specific dUTPase. This could conceivably account for the absence of uracil in DNA.

In the case of purine nucleotides, Larsson (41) has demonstrated

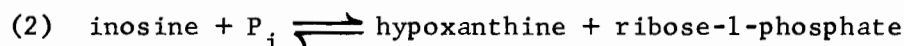
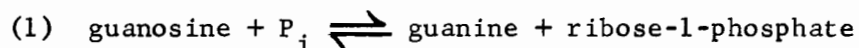
similar reductions at the dinucleotide level in E. coli. Reichard (42) has shown the reduction of guanosine to deoxyguanosine nucleotide in chick embryo.

In keeping with the deletion hypothesis mentioned earlier (6), which stresses the importance of feedback systems in maintaining control of cell division, Reichard et al (43) have found that the reductive conversion of CDP to dCDP is inhibited by the deoxynucleotides of guanine, adenine and thymine. Similarly, GDP to dGDP is inhibited by guanine and adenine nucleotides.

1.5 Nucleoside and Deoxynucleoside Phosphorylase

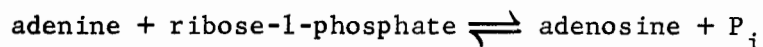
A) Nucleoside Phosphorylase:

Kalckar (44, 45) has shown that the following reactions occur in rat liver:

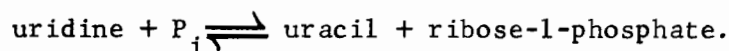


He has found that a high concentration of phosphate results in the phosphorylytic splitting of the nucleoside. The equilibrium is 80-90% displaced in favor of nucleoside formation, when equimolar amounts of base and deoxyribose-1-phosphate are present. The addition of guanase in reaction (1) or xanthine oxidase in reaction (2) results in increased phosphorolysis of the nucleoside.

Korn et al (46) have demonstrated a similar enzymic reaction in purified beef liver extract:



Page and Schlenk (47) have isolated an enzyme from E. coli catalysing the analogous reaction with uridine:



This enzyme is specific for uridine. Cytidine and thymidine are not split by this enzyme, nor does orotic acid react with ribose-1-phosphate in its presence.

The cleavage of inosine and deoxyinosine to hypoxanthine in the growth medium of tissue cultures of various tissues has been reported (48).

An enzyme has been purified from rabbit bone marrow which catalyses the phosphorolysis of guanosine and deoxyguanosine (49). The enzyme is inhibited by tris buffer and has no activity with adenosine, inosine or xanthosine. Pontis et al (50) have achieved a 250-fold purification of uridine phosphorylase from Ehrlich ascites tumour cells. They have reported identical maximum velocities for the phosphorolysis of uridine and deoxyuridine. However pH optima and inhibition data have indicated that the two reactions are catalysed by two different proteins or, conceivably, by two different active centers on one protein. The preparation also carries out the phosphorylase of thymidine and 5-halogen substituted nucleosides, but will not cleave cytidine or purine nucleosides.

Harrington (51, 52) has shown that in the presence of cytidine, there is a stimulation of incorporation of purine precursors into the nucleotides of Ehrlich ascites tumour cells. Only those precursors which depend on a linkage with pentose are stimulated. A high-speed fraction of these tumour cells produces uridine, uracil and ribose when incubated with cytidine and phosphate. She has proposed that the cytidine is deaminated to uridine which, because of the active uridine phosphorylase in these tissues, can transfer its ribose group to purine acceptors.

Gotto and co-workers (53) have purified the enzyme (or enzymes) which cleaves inosine, guanosine, adenosine and xanthosine. The system

requires phosphate or arsenate. Uridine is also split by this fraction, but cytidine and thymidine are not cleaved. A stimulation of uracil incorporation into nucleic acids by inosine has been reported (59).

The products of several of these phosphorylytic transfer reactions have been located in the extracellular medium of Ehrlich ascites cells. Patterson (55) has shown that upon incubation of the cells with uridine or guanosine in the presence of hypoxanthine, there is an increase of inosine in the medium. He has also obtained uridine from uracil in the same manner. This has led him to suggest a coupled reaction between two phosphorylases, in which the pentose moiety of a purine riboside is transferred to a pyrimidine base.

B) Deoxynucleoside Phosphorylase:

Similar studies have been carried out on the deoxynucleoside-splitting enzymes.

Friedkin and Kalckar (56) reported the phosphorolysis of hypoxanthine and guanine deoxyribosides with the concurrent formation of deoxyribose-1-phosphate, in purified calf and rat liver extracts and in calf thymus. They have also reported the synthesis of thymidine and deoxyuridine from thymine or uracil plus deoxyribose-1-phosphate in horse liver. The enzyme has no effect on cytidine.

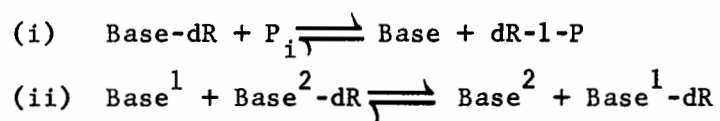
Purine nucleoside phosphorylase, which is active on deoxyinosine and deoxyguanosine, has been obtained from E. coli (57). The enzyme is dependent on the presence of phosphate or arsenate. Marinello and Martinelli (58) have reported the phosphorylytic cleavage of deoxyguanosine in rat liver homogenate.

Razzel and Khorana (59) have purified an enzyme from E. coli which,

while tolerating a variety of substitutions on the pyrimidine ring, has been found to be absolutely specific for deoxyribose-1-phosphate. The enzyme has no effect on deoxyguanosine, deoxyadenosine and deoxycytidine.

Rachmeler et al (60) have obtained extracts from E. coli grown in a thymidine-supplemented medium which contain much greater thymidine phosphorylase activity than similar extracts from bacteria grown in an un-supplemented medium. In this case, the enzyme's activity is of a catabolic nature, favouring the splitting of thymidine to thymine and deoxyribose-1-phosphate.

Zimmerman (61) has done extensive work on thymidine phosphorylase (i) and deoxyribosyl transferase (ii).



He has demonstrated a constant ratio of phosphorylase to transferase activity, as well as identical pH optima and substrate specificity in rat liver. This has led him to suggest that the transferase and phosphorylase activities are on the same protein molecule.

In continuing these studies, Zimmerman (62) has recently made a quantitative comparison of the effectiveness of different deoxyribosides in transferring their deoxyribose group to thymine and uracil. In rat liver, the most effective deoxyribose donors are thymidine and deoxyuridine, followed by deoxyguanosine. Deoxyadenosine, deoxycytidine and deoxynosine have little or no effect.

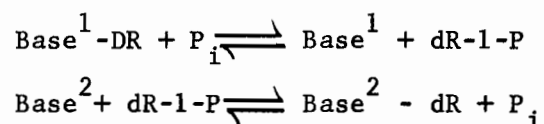
He has found no evidence of these enzymes in rat hepatoma, Ehrlich ascites carcinoma or Morris or Novikoff hepatomas. The greatest transferring

activity is in human spleen. The enzyme is inhibited by p-chloromercurobenzoate and sulphate.

De Verdier and Potter (63) have also carried out investigations on uracil and thymine metabolism in rat liver. They have demonstrated the ability of both uracil & thymine to accept a deoxyribose moiety from either deoxyuridine or thymidine.

Another enzyme system which accomplishes the same sort of exchange has been found in certain bacteria (64). It is immaterial which deoxyriboside or which base is provided. A mechanism exists which effects interconversion between any purine or pyrimidine base and any deoxyriboside.

The following reactions occur in these bacteria:



In these reactions, however, the purine or pyrimidine base plus deoxyribose-1-phosphate cannot be substituted for the nucleosides. Thus the bacteria contain specific enzymes which bring about these interconversions.

It has been shown in L. helveticus that hypoxanthine deoxyribose can donate its deoxyribose moiety to thymine, uracil, cytosine and 5-methyl cytosine, if an excess of these pyrimidines are present (65). This species can also transfer the deoxyribose from thymidine to adenine, guanine, hypoxanthine and xanthine.

The nature of these phosphorylases is such that they can serve as anabolic synthesizing nucleosides from their respective bases; they may also be catabolic, splitting the nucleosides to purines or pyrimidines. It would be interesting to see if their function is anabolic in proliferating

tissues and catabolic in less rapidly growing systems. In the experimental work to be presented here, the reactions catalyzed by these phosphorylases are most definitely anabolic reactions. However, this could be due to the extremely active kinase systems present in the Ehrlich ascites carcinoma. Any nucleoside or deoxynucleoside formed is rapidly phosphorylated, and the reaction is pulled in the direction of nucleoside synthesis instead of cleavage..

1.6 Uridine and Deoxyuridine Kinase

In systems which carry out 'de novo' biosynthesis of pyrimidine nucleotides, uridine-5-monophosphate is formed from orotidine-5-phosphate (the final product in the 'de novo' chain by orotidine-5-phosphate decarboxylase (66)).

As mentioned above (47), uridine phosphorylase can convert exogenous or reutilized uracil to uridine. Canellakis (67) has reported systems in rat liver which are capable of converting uracil to uridine and of phosphorylating the uridine to its mono-, di-, and tri-nucleotides. He has purified the enzymes involved, (uridine, uridylic acid and uridine diphosphate kinases), and has demonstrated their absolute dependence on Mg^{++} and on purine di- or tri-nucleotides such as ATP, ADP or ITP. Enzymes have also been found in rat liver which are capable of phosphorylating UMP to UDP in the presence of ATP and Mg^{++} . Cytidylic acid has been shown to be derived from UMP at the level of triphosphate to diphosphate (76, 76a).

Skold (21), in a study of these alternate routes of uracil metabolism, has found uridine and deoxyuridine kinase activity in a variety of different tissues. The highest activity reported is in Ehrlich ascites

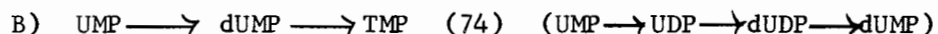
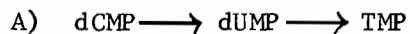
carcinoma cells. These studies indicate some correlation between the degree of proliferation of a tissue and the efficiency with which the tissue employs these alternate reserve pathways. A considerable increase in the activities of uridine phosphorylase and kinase takes place during liver regeneration, as compared with normal rat liver, where these activities are quite low.

In a more detailed study on uridine kinase (68) the enzyme has been purified 400-fold from the Ehrlich ascites tumour. The enzyme requires ATP and Mg^{++} for optimal activity. It can react with cytidine and 5-fluorouracil, but not with deoxyuridine. It is interesting to note, however, that Okazaki and Kornberg (69) have found thymidine kinase to be capable of phosphorylating uridine. It has also been reported (76) that the deoxyuridine kinase levels increase 10-fold during rat liver regeneration in a manner very similar to the thymidine kinase increase during this period. On the basis of these findings, it has been suggested that the phosphorylation of thymidine and deoxyuridine are carried out by the same enzyme.

It has been shown that uridine triphosphate causes a feedback inhibition of uridine kinase (71). The enzyme which phosphorylates UMP to UDP (72) is inhibited by alloxan.

1.7 Thymidylate Synthetase

Before discussing the action of thymidine kinase, it is necessary to mention the relation of uridine and deoxyuridine metabolism to thymidine metabolism. Again, the enzyme which phosphorylates thymidine to thymidylic acid is considered to be an alternate route. The general source of DNA-thymine in resting tissues is thought to be via two main pathways:



Labelled cytidylic acid has been shown to be a precursor of DNA thymidylic acid in rat tissues (75).

The conversion of deoxyuridine-5-phosphate to thymidine-5-phosphate ~~to thymidine-5-phosphate~~ in extracts of E. coli has been reported (77, 78).

Evidence has been presented indicating the source of the methyl group at the "5" position of the thymine ring to be N^{10} -hydroxymethyltetrahydrofolic acid (79). The suggested reaction is the combination of the N^{10} -hydroxymethyl group of tetrahydrofolic acid with deoxyuridine-5-phosphate at the "5" position (80). This is followed by cleavage of water and splitting of the condensation products to yield thymidine-5-monophosphate and oxidized tetrahydrofolic acid. The enzyme involved is termed, appropriately, thymidylate synthetase. Deoxyuridine has been shown to be the main methyl group acceptor, in regenerating rat liver and in intestinal mucosa (81).

1.8 Thymidine Kinase

Numerous studies have been conducted on thymidine kinase, for it has been shown to be implicated in the control of DNA synthesis. Most of the earlier work on thymidine kinase has taken the form of studies on thymidine incorporation into DNA.

The capacity of thymidine to serve as a precursor has been demonstrated in E. coli (82), in chick embryo (83), and in regenerating rat liver (84). There is very little diversion of radioactivity from labelled thymidine to any other DNA component or to RNA.

Mantsavinos and Cannelakis (10) have reported the existence of enzymes which can convert the deoxy-5-mononucleotides of adenine, guanine, cytosine and thymidine to the corresponding di- and tri-phosphates in a supernatant fraction of rat liver homogenate obtained after high speed centrifugation. The reaction depends on the presence of ATP and Mg^{++} . It has been shown (85) that in the presence of ATP, a "high-speed" supernatant fraction from regenerating rat liver will catalyse the reactions: thymidine $\rightarrow$ thymidine-monophosphate $\rightarrow$ thymidine triphosphate. Purified DNA polymerase will not incorporate thymidine- H^3 into DNA without the prior action of these kinases.

Bianchi et al (86) have studied thymidine kinase systems in a number of normal and malignant tissues and have suggested a direct phosphorylation of TMP to TTP in all the tissues studied. Their basis for this suggestion is the apparent absence of an appreciable amount of TDP as an intermediate. In another report (87), the authors report low activity or complete deficiency of thymidine kinase in several human tumours.

Several papers have been published which dispute the direct production of TTP from TMP and which give evidence against the proposed pyrophosphate transfer mechanism suggested by Bianchi (88). Various studies have been carried out which show that TMP and TDP always appear before TTP (89, 90, 91). In addition, investigations with P^{32} -labelled TDP have shown that it is rapidly converted to TTP without any observed intermediate formation of TMP. The kinase systems responsible for the incorporation of thymidine into DNA in extracts from the Ehrlich ascites carcinoma have been investigated (92). A sequential appearance of thymidine kinase, thymidine mononucleotide kinase and thymidine di-nucleotide kinase have been reported.

Okazaki and Kornberg (69) have purified thymidine kinase from E. coli and have conducted studies on its requirements, inhibitors, etc. They have shown that it is inhibited by the final product of these kinases: thymidine triphosphate (93).

It has been shown (94) that exogenously supplied thymidine stabilizes thymidine kinase in extracts of cultured mouse fibroblasts. Kit and co-workers (95) have carried out studies on stationary mouse fibroblast cells. They have shown that thymidine prevents the normal decrease in thymidine kinase activity in aging cells and have suggested that a conformational change in the enzyme to an inactive form occurs with age. This is based on their observations that thymidine kinase is rapidly inactivated when incubated at 38° at pH 7 to 8, but that TTP, ATP or thymidine can protect the enzyme against this thermal inactivation.

1.9 The Relation of Thymidine Phosphorylation to DNA Synthesis & Cell Division

Thymine is the only base specific for DNA and therefore it is not surprising that many investigators have studied its conversion to the polynucleotide form as a possible control of DNA synthesis. Very definite correlations have been shown to exist between the amount of DNA synthesis and the levels or activity of the thymidine kinases.

Gray and co-workers (96) have found high activity in the thymidine and thymidylate kinase systems in certain highly proliferating tissues such as Ehrlich ascites carcinoma, regenerating rat liver and rabbit bone marrow. These enzymes are much less abundant in such tissues as liver, kidney, etc. The addition of liver or kidney extracts greatly reduces the ability of the more highly proliferating tissue extracts to phosphorylate thymidine and to form DNA.

However, the non-growing tissue extracts will phosphorylate deoxyadenosine, deoxyguanosine and deoxycytidine to the triphosphate level. When these extracts are added to the rapidly growing tissue extracts, no inhibition of the other kinases is exhibited. Thus the resting tissues contain a factor capable of inhibiting the thymidylate system, which does not seem to be present in the more active cell systems. This would indicate a role of the thymidine kinases in regulating DNA biosynthesis.

In keeping with these results, Keilley (97) has found active thymidine phosphorylating systems in mouse ascites hepatoma, in contrast to low TTP synthesis in mouse liver. Various kinetic studies on the phosphorylation of TMP in the liver indicate that the relative lack of TTP synthesis is due to factors which suppress the synthesis of TTP, rather than a deficiency of the phosphorylating enzymes.

Investigations have been carried out on thymidine-deficient growth in HeLa cells that have been treated with 5-fluorouracil-2-deoxyribose which inhibits the phosphorylation of thymidine (98). Although cell division is abruptly stopped, there is continued accumulation of a DNA-like material. It has been suggested that this substance is an altered DNA which is unable to initiate cell division. When thymidine is restored to this system, there is a lag before the synthesis of new DNA. This would indicate that the cellular machinery is not poised for DNA synthesis. Possibly thymidine or its derivatives play an auxiliary role in DNA synthesis.

Bollum et al have shown that regenerating rat liver, X-irradiated in the early G₁ period (pre-DNA synthesis), does not synthesize these enzymes. Regenerating liver is an excellent tissue for studying the appearance of these systems. In this tissue, a marked increase in thymidine kinase at the

time when DNA synthesis begins has been reported (100). However, it has also been shown in the same study that thymidylate kinase does not appear until two hours after the peak of DNA synthesis. From these results, it appears that the control of DNA synthesis occurs at a metabolic step prior to TMP formation.

A study on the relationship of the thymidine kinase to the mitotic cycle has recently been carried out by Brent et al (101). They have reported a low level of thymidine kinase immediately after division (G_1 phase) which rises during the S period (the phase of DNA synthesis) and reaches a peak five hours after the peak of DNA synthesis. The levels then fall rapidly during the following division. A similar pattern is obtained when TMP is substituted for thymidine.

They have suggested that these enzymes may be present at all times in the cell, but in a form which is not apparent when the enzyme is assayed in the supernatant. Either they may be present in the form of a complex with the nuclear particulate fraction before or during DNA synthesis and are slowly released to the supernatant when their function is completed, or the observed increase may be due to actual enzyme synthesis. In this case, the subsequent decrease could be explained if the enzyme were bound to the nuclear particulate fraction as it is synthesized in preparation for the next cell cycle.

The plant Lilium longiflorum is another excellent species for such studies. In this plant's growth cycle, thymidine kinase appears at a defined time and lasts for twenty-four hours. This periodicity can be demonstrated in extracts as well as in intact cells (102). The thymidine kinase always appears before the synthesis of DNA and its formation has the characteristics of enzyme induction:

a) its appearance in the microspores is preceded by a transient pool of deoxyribonucleosides from outside the cells, where it is being synthesized.

b) its production can be suppressed by interference with RNA or protein synthesis (103).

Kit (104) has found an increase of the enzymes in animal cells infected with pox virus. Following inoculation there is a subsequent increase in the levels of thymidine and thymidylate kinases. The results indicate synthesis of these enzymes 'de novo', rather than activation of pre-existing systems. He has suggested that the information for the synthesis of the thymidine kinase is coded in the viral genome. This has been substantiated by examining the new enzymes on anion exchange columns, which indicate that they have several differences from the normal cellular enzyme proteins.

1.10 The Relation of DNA Synthesis to Mitosis

In normally dividing cells, the DNA content must double at some time before chromosome duplication. Autoradiographic, biochemical and Feulgen microphotometry techniques have made it possible to determine at what point in the cell cycle DNA, RNA and protein synthesis occur (105). Because DNA and histones are the most characteristic components of chromosomes, they can serve as useful tracers of chromosome substance in nuclei at stages when the chromosomes themselves are morphologically obscure (106, 107).

Swift (108) has shown that the time of DNA synthesis in the liver of mouse embryo occurs in late interphase, whereas it occurs in mid-interphase in mouse spermatocytes. Richards et al (109) have carried out studies on the

time of DNA synthesis in Krebs ascites cells, as compared with normal embryonic mouse cells. They have found that in both cases DNA synthesis takes place at a definite interval during interphase. In ascites cells, the DNA synthesis starts at mid-interphase and lasts until late interphase. In embryonic tissues the DNA synthesis begins in late interphase and lasts for a shorter interval of time than in the ascites cells, before the DNA content is doubled.

It has been found in mouse liver, where diurnal waves of mitosis can be followed, that a period of DNA synthesis precedes the wave of mitosis by six to twelve hours (110).

Edwards et al (111) have studied the time of the various cell phases in Ehrlich ascites tumour cells. They have shown that the average duration of interphase is 12.9 hours. DNA is constantly synthesized over an 8.5 hour period of the interphase. Prophase lasts 4.2 hours and metaphase, anaphase and telophase are 0.38, 0.23 and 0.36 hours respectively.

In nearly all tissues studied, DNA synthesis takes place during interphase. In fact, some studies have revealed that no DNA synthesis occurs during mitosis. When mitosis is blocked by colchicine in Vicia faba, DNA synthesis does not proceed, although this study suggests that during interphase, the drug has no effect on the synthesis of DNA (112).

Whether chromosome reproduction actually causes cell division has been the subject of many investigations. DNA synthesis is inhibited and cell division ceases in E. coli mutants, which are incapable of synthesizing thymine when they are grown in its absence (113). However, other metabolic processes are unaffected and the cells eventually die. Biesle (114), in a review on the modifications of mitosis, has reported that

specific DNA inhibitors cause a blockage of cell division.

However, there are several facts which suggest that the doubling of the DNA content of a cell is not the trigger mechanism which initiates mitosis. Many strains of tumour cells exist which contain a large range of polyploid mitotic species. Evidently the DNA content of the cells can double, and even quadruple, without the cell undergoing mitosis. The wide range of studies on the temporal relationship of DNA synthesis to the cell cycle has shown that in many species there can be a considerable lag or interval between the completion of DNA duplication and the onset of prophase.

The fact that DNA synthesis, reflected in chromosome duplication, is one of the requirements for division is undisputed. Thus DNA synthesis exists as one of the controlling factors of mitosis.

1.11 Studies on Colchicine

A) General Effects:

Investigation of the effects of colchicine on nucleic acid metabolism in Ehrlich ascites tumour cells has formed part of the research program to be described in this thesis.

Colchicine, as a mitotic inhibitor, cancer chemotherapeutic agent, and inducer of polyploidy, has been the subject of very many investigations. No one has yet elucidated its mode of action. Eigisti and Dustin have compiled a comprehensive book which reviews the research on and with the drug up to 1956 (115).

Colchicine is an alkaloid derived from the colchicum plant and has been mentioned in writings dating back to 1500 B.C. In these early

Egyptian papyrus records colchicine is described as an antidote for gout and a number of other rheumatic ailments. Its high toxicity has always made necessary the very cautious use of the drug. Extremely minute amounts (10 mg. in man) can be lethal (116).

In 1934, Dustin (117) discovered that colchicine causes metaphase arrest in all dividing cells. This is accompanied by gross morphological aberrances which he has termed "C-mitosis". These include: a short prophase, followed by disintegration of the nuclear membrane, cytoplasmic swelling and extreme clumping together of the chromosomes. The mitoses remain arrested in this state for up to 24 hours. The effect is reversible. It has been shown that colchicine's effect is on the spindle which forms at metaphase. The drug interferes with the formation of this structural element (118). Many investigators have tried to clarify the mechanism of the drug's action through biochemical studies.

B) General Biochemical Effects:

Various investigators (119, 120, 121) have been able to reverse somewhat the antimitotic effect of colchicine by the addition of ATP. They have suggested a possible interference of the drug in ATP synthesis.

Upon the administration of colchicine, an accumulation of free amino acids in regenerating rat liver, has been reported (122), presumably due to increased protein cleavage as a result of mitotic blockage. Affonso et al (123, 124) have shown an inhibitory effect of the drug on rat liver xanthine oxidase 'in vivo'. In a crude homogenate of the rat liver, colchicine has no effect on the enzyme, but when the enzyme is purified, the drug has an inhibitory effect at extremely low concentrations (10^{-9} M). They have suggested that some factor in the crude homogenate protects the

enzyme from the drug.

Taylor (125) has conducted studies using radioactive colchicine (tritium labelled) in human cell cultures. There is no direct effect on macromolecular synthesis or cell cycle duration at a concentration of colchicine which completely inhibits mitosis. The work shows that colchicine is bound to unidentified sites in the cells, in amounts proportional to the colchicine concentration. This binding is irreversible. He has suggested a mechanism of colchicine's action based on the correlation between the appearance of blocked mitoses and the radioactivity per cell. When 3-5% of these sites are bound with colchicine, the cell is unable to form a functional mitotic spindle.

Another interesting mechanism of action for colchicine has been proposed, based on studies of melanocyte darkening of frog skin. Malawista (126, 127) has observed a potentiated MSH-stimulated darkening of frog skin in the presence of colchicine. The normal lightening of color after removal of MSH is inhibited by colchicine. The drug by itself causes a gradual, dose-dependent darkening. He has suggested that colchicine alters the sol/gel equilibrium in the cell by maintaining the sol state. This would explain colchicine's effect on the mitotic spindle, which is an oriented, gelated, birefringent structure.

Various investigations on the effect of colchicine on protein synthesis have been carried out. The drug appears to inhibit protein synthesis (128), but any connection between this effect and mitosis has not yet been shown. An inhibition of incorporation of labelled methionine into ascites tumour cells has been shown, at concentrations which inhibit mitosis (129).

Kohen and Busch (130) have demonstrated a similar inhibition of incorporation of arginine-U-C¹⁴ 'in vitro', into Ehrlich ascites tumour-bearing mice which had been preinjected 12-24 hours previously with colchicine. The suppression of labelling of the mitochondrial protein is greater than the general inhibition of protein labelling. The suggestion has been made that the antimitotic effect may be due to suppression of mitochondrial biosynthesis of substances necessary for completion of metaphase.

C) Colchicine and RNA:

Recently, considerable interest has been focused on the effects of colchicine on RNA metabolism.

RNA synthesis in Chinese hamster cells has been found to be confined to the nucleus (131). When the cells are treated with colchicine, much of the tritium-labelled RNA which is synthesized in the nucleus remains associated with the chromosomes in the blocked metaphase, instead of migrating to the cytoplasm. The drug inhibits the synthesis of RNA in the nuclei of cells which are blocked in mitosis, but it has no effect on nucleoside incorporation into the RNA of interphase cells.

When colchicine is administered to partially hepatectomized rats, there is a decrease in ATP and UTP levels, with a rise in the corresponding mononucleotides, suggesting either an increased de-phosphorylation or decreased phosphorylation of the mononucleotides (136). Sandburg et al (132) have demonstrated an inhibition by colchicine of tritiated uridine or cytidine into cytoplasmic and nucleolar RNA. Chromosomal RNA is not affected. Cells in C-mitosis continue to show some RNA synthesis. The authors have suggested that RNA synthesis is initiated by chromosomes and transferred first to the nuc-

leolus and then to the cytoplasm.

Love (133) has carried out several morphological studies on Ehrlich ascites cells in mice pretreated with colchicine. Amorphous ribonucleoprotein of the nucleoplasm normally diffuses 'into the spindle' after prophase. In C-mitosis, this RNA is dispersed throughout the cytoplasm which could account for failure of spindle material to assemble. Colchicine prevents the transfer of ribonucleoproteins, synthesized in the nucleoli, to the cytoplasm. Several other mitotic inhibitors show this inhibition, for example 5-fluorodeoxy-uridine.

Creasey and Markiw (134) have recently investigated the effects of colchicine, on RNA synthesis 'in vivo' in the Ehrlich ascites carcinoma. Trisubstituted uridine, injected into the mice after prior treatment with colchicine, is incorporated into soluble RNA to a lesser extent than in normal mice. The incorporation of valine -C¹⁴ into the protein fraction is also inhibited. These inhibitory effects can be reversed by massive doses of glutamate.

Similar experiments have also been carried out 'in vitro' (138). The amount of colchicine necessary to produce a similar inhibition of RNA is many-fold higher than 'in vivo'. This could possibly be explained if some kind of activation of colchicine is necessary for it to have effect and if this activation occurs more easily in the host, than in tumour cells 'in vitro'. The authors have found that in the 'in vivo' experiments, there is a necessary lag of 6 hours before the colchicine has an effect, while the 'in vitro' effect can be obtained after an hour's incubation.

Several investigations have been reported on the effect of colchicine on the incorporation of RNA precursors. A decrease in ATP with a concurrent increase in RNA utilization has been reported (135). Wang et al (136) have shown similar decreases in ATP and UTP in rat liver. Weinfeld and

Sandberg (137) have also demonstrated an increase in utilization of all precursors which are dependent on amination reactions. They have suggested a stimulatory effect by colchicine on the nucleotide aminating enzymes of rat liver.

D) Colchicine and DNA:

Various conflicting reports have been published on the effect of colchicine on DNA synthesis. If the sole effect of the drug is a direct inhibition of spindle formation, any inhibition of DNA synthesis would be a secondary effect. One would attribute such an inhibition to an increase in the number of cells in the non-DNA-synthesizing metaphase.

Klein et al (139) have obtained no increase in chromosome ploidy in colchicine-treated Ehrlich ascites cells as a normal consequence of metaphase arrest. Thus the cells fail to double the DNA. They have explained this by stating that either the cells do not double their DNA content until early metaphase or else the doubling is inhibited by colchicine.

Hell and Cox (140) have studied the effect of colchicine on the initiation and rate of DNA synthesis in guinea pig epidermal cells 'in vitro'. When the cells are incubated for 2 hours in colchicine, a 14% inhibition of DNA synthesis is obtained. This would suggest that the number of metaphases blocked by colchicine would be an underestimate of the mitotic activity. However, other studies have shown no colchicine inhibition of the incorporation of tritiated thymidine into micronucleated cells (141, 142). Also, in adult mouse epithelia treated with the drug, there is no difference in the duration of DNA synthesis from that in non-treated cells (143). Skipper et al (144) have found a continuous incorporation of carbon-14 into the nucleic

acids of tumour cells treated with colchicine. Bloch (145) has reported that in rat fibroblast cells treated with colchicine, aside from mitotic blockage, there is no direct inhibition of DNA synthesis.

Colcemid (N-deacetyl-N-methyl colchicine) has been used for investigations in Chinese hamster cell cultures. Some indication of a primary effect on DNA synthesis in earlier stages has been obtained (146). Moss and Quastel (147) and Ilan and Quastel (148) have shown a considerable inhibition by colchicine of DNA synthesis in Ehrlich ascites tumour cells 'in vitro'. The levels of colchicine used are much higher than that needed to obtain a similar 'in vivo' effect.

The effects of this widely used drug, then, are reasonably constant with regard to protein and RNA synthesis, causing definite inhibition of biosynthesis. However, the effects of the drug on DNA synthesis are of a more variable nature. The experimental results presented in this thesis indicate an inhibitory effect of colchicine on DNA synthesis both 'in vivo' and 'in vitro'.

1.12 The Vinca Alkaloids

In recent years, another class of anti-mitotic agents with similar properties to those of colchicine has come to light. The vinca alkaloids, in particular vincristine, vinblastine, and leurosine, have been shown to have definite anti-tumour properties with less toxicity than colchicine (149, 150).

Cutts (151) and Johnson et al (153) have described the antimitotic properties of vinblastine 'in vivo'. Like colchicine, it is a spindle poison and produces C-mitosis in Ehrlich ascites cells, but the mitotic effect is more lasting and more complete. Vinblastine stops every cell that enters mitosis from continuing past metaphase, while with colchicine, some

cells progress to anaphase and telophase. The effects of vinblastine are dose-dependent; low concentrations block spindle formation while high concentrations cause shrinkage and coalescence of chromosomes.

Several studies on the effects of vinblastine on nucleic acid synthesis have been conducted (152, 153). In regenerating rat liver, this agent has very little effect on incorporation of formate -C¹⁴ into RNA or DNA. In Ehrlich ascites, an 18% inhibition of formate incorporation into DNA is obtained. In the actual bases, adenine and guanine are significantly lower in radioactivity. There is little effect on cytosine or thymine, which is actually slightly increased.

In bone marrow, the action of vinblastine on DNA synthesis as measured by formate -C¹⁴ incorporation is very pronounced, causing upwards of a 90% inhibition, which is reflected in all the component bases of the DNA (152).

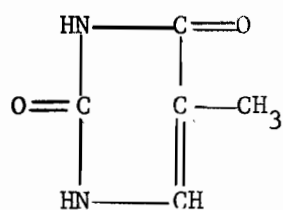
Creasey and Markiw (154) have recently carried out investigations on the effects of colchicine, vinblastine and vincristine on RNA synthesis in Ehrlich ascites cells 'in vivo' (127) and 'in vitro' (128). With colchicine and vinblastine, there is a marked discrepancy in the doses necessary to produce inhibition 'in vivo' and 'in vitro', but vincristine will inhibit the RNA synthesis 'in vitro' with an equivalent dose to that necessary 'in vivo'.

OBJECTIVES

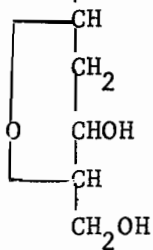
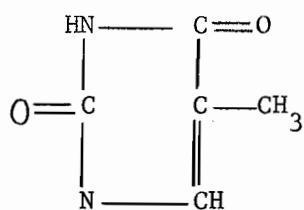
The topics discussed in Chapter 1 have been chosen with regard to their relevance to the experimental data presented in this thesis. The objectives of this research project have been the further elucidation of salvage pathways which exist in Ehrlich ascites tumour cells, and the effect of colchicine thereon.

The first part of the experimental work has dealt with the utilization of preformed precursors for DNA biosynthesis in tumour cells. This has involved studies of the activities of certain of the enzymes discussed in Chapter 1.3. The second part is concerned with studies on colchicine with regard to the effect of this drug on DNA biosynthesis. By investigating the effect of colchicine on nucleic acid metabolism, it is hoped that the antimitotic effects of the drug can be more clearly understood.

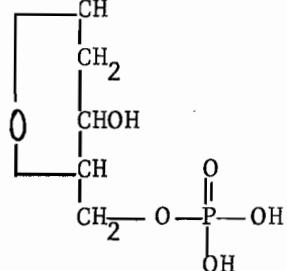
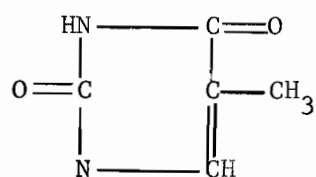
The formulae of the compounds most frequently discussed in this thesis are shown on the following page.



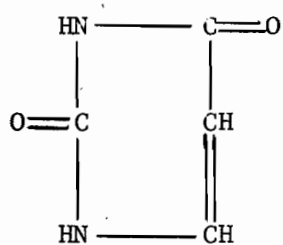
thymine



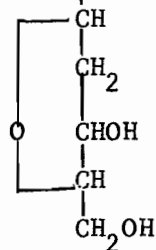
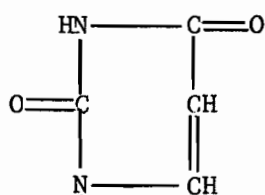
thymidine



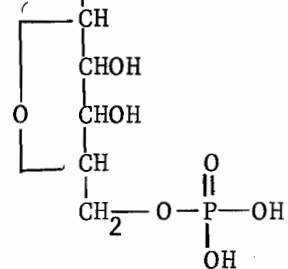
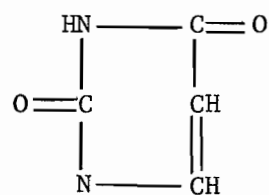
thymidine-5'-monophosphate



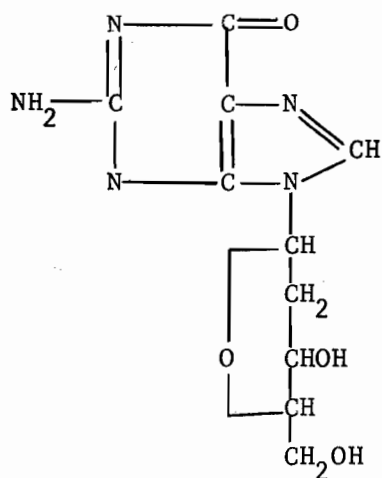
uracil



deoxyuridine



uridine-5'-monophosphate



deoxyguanosine

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintenance of tumour cells

All experiments, unless otherwise stated, were carried out on the Ehrlich ascites carcinoma. The tumour cells were harvested from male white mice from Robidoux and C.F.W. farms. These cells were obtained in the "liquid" form which was sustained in the following manner (155):

Solid tumours, growing in the axillary region, were maintained by weekly subcutaneous injections of minced solid tumour suspended in sterile isotonic saline in a ratio of one to one. When the solid tumours reached 8-10 days of growth, they were removed from the mice, minced very finely and injected intraperitoneally, after being mixed one to five in sterile saline. This injected minced suspension grew in the liquid ascitic form in the peritoneal cavity. These cells were then removed from the mice, glycerol added to a concentration of 6%, and stored in 5 ml. vials on dry ice at -20°C . The contents of these vials were used for subsequent liquid to liquid transfers. Only cells obtained thus (solid to liquid to liquid) were used.

2.2 Chemicals

The common chemicals used in all experiments were of "Reagent Grade" quality. No further purification was carried out.

The radioactive chemicals: adenine-8- C^{14} , guanine-8- C^{14} , thymidine-2- C^{14} , glycine-1- C^{14} , thymine-2- C^{14} and uracil-2- C^{14} , were obtained from the Radiochemical Centre, Amersham, England. Uridine-2- C^{14} was obtained from New England Nuclear Corporation, Boston, Massachusetts. Tritiated deoxy-

guanosine was obtained from Volk Radiochemical Co., Chicago. Colchicine was purchased from Abbott Laboratories in Montreal. Vincristine and vinblastine were obtained from the Ely Lilly and Co., Indianapolis, Indiana. Demecolcin was a gift from Dr. N. MacDonald of the Royal Victoria Hospital. Calf thymus DNA was a generous gift from Dr. S.C. Sung. It was prepared according to Kay, Simmons and Dounce (156).

All deoxynucleosides and deoxynucleotides were obtained from the Calbiochem Corp., Los Angeles. The purines, pyrimidines and nucleosides used were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

2.3 Preparation of solutions

Each solution was prepared in the appropriate concentration in distilled water. The various Krebs Ringer salt solutions were made up at five times isotonic concentrations, and stored at 4°C. The sodium bicarbonate, phosphate, and tris-hydrochloride buffer solutions were also stored at 4°C. All other solutions were prepared on the same days as the experiment. In the 'in vivo' experiments, colchicine, vincristine and vinblastine were dissolved in sterile isotonic saline, and were prepared on the day of use.

2.4 Preparation of tumour cells

The cells were prepared in a manner similar to that described by Johnstone and Scholfield (157). The cells were harvested in liquid suspension from the mice, six or seven days after inoculation. The ascitic fluid was diluted one to five in ice-cold isotonic Krebs-Ringer solution, and the suspension was centrifuged at maximum speed in the 6-place head of

the International Clinical Model centrifuge for 30 seconds. The supernatant containing most of the red blood cells was decanted. This was repeated until a clear, straw-coloured supernatant was obtained. The cells were then centrifuged at maximum velocity for two minutes to obtain the packed cell volume.

When whole-cell systems were used, the packed cells were resuspended in ice-cold isotonic Krebs-Ringer solution to yield a 1:10 dilution. 0.5 ml. of the suspension were then added to 2.5 ml. of incubation medium resulting in 3.0 ml. of isotonic, buffered solution. This yielded 5 mg. dry weight of tissue, or 50 mgm. wet weight.

To obtain cell-free extracts, a method of osmotic disruption similar to that described by Smellie (158) was employed. The packed cells were suspended in ice-cold distilled water in a ratio of one to ten, for 15 minutes. The suspension was then homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle for 1.5 minutes. It was found that there was no significant difference in the systems studied whether or not sucrose was added to the homogenate to a concentration of 0.25 M (isotonic).

The ruptured cells were centrifuged at 18,000 x g. in the International Portable Refrigerated Centrifuge for 30 minutes. The supernatant obtained was incubated in a buffered solution containing the appropriate additions.

To test the whole-cell suspensions for viability, and the homogenates for completion of membrane-rupture, both preparations were stained with 1% eosin and examined under a microscope. If the intact cells were viable, they would not take up the dye. In the homogenates, if the cell membranes were ruptured completely, staining with 0.08% crystal violet in 6% acetic acid would reveal only nuclei, and no membrane-bounded cytoplasm

around the nuclei. This homogenization process did not disrupt the cell nuclei.

2.5 Incubation Methods

Incubations of intact cell suspensions were carried out in isotonic Krebs-Ringer medium (159). The amount of CaCl_2 was halved in order to avoid precipitation with phosphate. The pH was maintained at 7.4 by phosphate buffer (0.02 M), by bicarbonate buffer (0.02 M) or by Tris-HCl (30 mM). When bicarbonate buffer was used the flasks were gassed with O_2/CO_2 (95:5) for 10 minutes prior to incubation. When glucose was used, it was added in an amount of 10 mMoles. The final volume of incubation medium was always 3.0 mls.

In the case of cell-free extracts, the pH was maintained at 7.4 by phosphate buffer (0.05 M) or by tris buffer (0.01 M). The final volume in most cases, was 1.0 ml., 0.5 ml of extract were used per vessel. Most incubations of extracts were carried out in 10 ml Ehrlenmeyer flasks stoppered with Parafilm. The flasks were incubated in a Research Specialties water bath shaker maintained at 37°C. In order to study synthesis of DNA in a cell-free extract, the supernatant of the cell homogenate was incubated as above, with ATP (2 μmoles), MgCl_2 (2 μmoles), and calf thymus DNA (0.02 mg per flask).

2.6 Radioactivity measurements

All non-chromatographed samples were counted with a Geiger-Mueller thin window gas counter (20% efficiency) attached to a Tracerlab Scaler. All samples were corrected for background. Self-absorption corrections were not applied, as the plated samples were infinitely thin.

Radioactive spots obtained by paper chromatography were counted directly on the paper with a shielded Geiger probe. The efficiency was 10% of that obtained by elution of the spots from the paper, but elution of radioactive spots proved to give less consistent results than direct counting. In experiments where tritium was the label, the paper spots were cut into $1\frac{1}{2} \times 3/4$ " rectangles and placed in counting vials containing 20 mls of scintillation counting fluid (2 litres toluene, 600 mg dimethyl oxazole, 10 gm 2, 5-diphenyloxazole) and counted in a Packer Tri-Carb Liquid Scintillation counter.

2.7 Determination of Radioactivity in the Acid Soluble, RNA and DNA Fractions

In all experiments where the separation of the acid-soluble, RNA and DNA fractions were required, a modification of the Schmidt and Tannhauser method (166) was employed. When estimation of the total nucleic acids was necessary, this portion was isolated according to Schneider's procedure (161).

In order to separate RNA from DNA and both from the acid-soluble nucleotides, the following procedure was employed:

Following termination of the incubation by immersing the vessels in ice, the contents of each vessel were decanted into centrifuge tubes and spun at maximum speed in the International Clinical centrifuge for 2 minutes.

The supernatant consisting of the incubation medium, cellular metabolites, and radioactive compounds was discarded. The cells were washed once in 5 ml of ice-cold isotonic Krebs-Ringer solution and subsequently suspended in 5 ml of 5% trichloroacetic acid for 30 minutes at 0°C. The trichloroacetic acid suspension was then centrifuged for two minutes at maximum speed. The supernatant containing all acid-soluble nucleotides and derivatives

was shaken several times with 10 ml of ether in order to remove the trichloroacetic acid. 0.05 ml of this acid-soluble portion was then plated on aluminum planchets and counted as described previously. If individual components of the acid-soluble portion were desired, an aliquot of the supernatant was chromatographed, and the separated components were counted directly on the paper.

To obtain the nucleic acid fractions, the trichloroacetic acid precipitate was washed once with 5 mls of 5% trichloroacetic acid, once with 5 mls of 95% ethanol and once with 5 mls of 95% ethanol/diethyl ether (3/1). The washed precipitates were then suspended in 2.0 mls of 0.2N KOH and kept for 18 hours at 37°C. This effectively hydrolysed the RNA, while leaving the DNA intact. After the incubation, the DNA and proteins were precipitated by the addition of 0.05 mls of 70% perchloric acid. The precipitate was centrifuged down, and the supernatant decanted into other tubes where its pH was brought to neutrality by the dropwise addition of 1.0N KOH and 0.2M perchloric acid. This neutralized supernatant was then plated and counted as the RNA portion of the cells. The precipitate containing the DNA was washed twice with 5 mls of 5% trichloroacetic acid, suspended in 0.6 ml of acetone, plated and counted.

In the 'in vivo' studies the mice were injected with the radioactive precursor and were sacrificed after one hour. The cells obtained were washed several times with ice-cold isotonic Krebs-Ringer, suspended in ice-cold 5% trichloroacetic acid for one hour and treated in an identical manner to the 'in vitro' systems.

2.8 Estimation of Radioactivity in the Component Bases of DNA

In order to obtain the distribution of the radioactivity which had been incorporated into the DNA, the DNA-containing precipitate was dried and heated in 0.1 ml 70% perchloric acid for 60 minutes in a boiling water bath. The digest was then neutralized with 10 N KOH and subsequently acidified to pH 2 with 6 N HCl. The resulting suspension was centrifuged and a 100 μ l microlitre aliquot of the supernatant was spotted on 18 x 24" sheets of Whatmen # 1 paper. This paper was then run two-dimensionally in the following solvent systems:

- a) descending butanol: $\text{H}_2\text{O}:\text{NH}_3$ (86:9:5)
- b) ascending isopropanol: $\text{HCl}:\text{H}_2\text{O}$ (65:16.5:18.5)

This gave clear separation of the component bases which could be identified under on ultraviolet lamp and compared to standard solutions run in the identical manner.

2.9 Estimation of Radioactivity in the Bases, Deoxynucleosides and Deoxynucleotides of Acid-Soluble Portions of Intact Cell Systems and of Cell-Free Extracts

In whole-cell preparations, when the distribution of the radioactivity in the acid-soluble components was desired, the trichloroacetic acid-free supernatant was brought to dryness by flash evaporation in a Buchler universal flask evaporator and redissolved in 0.5 ml of H_2O . 0.2 ml of this was chromatographed. The reduction of volume was necessary because of the low counts obtained in the whole-cell systems' acid-soluble portions. In intact cells the bases and nucleosides can freely exchange with the extracellular medium (163). In addition, a large proportion of the nucleotides

formed are used for the synthesis of macromolecules. Both factors account for loss of radioactivity in whole cell preparations. Conversely, in cell-free preparations, where these factors play no part, the acid-soluble radioactivity reflects all the nucleoside and nucleotides formed. Unless ATP, Mg^{++} and a primer are present, no appreciable nucleic acid synthesis occurs (9). Even when they are present, the amount synthesized is many times less than that formed in whole cells. Thus in cell-free extracts, a direct aliquot of the trichloroacetic acid-free supernatant was sufficient to give quantitative counts of the radioactivity distribution in the nucleic acid precursors.

In both whole-cell and cell-free extract preparations, the same chromatographic systems were used. In order to separate pyrimidine nucleotides from the bases and deoxynucleosides, the following solvent system was employed:

Ethanol/1.0M ammonium acetate pH 7.5 (7/3) (164)

When run for 18 hours on Whatman # 3 this technique gave effective separation of bases plus deoxynucleosides from the deoxynucleotide portion. In addition, the mononucleotides were separated from the di- and tri-deoxynucleotide portion which ran as one spot. However the bases and deoxynucleosides were indistinguishable.

In order to obtain clear separation of the pyrimidine bases from their deoxynucleosides, the samples were spotted on 1 inch strips of Whatman # 1 and placed in a descending trough containing the upper phase of the following solvent system, and run for 12 hours:

Ethyl acetate/ H_2O /formic acid (60/35/5), (165)

In this system all deoxynucleotides remained at the origin.

2.10 Variations in the Extent of Incorporation of Radioactive Precursors

In the 'in vitro' experiments the difference in radioactivity between duplicates did not exceed 10 per cent. Although there were considerable differences in the degree of biochemical activity in different animals, the per cent effects of all compounds on the biochemical systems studied, and the relative operation of these biochemical systems remained the same throughout.

In the 'in vivo' experiments, each result represents an average value obtained from four to six mice because of these individual differences in biochemical activity. The results can still be considered significant, as the differences in incorporation between the various groups varied from 50% to many fold. In all cases where 4 or more animals were used, the results were treated statistically. A standard error of the mean (s.e.m.) is given and the P value, if necessary is indicated (166).

CHAPTER 3.

STUDIES ON METABOLIC TRANSFORMATIONS BETWEEN PYRIMIDINE BASES AND PURINE DEOXYNUCLEOSIDES IN INTACT EHRLICH ASCITES TUMOUR CELLS

The utilization of preformed precursors for nucleic acid biosynthesis has been mentioned in Chapter 1.2. As has been discussed, these alternate pathways tend to be more active in rapidly growing tissues. It has been decided that a necessary starting point in a study of these routes would be a comparative survey of the incorporation of various purine and pyrimidine bases into the DNA of Ehrlich ascites tumour cells.

3.1 The Effects of Various Deoxynucleosides on the Incorporation of Radio-activity from C¹⁴-labelled Purine and Pyrimidine Bases into the DNA of Ehrlich Ascites Carcinoma Cells

Table 1 shows the magnitude of incorporation of radioactivity from the more common nucleic acid bases into DNA, as well as the effect of various deoxynucleosides thereon. The purine bases, as such, tend to be superior to pyrimidine bases in incorporation into DNA. The extent of incorporation of free thymine is extremely low (167). The level of cytosine incorporation is also negligible. The utilization of radioactivity from uracil-2-C¹⁴ for DNA synthesis is slightly higher than that with labelled thymine or cytosine. As will be subsequently discussed, the uracil must be converted at some stage to one or more of the component bases of DNA.

With the exception of cytosine, the deoxynucleosides all exert significant inhibitory or stimulatory effects on the incorporation of each base into DNA.

TABLE 1

Effects of Various Deoxynucleosides on the Incorporation of Radioactivity from Carbon¹⁴-labelled Purine and Pyrimidine Bases into the DNA of Ehrlich Ascites Tumour Cells

A d d i t i o n s	mu Atoms Carbon ¹⁴ Incorporated Into DNA				
	Adenine-8-C ¹⁴	Guanine-8-C ¹⁴	Thymine-2-C ¹⁴	Uracil-2-C ¹⁴	Cytosine-2-C ¹⁴
None	0.23	0.36	0.009	0.014	0.006
Deoxyadenosine	0.03	0.04	0.007	0.020	0.006
Deoxyguanosine	0.13	0.05	1.290	0.490	0.007
Thymidine	0.19	0.24	0.007	0.012	0.006
Deoxycytidine	0.88	0.78	0.026	0.003	0.005
Deoxyuridine	0.23	0.22	0.143	-	-

Input: counts/minute/vessel

concentration (mM)

adenine-8-C ¹⁴	400,000	0.1	
guanine-8-C ¹⁴	375,000	0.15 (carrier-free)	
thymine-2-C ¹⁴	400,000	2.9×10^{-2}	"
uracil-2-C ¹⁴	800,000	4.5×10^{-2}	"
cytosine-2-C ¹⁴	550,000	0.1	"

Ehrlich ascites tumour cells have been incubated in isotonic Krebs-Ringer solution containing 0.02 M phosphate buffer, pH 7.4, and 10 mM glucose (except for thymine-2-C¹⁴ + uracil-2-C¹⁴ where glucose has been omitted). Final volume: 3.0 ml. Duration of incubations: 1 hr. Temp. of Warburg apparatus: 37°C. Gas phase: air. Results expressed per 50 mg wet weight tissue. Each deoxynucleoside 1 mM.

Cytosine, however, is poorly incorporated throughout. Although it has not been recorded in tabular form, the accumulation of radioactivity from cytosine into the acid-soluble pool is 50 to 80% lower than that found with the other purines and pyrimidines. On the other hand, cytidine and deoxycytidine have been shown to be effective sources of RNA and DNA respectively (75, 82).

Deoxyadenosine inhibits the incorporation of labelled adenine and guanine into DNA. This may be due to isotopic dilution, or to the previously reported inhibitory effect of this compound on DNA synthesis (168, 169).

The most striking result evident from Table 1 is the great stimulatory effect of deoxyguanosine on labelled thymine and uracil incorporation into DNA. In the case of thymine-2-C¹⁴, the deoxyguanosine stimulation is 140-fold. This signifies greater incorporation of radioactivity from thymine than from any other purine or pyrimidine base. There is also a considerable increase of incorporation of uracil into DNA, in the presence of deoxyguanosine. Cytosine incorporation is not affected at all by deoxyguanosine.

Thymidine exerts very little effect on any of the systems studied, except for a 20% and 25% inhibition of incorporation of labelled adenine and guanine respectively, into DNA.

Deoxycytidine, on the other hand, does exert certain stimulatory effects. Marked enhancement of the incorporation of adenine-8-C¹⁴ and guanine-8-C¹⁴ are obtained. Deoxycytidine also slightly enhances thymine-2-C¹⁴ incorporation into DNA but inhibits the utilization of radioactivity from uracil-2-C¹⁴. This inhibition of the incorporation of labelled uracil might be ascribed

TABLE 2

Incorporation of Radioactivity into DNA from: Thymine-2-C¹⁴, Thymine-2-C¹⁴
plus 1 mM Deoxyguanosine, Thymidine-2-C¹⁴ into Intact Cells and Cell-Free
Extracts of Ehrlich Ascites Tumour Cells

P r e c u r s o r	mu Atoms C ¹⁴ Incorporated Into DNA	
	Intact Cells	Cell Extracts
Thymine-2-C ¹⁴	0.009	0
Thymine-2-C ¹⁴ plus 1 mM dG	1.432	0.068
Thymidine-2-C ¹⁴	3.142	0.117

Input: Thymine-2-C¹⁴ 400,000 counts/min./vessel, 0.15 mM
Thymidine-2-C¹⁴ 220,000 " " , 1.5 x 10⁻² mM

Whole cell systems prepared and incubated as described in Table 1, with glucose omitted.

Cell Extracts: cell-free extracts prepared as described in Chapter 2, and incubated with 0.02 M phosphate buffer, ATP (2 μmoles/vessel) MgCl₂ (2 μmoles/vessel) 0.1 ml calf thymus DNA (1.0 mg/ml).

to isotopic dilution. Maley and Maley (170) working with several mammalian tissues, and Scarano (171, 172) with sea urchin eggs, have reported the conversion of dCMP to dUMP which would result in dilution of radioactivity from uracil-2-C¹⁴. No direct conversion of dCMP to thymine deoxynucleotides has been reported.

Deoxyuridine exerts little or no effect on purine utilization, but markedly increases the incorporation of thymine-2-C¹⁴. As mentioned in Chapter 1.5, the conversion of thymine to thymidine in the presence of deoxyuridine in extracts of rat liver and hepatoma has been demonstrated.

It can be concluded from these results that mechanisms exist in the Ehrlich ascites tumour which result in utilization of all preformed bases, with the exception of cytosine. It has previously been thought that thymine is not utilized at all, and there has, to our knowledge, been no data on its incorporation into DNA. However, as can be seen from Table 1, deoxyguanosine, and to a much lesser extent, deoxyuridine can greatly increase thymine incorporation.

3.2 Comparison of (1) Thymine plus Deoxyguanosine with (2) Thymidine Incorporation into DNA

Unlike thymine, thymidine is an extremely effective precursor of DNA (83, 173, 82). As has been discussed in Chapter 1 the phosphorylation of thymidine to TMP is rapidly carried out by thymidine kinase which is abundant in most rapidly proliferating tissues.

A comparative study of the incorporation into DNA of thymine plus deoxyguanosine, with that of thymidine has been carried out. The results shown in Table 2 indicate that thymine-2-C¹⁴, in the presence of

TABLE 3

Effect

of 10 mMolar Glucose on Incorporation of Radioactivity from: Uracil-2-C¹⁴,
Uracil-2-C¹⁴ plus 1 mM Deoxyguanosine, Uridine-2-C¹⁴, Thymine-2-C¹⁴, Thymine-
2-C¹⁴ plus 1 mM Deoxyguanosine, Thymidine-2-C¹⁴ Into Acid-Soluble Nucleotides
and DNA of Ehrlich Ascites Tumour Cells

P r e c u r s o r	Per Cent Effect of Glucose	
	Acid-Soluble Nucleotides	D N A
Uracil-2-C ¹⁴	+ 190	+ 410
Uracil-2-C ¹⁴ plus dG	- 7	0
Uridine-2-C ¹⁴	+ 25	- 40
Thymine-2-C ¹⁴	- 8	0
Thymine-2-C ¹⁴ plus dG	- 8	- 32
Thymidine-2-C ¹⁴	- 9	- 25

Cells incubated as described in Table 1, $\pm$ 10 mMolar glucose

deoxyguanosine, is approximately 50% as effective a source of DNA thymine as is thymidine-2-C¹⁴. This is also evident in a cell-free extract where DNA synthesis has been studied. The incorporation of thymine-2-C¹⁴ alone into DNA is negligible both in intact cells and in cell-free extracts.

3.3 The Effect of Glucose on the Incorporation of Various Pyrimidines and Pyrimidine Derivatives into Nucleotides and DNA

Table 3 describes the incorporation of radioactivity into the acid-soluble and DNA fractions of Ehrlich ascites cells from uracil-2-C¹⁴ and thymine-2-C¹⁴ both in the presence and absence of glucose. The effects of this sugar on incorporation of thymidine-2-C¹⁴ and uridine-2-C¹⁴ are also presented. In all cases involving thymine and thymidine, the glucose exerts a slight inhibitory effect. This finding is in keeping with the observations of Sung (174). Further studies on labelled thymine and thymidine incorporation have therefore been carried out in the absence of glucose.

Conversely, a marked increase in uracil-2-C¹⁴ incorporation into both fractions, in the presence of 10 mM glucose, has been obtained. Many earlier investigations have been carried out which demonstrate that glucose is a source of the ribose moiety of nucleosides and nucleotides. Horecker and Smyrniotis (175) have shown that the key intermediate in the hexose monophosphate shunt is ribulose-5-phosphate, which is formed from 6-phosphogluconic acid by oxidation. Investigations carried out on yeast and liver have shown that ribulose-5-phosphate is easily converted to ribose-5-phosphate (176). The latter compound can then form ribose-1-phosphate by the action of phosphoribomutase. In this manner glucose functions as a source of ribose-1-phosphate.

The direct conversion of uracil to UMP (through combination with PRPP) has been observed only in bacteria (18, 24). Uridine phosphorylase is active in liver (63), Ehrlich ascites tumour cells (24), and several other mammalian tissues. This is the main route of uracil incorporation into higher derivatives. Glucose acts to provide ribose-1-phosphate, which, through the mediation of this enzyme, will convert uracil to uridine, an effective precursor of nucleotides and nucleic acids.

A slight stimulation of uridine-2-C¹⁴ incorporation into the acid-soluble nucleotides in the presence of glucose is also obtained. Since the catabolism of uridine to uracil is an active pathway in these and other tissues (67), the functioning of glucose as a source of the ribose moiety of uridine would reverse this catabolic step. This would result in the reconversion of any uracil formed from uridine breakdown back to uridine.

However, in the presence of glucose, the incorporation of radioactivity from labelled uridine into DNA is inhibited. Presumably the uridine moiety is changed to thymidine or to another DNA component which, like thymidine, is inhibited by glucose.

Whatever stimulatory effect glucose has on uracil incorporation alone, is not observable in the presence of 1 mM deoxyguanosine, which enhances uracil utilization in its own right, and to a much greater degree than does glucose.

When the stimulations of uracil incorporation into DNA by deoxyguanosine (Table 1) and by glucose (Table 3) are compared, it can be seen that the presence of deoxyguanosine results in a 30-fold increase compared to a 2- to 4-fold stimulation in the presence of glucose.

It is evident from the data presented in Table 3 that, except in the case of uracil-2-C¹⁴, glucose exerts either an inhibitory effect, or none

TABLE 4

Effect of Varying the Concentration of Deoxyguanosine on the Incorporation of Thymine-2-C¹⁴ Into the DNA of Ehrlich Ascites Tumour Cells

Deoxyguanosine Concentration mM	DNA counts/minute
Nil	65
0.05	2006
0.10	6849
0.50	19425
1.00	25550
5.00	8438

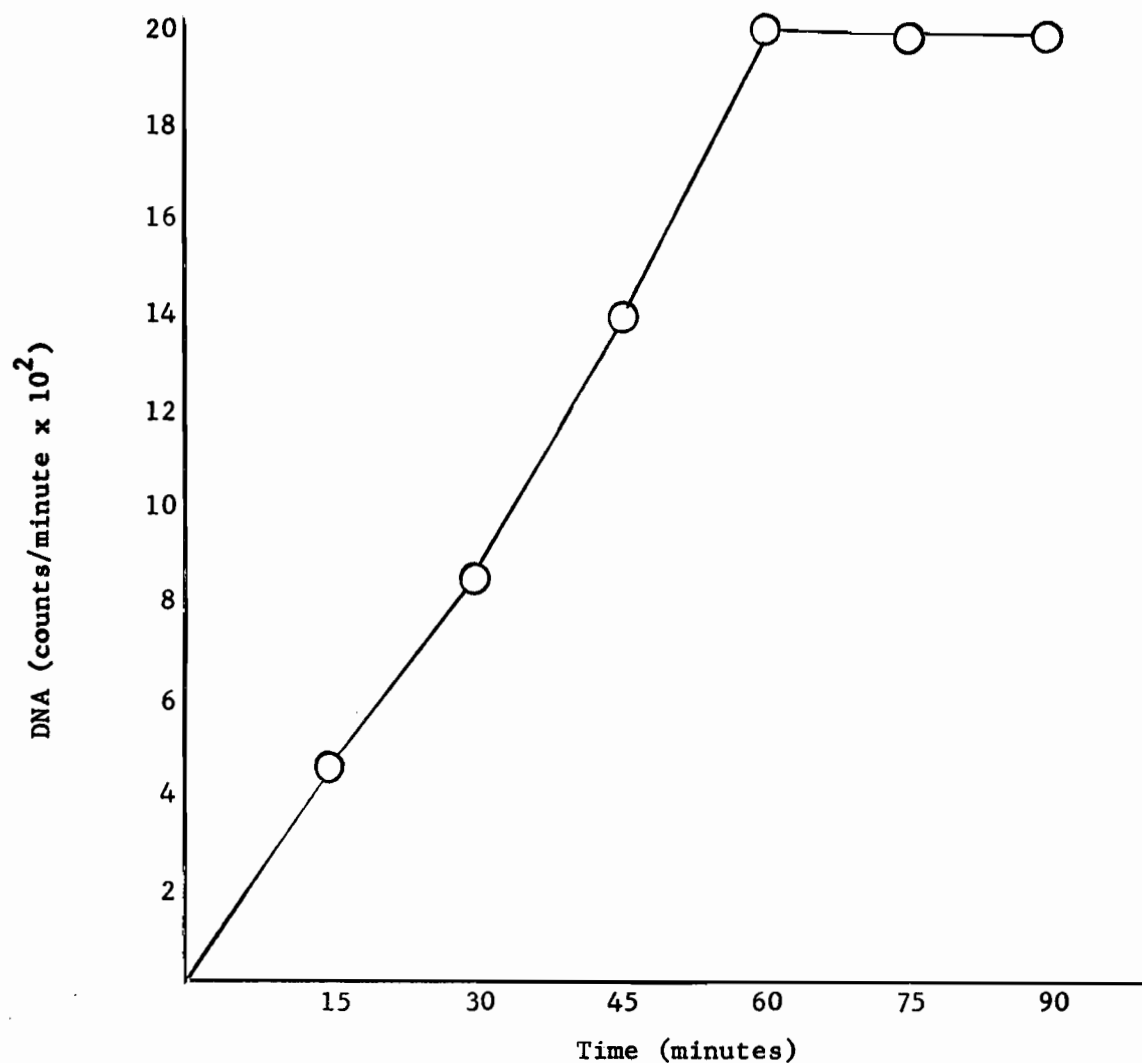
Input: Thymine-2-C¹⁴: 500,000 counts/minute/vessel, 1.2×10^{-2} mM

Results expressed as counts/minute/50 mg wet weight tissue.

Cells incubated as described in Table 1 with 10 mMolar glucose omitted.

Figure 1

Effect of Duration of Incubation on the Incorporation of Thymine-2-C¹⁴
into DNA in the Presence of Deoxyguanosine



Thymine-2-C¹⁴: 75,000 counts/minute/vessel/0.3 x 10⁻² mM.

Deoxyguanosine: 1 mM.

Results expressed as counts/minute/50 mg wet weight tissue.

Cells incubated as described in Table I, with 10 mM ~~glucose~~ glucose omitted.

none at all, on the incorporation of these precursors into DNA. In subsequent experiments involving these precursors, glucose has been omitted.

3.4 Effects of Time of Incubation, and Concentration of Deoxyguanosine, on Incorporation of Thymine-2-C¹⁴ into DNA

Table 4 and Figure 1 are the results of concentration and time studies respectively, of the deoxyguanosine-stimulated incorporation of thymine-2-C¹⁴ into DNA. As can be seen from Table 4, the optimum concentration of deoxyguanosine for this stimulatory effect is 1 mM. Above this concentration the deoxynucleoside begins to exert an inhibitory effect. Even at concentrations of deoxyguanosine as low as 0.05 mM (1/20 the optimum concentration), the incorporation into DNA is many times greater than that obtained with labelled thymine alone.

In the time study (Figure 1), it is evident that by one hour after the start of the incubation, the rate of thymine-2-C¹⁴ incorporation in the presence of 1.0 mM deoxyguanosine has become constant. Accordingly all subsequent studies of the deoxyguanosine effect have been carried out at 1 mM concentration of the deoxynucleoside, and for one hour duration.

3.5 Effect of Deoxyguanosine on the Incorporation of Uridine-2-C¹⁴ and Thymidine-2-C¹⁴ into the Acid-Soluble Nucleotides and DNA of Ehrlich Ascites Tumour Cells

In view of the extensive incorporation of radioactivity from uracil-2-C¹⁴ and thymine-2-C¹⁴ into DNA in the presence of deoxyguanosine, studies of the effect of this deoxynucleoside on the utilization of labelled uridine and thymidine for DNA biosynthesis have been carried out.

TABLE 5

Effect of 1 mM Deoxyguanosine on Incorporation of Thymidine-2-C¹⁴ and Uridine-2-C¹⁴ into Acid-Soluble Nucleotides and DNA of Ehrlich Ascites

Tumour Cells

P r e c u r s o r	Acid Soluble Nucleotides counts/minute	DNA counts/minute
Thymidine-2-C ¹⁴	3,475	41,882
Thymidine-2-C ¹⁴ plus Deoxyguanosine	10,575	24,704
Uridine-2-C ¹⁴	19,375	2,433
Uridine-2-C ¹⁴ plus Deoxyguanosine	27,750	1,506

Thymidine-2-C¹⁴: 41,882 counts/minute represent 1.26 μ atoms carbon¹⁴
incorporated into DNA

Input: 220,000 counts/minute/vessel, 1.5×10^{-2} mM

Uridine-2-C¹⁴: 2,433 counts/minute represent 0.31 μ atoms carbon¹⁴
incorporated into DNA

Input: 500,000 counts/minute/vessel 3×10^{-2} mM

Results expressed as counts/minute/50 mg wet weight tissue.

Concentration of Deoxyguanosine: 1 mM.

Cells incubated as described in Table 1, 10 mMolar glucose omitted.

The effects of 1 mM deoxyguanosine on the incorporation of radioactivity from uridine-2-C¹⁴ and thymidine-2-C¹⁴ into the acid-soluble and DNA fractions of Ehrlich ascites tumour cells are shown in Table 5. In each case, a definite inhibition of incorporation of radioactivity into the DNA is obtained.

There is also increase in the incorporation of radioactivity into the acid-soluble portion. This would seem to indicate a blockage of incorporation of the precursors into DNA, resulting in an accumulation of acid-soluble nucleotides. The inhibition of DNA synthesis in cultures of Ehrlich ascites tumour cells by deoxyguanosine, at concentrations of 1 mM, has been previously reported (187).

3.6 Distribution of Radioactivity in Thymine Derivatives in the Acid-Soluble Pool after Incubation of Ehrlich Ascites Tumour Cells in the Presence and Absence of Deoxyguanosine

From the preceding results it has become increasingly clear that the mechanism of stimulation of thymine-2-C¹⁴ incorporation into DNA is based on the direct transfer of the deoxyribose group from deoxyguanosine to thymine. This results in the formation of thymidine which, as mentioned previously, is an excellent precursor of DNA. The fact that (1) thymine incorporation into DNA in the presence of deoxyguanosine becomes quantitatively comparable to the incorporation of thymidine, and also that (2) this deoxynucleoside has no such stimulatory effect on thymidine itself, would support such a supposition. Table 6 gives further indication that this is the probable mode of action of deoxyguanosine. Using the ethyl acetate: formic acid: water solvent system described in Chapter 2.9, the acid-soluble nucleotides and nucleosides of the cells incubated with labelled thymine in the presence and absence of deoxy-

TABLE 6

Effect of Deoxyguanosine on Incorporation of Thymine-2-C¹⁴ into Acid-Soluble Nucleotides and DNA of Intact Ehrlic Ascites Tumour Cells

A d d i t i o n s	Thymidine	Thymidine Nucleotides	D N A
	c o u n t s	p e r m i n u t e	
Nil	120(⁺ ₋ 30)	95	119
Deoxyguanosine	315(⁺ ₋ 48)	1540	11,347

Input: thymine-2-C¹⁴ : 400,000 counts/minute/vessel, 2.9×10^{-2} mM

Concentration of deoxyguanosine: 1 mM

Results expressed as: counts/minute/100 mg wet weight tissue.

Cells incubated as described in Table 1, 10 mMoles glucose omitted.

have been separated.
guanosine_A This increase becomes much more marked in the thymidine nucleotides. If deoxyguanosine increases the formation of thymidine from thymine, the active thymidine kinase system present in the Ehrlich ascites cells (91) can convert whatever thymidine is produced to TMP and eventually to TTP. The availability of thymidine would then be the limiting step.

Thus the results of Table 6 lend further weight to the supposition that deoxyguanosine functions as a deoxyribose donor, converging thymine to thymidine. As discussed in Chapter 1, several instances of such deoxyribose transfer have been reported in mammalian tissues (61, 63) and in bacteria (64).

3.7 The Effect of Purines, Purine Nucleosides, and Purine Deoxynucleosides on Thymine-2-C¹⁴ Incorporation into Ehrlich Ascites Tumour Cells

The effects of a number of purines, their ribosides and deoxyribosides on the utilization of thymine for acid-soluble nucleotide and DNA synthesis have been studied and are shown in Table 7. Deoxyinosine very greatly enhances the incorporation of thymine into DNA. Both deoxyguanosine and deoxyinosine also cause some increase in acid-soluble radioactivity. Deoxyadenosine brings about no such increase in either fraction. As has been mentioned previously, this deoxynucleoside is a potent inhibitor of DNA biosynthesis.

Guanosine, inosine and adenosine have no stimulatory effect of thymine incorporation into DNA; conversely some inhibition of incorporation is indicated in their presence.

3.8 The Distribution of Radioactivity in the DNA Fraction Following the Incorporation of Uracil-2-C¹⁴ into Ehrlich Ascites Tumour Cells in the Presence and Absence of Deoxyguanosine

TABLE 7

Effects of Purines, Purine Nucleosides and Purine Deoxynucleosides on Incorporation of Thymine-2-C¹⁴ Into the Acid-Soluble Nucleotides and DNA of Ehrlich Ascites Tumour Cells

A d d i t i o n	Acid-Soluble Nucleotides counts/minute	DNA counts/minute
None	7,800	168
Guanine	8,338	125
Guanosine	10,612	55
Deoxyguanosine	11,538	14,181
Hypoxanthine	7,712	139
Inosine	6,375	53
Deoxyinosine	9,425	13,589
Adenine	6,238	120
Adenosine	8,175	97
Deoxyadenosine	8,263	131

Input: Thymine-2-C¹⁴ : 400,000 counts/minute/vessel, 2.9×10^{-2} mM

Concentration of each added compound: 1 mM

Cells incubated as described in Table 1, 10 mMolar glucose omitted

Results expressed as: counts/minute/50 mg wet weight tissue.

TABLE 8

Incorporation of Radioactivity from Uracil-2-C¹⁴ Into the Component Bases of
DNA of Ehrlich Ascites Tumour Cells

Additions	Adenine	Guanine	Cytosine	Thymine
None	40	15	100	130
Deoxyguanosine	0	50	250	1260

Input: Uracil-2-C¹⁴ : 800,000 counts/minute/vessel, 4.5×10^{-2} M

Deoxyguanosine: 1 mM

Results expressed as: counts/minute/50 mg wet weight tissue.

Cells incubated as described in Table 1.

In view of the extensive incorporation of radioactivity from uracil-2-C¹⁴ into the DNA of these tumour cells, a study of the distribution of label in DNA has been undertaken. The DNA has been hydrolysed and chromatographed according to the procedure described in Chapter 2.8.

In the absence of deoxyguanosine, radioactivity from uracil-2-C¹⁴ is distributed mainly between the cytosine and thymine components of DNA. It has been reported that UMP can act as a source of CMP in rat liver (178) and in rat hepatoma (179). By the action of cytidine nucleotide reductase, an enzyme present in most tissues, CMP can be converted to dCMP which is a source of DNA cytosine.

Uracil can also serve as a source of DNA thymine by the following pathway: the uracil is phosphorylated to uridine diphosphate which can be reduced to deoxyuridine diphosphate (36), and then dephosphorylated to dUMP. This compound then be methylated to form TMP, the reaction being catalysed by thymidine synthetase (80).

As shown in Table 8, deoxyguanosine stimulates the incorporation of radioactivity from uracil-2-C¹⁴ into the thymine component of DNA only. It can therefore be assumed that the deoxyguanosine can transfer its deoxyribose group to uracil, as it can to thymine. The resulting deoxyuridine can be converted to dUMP, a reaction mediated by deoxyuridine kinase (69). This would explain the extensive increase in radioactivity in the thymine moiety of DNA subsequent to incubation of uracil-2-C¹⁴ in the presence of deoxyguanosine.

3.9 The Effects of Purine, Purine Nucleosides and Purine Deoxynucleosides on the Incorporation of Radioactivity from Uracil-2-C¹⁴ into the Acid-Soluble Nucleotides and DNA of Ehrlich Ascites Tumour Cells

TABLE 9

Effects of Purines, Purine Nucleosides and Purine Deoxynucleosides on Incorporation of Uracil-2-C¹⁴ Into the Acid-Soluble Nucleotides and DNA of Ehrlich Ascites Tumour Cells

A d d i t i o n	Acid-Soluble Nucleotides counts/minute	DNA counts/minute
None	9,600	74
Guanine	11,825	65
Guanosine	53,150	787
Deoxyguanosine	22,245	971
Hypoxanthine	10,425	56
Inosine	64,906	3,299
Deoxyinosine	15,350	3,061
Adenine	10,825	63
Adenosine	33,425	1,276
Deoxyadenosine	16,200	97

Input: Uracil-2-C¹⁴ : 650,000 counts/vessel, 3×10^{-2} mM.

Concentration of each compound added: 1 mM.

Cells incubated as described in Table 1, 10 mMolar glucose omitted.

Results expressed as counts/minute/50 mg wet weight.

As with thymine-2-C¹⁴, effects of the purine bases, nucleosides and deoxynucleoside on the incorporation of uracil-2-C¹⁴ into DNA have also been studied. The nucleosides bring about extensive stimulation of uracil into the acid-soluble fraction. Gotto et al (53) have reported the presence of active purine nucleoside phosphorylases in Ehrlich ascites tumour cells. These catalyse the following reactions:

- 1) $\text{guanosine} \xrightarrow{P_i} \text{guanine} + \text{ribose-1-phosphate}$
- 2) $\text{adenosine} \xrightarrow{NH_3} \text{inosine} \xrightarrow{P_i} \text{hypoxanthine} + \text{ribose-1-phosphate}$
- 3) $\text{xanthosine} \xrightarrow{P_i} \text{xanthine} + \text{ribose-1-phosphate}$

The ribose-1-phosphate can combine with uracil-2-C¹⁴ by the action of a phosphorylase. The resulting labelled uridine can be phosphorylated to UMP (68) in a reaction catalysed by uridine kinase. UMP can serve indirectly as a source of dUMP as shown previously (Chapter 3.8).

Both the nucleosides and deoxynucleosides stimulate the incorporation of radioactivity from uracil-2-C¹⁴ into DNA. Only deoxyadenosine has no such effect. In this case the fact that there is an increase in acid-soluble radioactivity in the presence of deoxyadenosine would indicate that there is a blockage of DNA synthesis by this deoxynucleoside.

It would be expected that the incorporation of radioactivity from labelled uracil into DNA in the presence of deoxynucleosides would be considerably greater than that obtained in the presence of the nucleosides. The route of uracil incorporation into DNA involves far less transformations in the presence of deoxynucleosides where the conversion of uracil to TMP would involve 3 main steps: uracil → deoxyuridine → dUMP → TMP. In the

TABLE 10

Incorporation of Radioactivity from Thymine-2-C¹⁴, Thymine-2-C¹⁴ plus 1.0 mM Deoxyguanosine, Thymidine-2-C¹⁴, Into the DNA of Various Tissues

Precursor	DNA counts/min./50 mg tissue (wet weight)			
	Rat Small Intestine	Rat Thymus	S-37-tumour	Ehrlich ascites tumour
Thymine-2-C ¹⁴	160	162	160	136
Thymine-2-C ¹⁴ plus 1.0 mM dG	294	56	7767	3854
Thymidine-2-C ¹⁴	200	6760	10420	17520

Inputs: Thymine-2-C¹⁴ : 400,000 counts/vessel, 1.2×10^{-2} mM
 Thymidine-2-C¹⁴ : 220,000 counts/vessel, 1.5×10^{-2} mM

For the S-37 ascites tumour and Ehrlich ascites tumour, the cells were incubated as described in Table 1. 10 mMolar glucose omitted.

Rat small intestine was prepared by everting a loop of rat small intestine and slicing rings of tissue weighing 50-100 mgs. The rings were incubated in identical conditions as the tumour cells.

Rat thymus was obtained by removing the thymus from a young rat, mincing it finely and passing it over several layers of gauze into a beaker containing ice-cold isotonic saline. This resulted in a suspension of minced thymus similar in consistency to ascites cells suspensions, and was treated in the identical manner (Table 1).

presence of purine nucleosides, uracil incorporation into DNA would necessitate twice the number of reactions: uracil $\rightarrow$ uridine $\rightarrow$ UMP $\rightarrow$ UDP $\rightarrow$ dUDP $\rightarrow$ dUMP $\rightarrow$ TMP. It would be assumed that the incorporation of uracil via the latter pathway would be far less efficient.

In fact, however, there is almost equal incorporation of uracil into DNA in the presence of nucleosides and deoxynucleosides. This becomes more plausible by the fact that there is a much greater incorporation of this pyrimidine into the acid-soluble nucleotides when incubated in the presence of nucleosides, than in the presence of deoxynucleosides. Possibly this higher incorporation results in a sufficient increase in labelled UMP from uracil-2-C¹⁴ to cancel out the loss of efficiency of the less direct pathway.

The fact that the purine bases exert no influence whatsoever on uracil incorporation further strengthens the supposition that the ribose and deoxyribose moieties are transferred to uracil and thymine.

3.10 Studies of Labelled Thymine and Thymidine Incorporation into the DNA of Various Tissues

A limited study of the incorporation of thymine-2-C¹⁴, thymine-2-C¹⁴ plus deoxyguanosine, and thymidine-2-C¹⁴ into the DNA fraction of various tissues has been carried out. Two tumour cell systems, sarcoma-37 and the Ehrlich ascites carcinoma have been investigated along these lines. Both show the deoxyguanosine-stimulated incorporation of thymine into DNA. Rat thymus is able to incorporate thymidine-2-C¹⁴ to a considerable extent, but shows no stimulation of thymine incorporation by deoxyguanosine. Tissue rings prepared from everted rat small intestine do not incorporate either thymine in the presence of deoxyguanosine, or thymidine to any great extent.

However, there is some indication from these results that the deoxyguanosine transference of deoxyribose to thymine might have some activity in rat small intestine.

Summary of Chapter 3

(A) Studies on Thymine-2-C¹⁴ and Thymidine-2-C¹⁴ Metabolism in Ehrlich Tumour Cells

1. In studying the incorporation of preformed purines and pyrimidines into the nucleic acids of Ehrlich ascites tumour cells, it has been found that thymine-2-C¹⁴ is not utilized to any appreciable extent. This has been found to be true for most tissues (63).

2. However, deoxyguanosine and deoxyinosine, at concentrations of 1 mM, greatly stimulate the incorporation of thymine-2-C¹⁴ into DNA. Deoxyuridine also enhances the incorporation of labelled thymine into DNA, but to a lesser extent. The other deoxynucleosides have no effect on thymine utilization in intact Ehrlich ascites tumour cells.

3. Guanosine and inosine (at 1 mM concentrations) do not stimulate incorporation of thymine-2-C¹⁴ into DNA, nor do guanine and hypoxanthine.

4. In the presence of deoxyguanosine, the extent of thymine-2-C¹⁴ incorporation into DNA is approximately half that obtained with thymidine-2-C¹⁴, which is considered an excellent precursor of DNA.

5. Deoxyguanosine inhibits the incorporation of thymidine-2-C¹⁴ into DNA. However, this purine deoxynucleoside increases the incorporation of labelled thymidine into the acid-soluble portion. This would indicate some blockage of DNA synthesis which results in accumulation of precursor deoxynucleotides.

6. Chromatography of the acid-soluble fraction, after incubation of thymine-2-C¹⁴ with (1 mM) deoxyguanosine, reveals an increase in radioactivity in thymidine over control values. This increase becomes more pronounced in thymidine nucleotides.

7. It is suggested that the deoxyguanosine and deoxyinosine function as donors of deoxyribose, converting thymine to thymidine, which is an extremely good DNA precursor. Thus mechanisms in Ehrlich ascites tumour cells do exist for the utilization of thymine. This represents a new "salvage" pathway for DNA synthesis in the Ehrlich ascites carcinoma.

(B) Studies on Uracil-2-C¹⁴ and Uracil Derivatives in Ehrlich Ascites Tumour Cells

1. Many of the results obtained from studies on the incorporation of thymine-2-C¹⁴ have been found to be similar to those observed with uracil-2-C¹⁴. Uracil alone is very poorly utilized. The addition of deoxyguanosine or deoxyinosine increases greatly the incorporation of radioactivity from uracil-2-C¹⁴ into DNA.

2. However, unlike experiments with thymine-2-C¹⁴, guanosine and inosine are also extremely effective in enhancing the incorporation of label from uracil-2-C¹⁴ into DNA. These purine nucleosides also bring about extensive increases in the incorporation of labelled uracil into the acid-soluble nucleotides.

3. Adenosine is also very effective in stimulating uracil-2-C¹⁴ incorporation into acid-soluble nucleotides, and DNA. Deoxyadenosine has no effect on the utilization of radioactivity from labelled uracil for DNA synthesis. This is presumably due to the inhibition of DNA synthesis caused by deoxyadenosine (168).

4. Guanine and hypoxanthine have no effect on the utilization of uracil. This is similar to results obtained with thymine.

5. Glucose (10 mMolar) increases the incorporation of radioactivity from labelled uracil into DNA, but inhibits slightly that of uridine-2-C¹⁴. The stimulation of uracil incorporation is presumably due to the function of glucose as a source of ribose-1-phosphate, which can combine with uracil to form uridine, a more efficient precursor of nucleic acids.

6. As with labelled thymidine, the incorporation of radioactivity from uridine-2-C¹⁴ into DNA in the presence of 1 mM deoxyguanosine is inhibited. There is a concurrent increase in radioactivity in the acid-soluble fraction. This increase in the presence of this purine deoxynucleoside, is presumably due to accumulation of deoxynucleotides which have been blocked from incorporation into DNA.

7. After incubation of the ascites cells with uracil-2-C¹⁴ in the presence and absence of deoxyguanosine, the DNA has been hydrolysed and the liberated bases separated chromatographically. Such experiments have revealed that in the absence of deoxyguanosine, radioactivity from uracil-2-C¹⁴ is distributed equally between DNA-thymine and DNA-cytosine. When deoxyguanosine is present, the extent of incorporation of label from uracil-2-C¹⁴ into DNA is greatly increased, and the location of this radioactivity is entirely in the thymine moiety.

8. All these results can be related to the necessity of converting uracil to uridine or deoxyuridine prior to incorporation into nucleotides and nucleic acids. It is suggested that deoxyguanosine and deoxyinosine can donate their deoxyribose groups to uracil forming deoxyuridine, which can then be converted to deoxyuridine monophosphate (dUMP). This dUMP can be

converted to TMP (see Chapter 1.7) by the action of thymidylate synthetase. This would explain the increase in radioactivity in the thymine portion of DNA when uracil-2-C¹⁴ is incubated with deoxyguanosine.

The nucleosides of guanine, hypoxanthine and adenine are also capable of transferring their ribose group to uracil (53). The resulting uridine can also be converted to thymidine monophosphate by a less direct route and thus it can be incorporated into DNA. The stimulation of uracil incorporation by glucose is also (indirectly) due to conversion of uracil to uridine.

These studies on the increased utilization of uracil for nucleic acid synthesis indicate further alternate pathways for the utilization of preformed pyrimidines for nucleic acid biosynthesis in Ehrlich ascites tumour cells.

CHAPTER 4

ASPECTS OF METABOLISM OF DNA PRECURSORS IN CELL-FREE EXTRACTS OF EHRLICH ASCITES TUMOUR CELLS

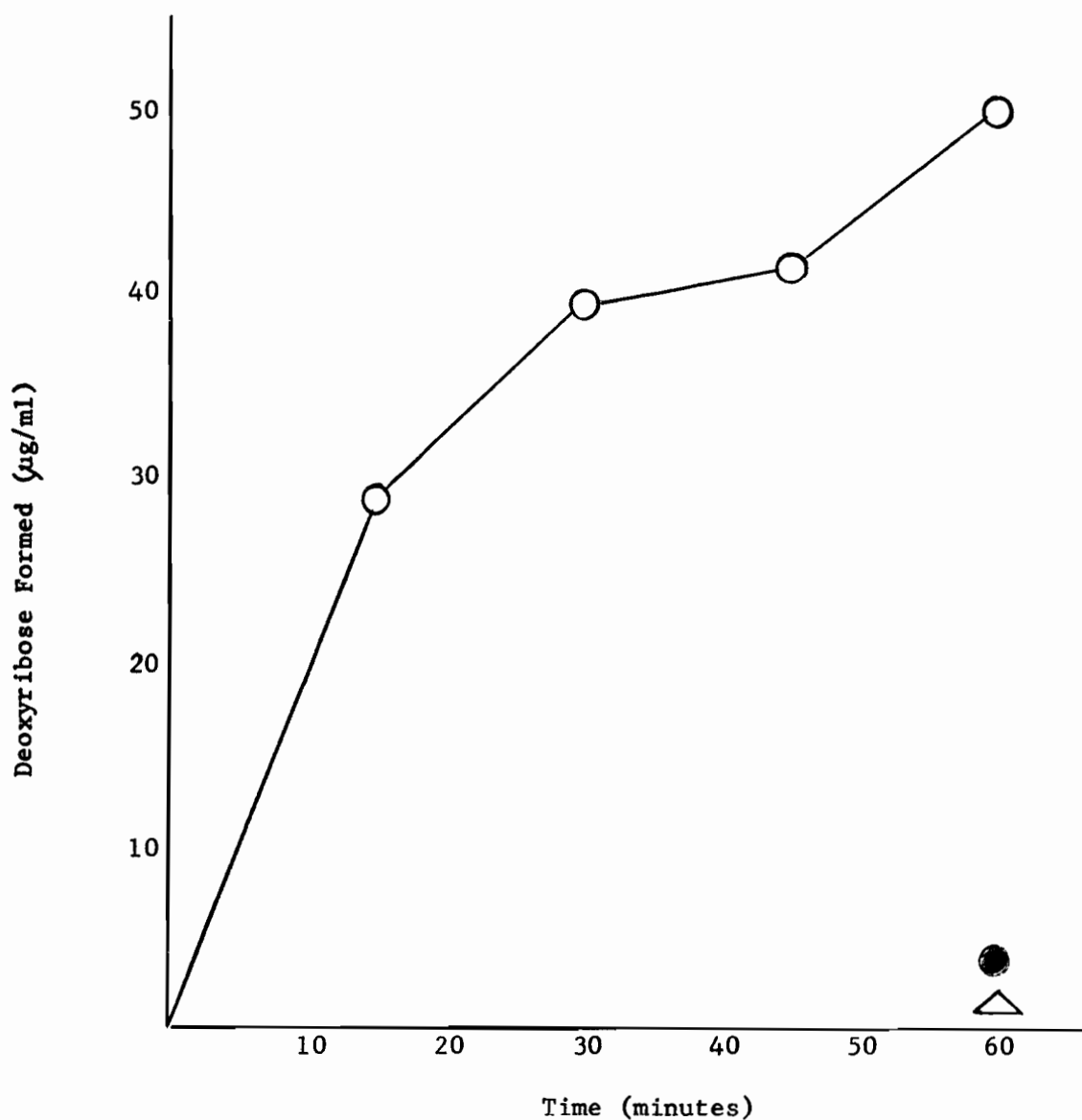
This chapter describes studies with cell-free extracts prepared from osmotically disrupted Ehrlich ascites tumour cells. These studies have been undertaken in order to observe the effects of certain phosphorylated compounds on these systems, and to further elucidate the mechanisms of the transformations observed in Chapter 3.

The occurrence of uridine phosphorylase has been previously described by Skold (21), in Ehrlich ascites tumour cells. The presence of purine nucleoside phosphorylase in this tissue has also been demonstrated (52). Zimmerman (61) has shown that thymidine phosphorylase may be the same enzyme as thymidine deoxyribose transferase. The interaction of the latter enzyme with uracil has been described (63). The results presented in this chapter indicate that one or several deoxynucleoside phosphorylases exist in Ehrlich ascites tumour cells, and that these bring about several inter-conversions between bases and deoxynucleosides. These studies indicate a high degree of activity of the "salvage pathways" discussed in Chapter 1, and support the existence of extensive routes for the utilization of pre-formed precursors in proliferating systems.

In these experiments, the cells have been disrupted and centrifuged as described in Chapter 2. All studies, with the exception of that described in Figure 2, have called for the separation of thymine, thymidine and thymidine nucleotides by paper chromatography.

Figure 2

Formation of Deoxyribose from Deoxyguanosine with Increasing Time Intervals
in an Extract of Ehrlich Ascites Tumour Cells.



- Extract prepared as described in Chapter 2, and incubated with deoxyguanosine (267 µg/ml), 0.02 M. phosphate buffer, pH 7.4 and water.

Deoxyribose estimated as described in Chapter 4.1.

△ - boiled extract

● - extract at 0°C.

○ - normally prepared extract at 37°C.

The solvent used (ethyl acetate/formic acid/water//60/35/5//V/V/V/) is very effective in carrying out these separations. Although it is one of the few systems which will effectively separate thymine from thymidine, phosphorylated thymine derivatives do not run in these solvents, and are left at the origin. Thus by necessity, the thymidine nucleotides (mono-, di-, tri) have been treated as one group, and compared as such with thymine, and thymidine. Studies on labelled thymine derivatives have the advantage that there is relatively small conversion of thymidine or thymidine nucleotides to any other DNA precursors (16). It has therefore been assumed that the radioactivity from thymine-2-C¹⁴ is solely due to thymine derivatives.

The sum of phosphorylated derivatives of thymidine is considered to be an index of thymidine kinase activity, as the rate of their formation is limited by this enzyme.

4.1 The Cleavage of Deoxyguanosine with the Resulting Formation of Deoxyribose by Cell-Free Extracts of Ehrlich Ascites Tumour Cells

An extract of tumour cells has been prepared as described in Chapter 2.4. The method of determining the amount of deoxyribose formed from cleaved deoxyguanosine is as follows: the extract is incubated for various time intervals with the additions described in Figure 2, after which the reaction is terminated by immersing the vessel in a boiling water bath for 10 minutes. Arresting the reaction by boiling avoids the cleavage of any deoxyguanosine which would occur if trichloroacetic acid were used (180).

The boiled extract is centrifuged and the supernatant is shaken with 250 mg of "activated" Norit (181). The charcoal removes by absorption any cyclic compounds, such as intact deoxyguanosine, from the solution. This

procedure is repeated three times, after which the supernatant is tested for the presence of deoxyguanosine by reading an aliquot in a Beckman Spectrophotometer at 255 m μ , the λ_{max} of deoxyguanosine (182). The supernatant is then subjected to the diphenylamine color reaction which is specific for deoxyribose (183).

Figure 2 illustrates the course of the enzymatic cleavage of deoxyguanosine to deoxyribose over a period of one hour. This does not indicate whether the deoxyribose is in the form of deoxyribose-1-phosphate, which would be the reaction product if the deoxyguanosine were split by a phosphorylase.

Friedkin (184) has demonstrated the cleavage of deoxyguanosine to guanine and deoxyribose-1-phosphate, which he has subsequently isolated (185). A phosphate-dependent enzymatic splitting of deoxyguanosine in rat liver, as measured by the formation of deoxyribose, has also been reported (58).

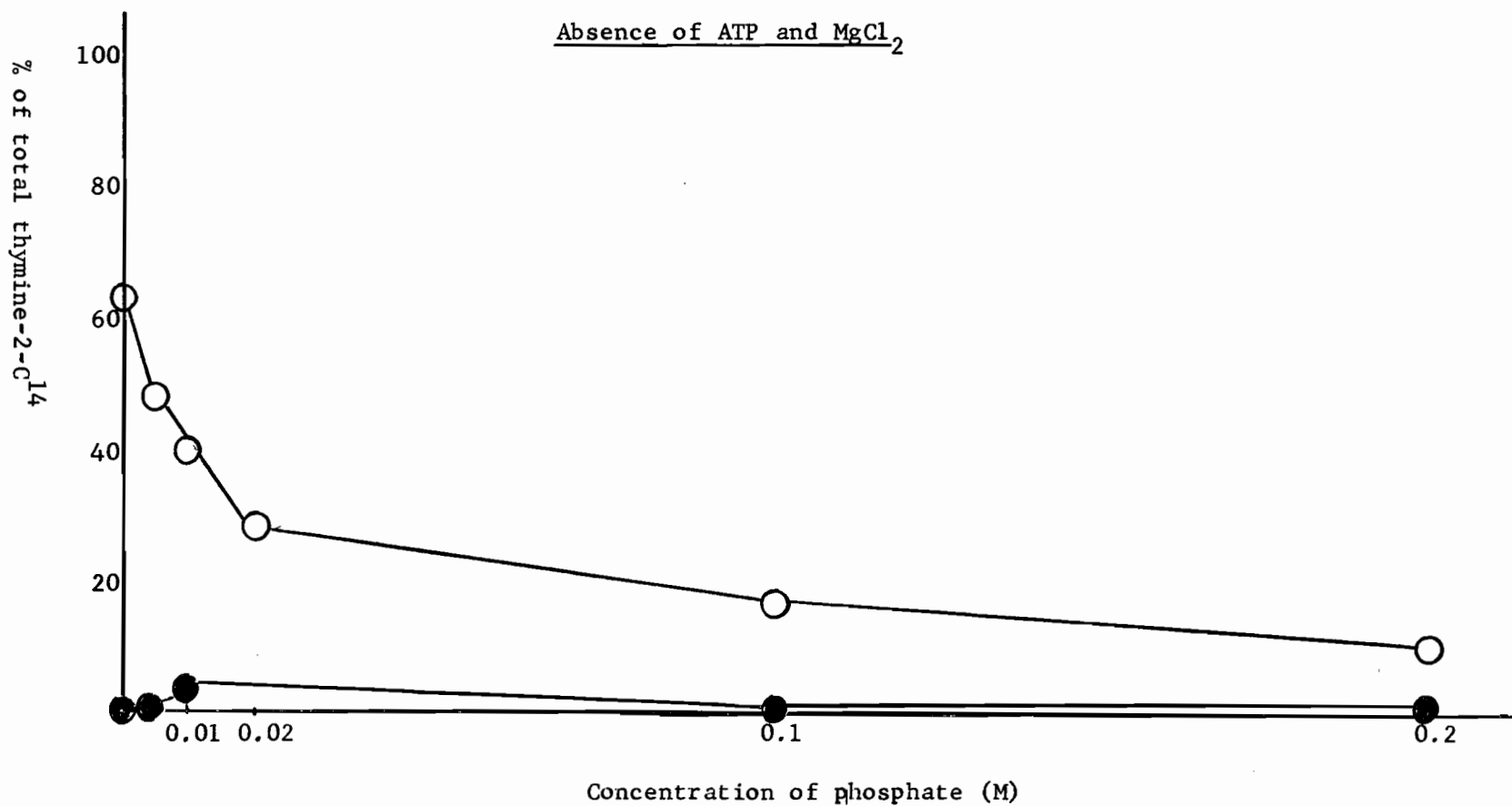
As can be seen in these results, the cleavage of this deoxynucleoside is fairly extensive. In the presence of 0.02 M phosphate buffer, 52 $\mu\text{g/ml}$ deoxyribose are formed after one hour. The maximum deoxyribose which could be split from deoxyguanosine is 116 $\mu\text{g/ml}$; thus approximately half the deoxynucleoside is cleaved in this time.

4.2 Effect of Concentration of Phosphate Ions on the Conversion of Thymine to Thymidine and to Thymidine Nucleotides in the Presence and Absence of ATP and MgCl_2

Figure 3 indicates a definite inhibitory effect of phosphate on the formation of thymidine from thymine, which increases in proportion to the concentration of phosphate ions. As these experiments have been carried

Figure 3

Effect of Phosphate Ion Concentration on the Deoxyguanosine-stimulated conversion of
Thymine-2-C¹⁴ to Thymidine and TMP in an Extract of Ehrlich Ascites Tumour Cells in the



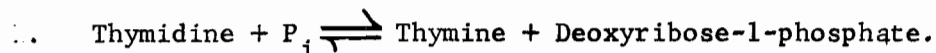
Thymine-2-C¹⁴: 150,000 counts/minute/vessel, 0.7×10^{-2} M.

Extract prepared as described in Table 11 with 30 mM tris-hydrochloride buffer, deoxyguanosine, 1 mM.

○ - thymidine

● - thymidine nucleotides

out in the absence of ATP and Mg^{++} ions, there is virtually no phosphorylation of the thymidine formed to thymidine monophosphate. These results indicate strongly the presence of thymidine phosphorylase in the Ehrlich ascites tumour cells. If so, any thymidine formed would be split to thymine and deoxyribose-1-phosphate:



The interaction of thymine with deoxyguanosine could be the result of the coupled action of two different phosphorylases which act together to transfer the deoxyribose moiety from deoxyguanosine to thymine. These two enzymes would be:

1. deoxyguanosine phosphorylase, which, in the presence of phosphate ions, splits deoxyribose-1-phosphate from deoxyguanosine. A high phosphate concentration would favor this reaction.

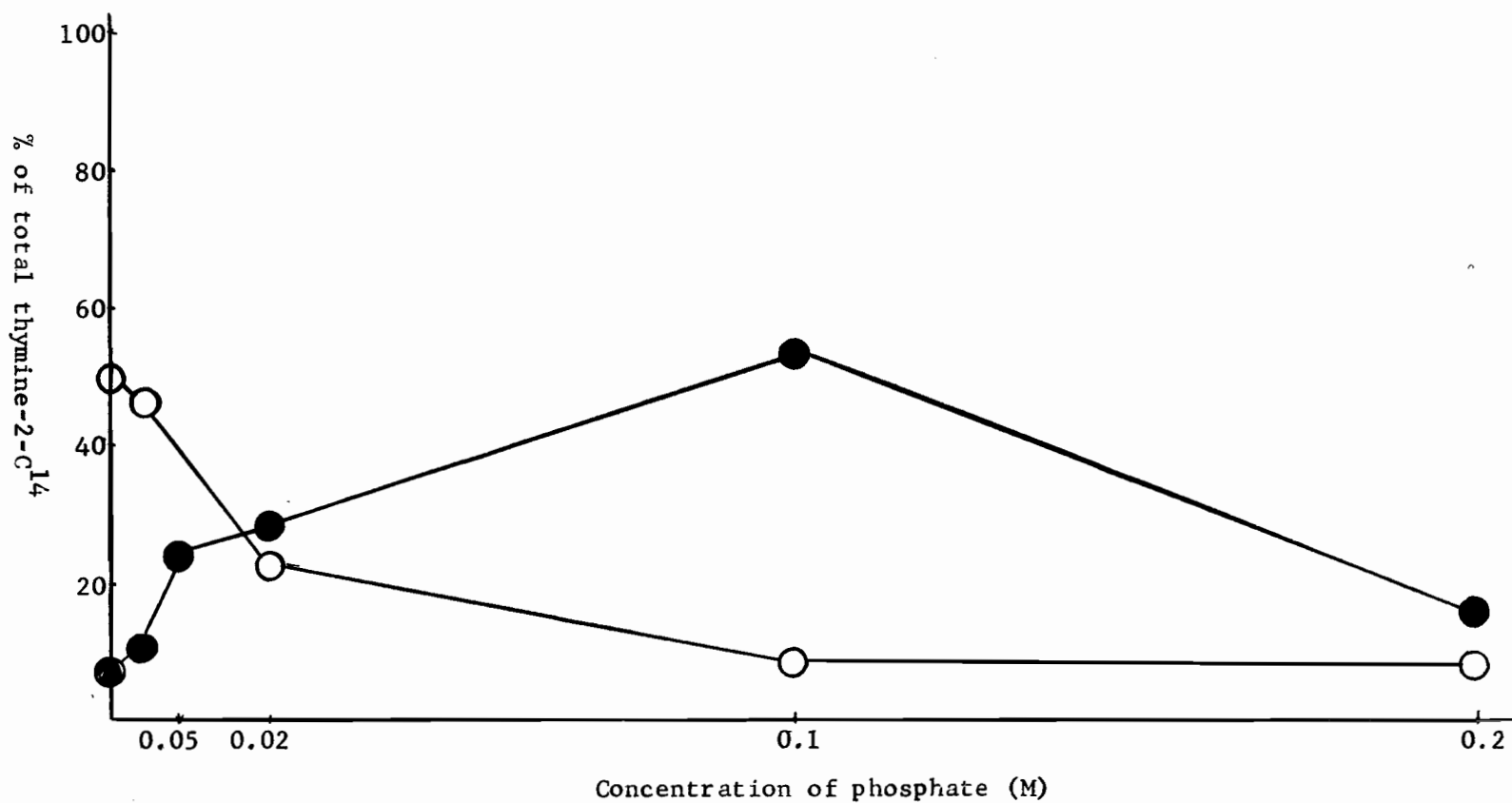
2. Thymidine phosphorylase, which catalyses the formation of thymidine from thymine and deoxyribose-1-phosphate. Excess phosphate would reverse this reaction. This would serve to explain the decreased formation of thymidine from thymine with increasing concentrations of phosphate.

There is nothing in these results to suggest that the deoxyribose transfer is the result of two different enzymes. However most studies on these two enzymes have demonstrated that they are fairly specific for their own substrates (56, 59).

The results shown in Figure 4, where the conditions are identical with those in Figure 3, except for the presence of ATP and $MgCl_2$, illustrate the formation of thymidine nucleotides from thymidine. Increased phosphate concentrations, although inhibiting the formation of thymidine from thymine,

Figure 4

Effect of Phosphate Ion Concentration on the Deoxyguanosine-stimulated Conversion of Thymine-2-C¹⁴ to Thymidine and TMP in an Extract of Ehrlich Ascites Tumour Cells in the Presence of ATP and MgCl₂



Thymine-2-C¹⁴: 150,000 counts/minute/vessel, 0.7×10^{-2} M.

Extract prepared as described in Table 11 with 30 mM tris-hydrochloride buffer, 1mM deoxyguanosine, 0.2mM ATP, 0.2mM MgCl₂.

○ - thymidine

● - thymidine nucleotides

markedly increase the synthesis of thymidine nucleotides in the presence of ATP. This might be explained by the fact that, as discussed previously, the presence of phosphate ions also stimulates the cleavage of deoxyguanosine to deoxyribose-1-phosphate, which can combine with thymine to form thymidine. The thymidine would also break down in the presence of phosphate ions except for the fact that when ATP and MgCl_2 are present, thymidine kinase is active, and converts whatever thymidine is formed to TMP; before it can break down. These results would seem to suggest that the low level of thymidine and correspondingly high level of thymidine nucleotides are interrelated. Thus if the kinase system is stimulated by phosphate ions, the rate of conversion of thymidine is increased, and the result is a smaller yield of deoxynucleoside and subsequently more deoxynucleotide. However, the results depicted in Figure 3 show that there is inhibition of thymine conversion to thymidine with increasing phosphate concentrations, in a situation where no TMP is being formed.

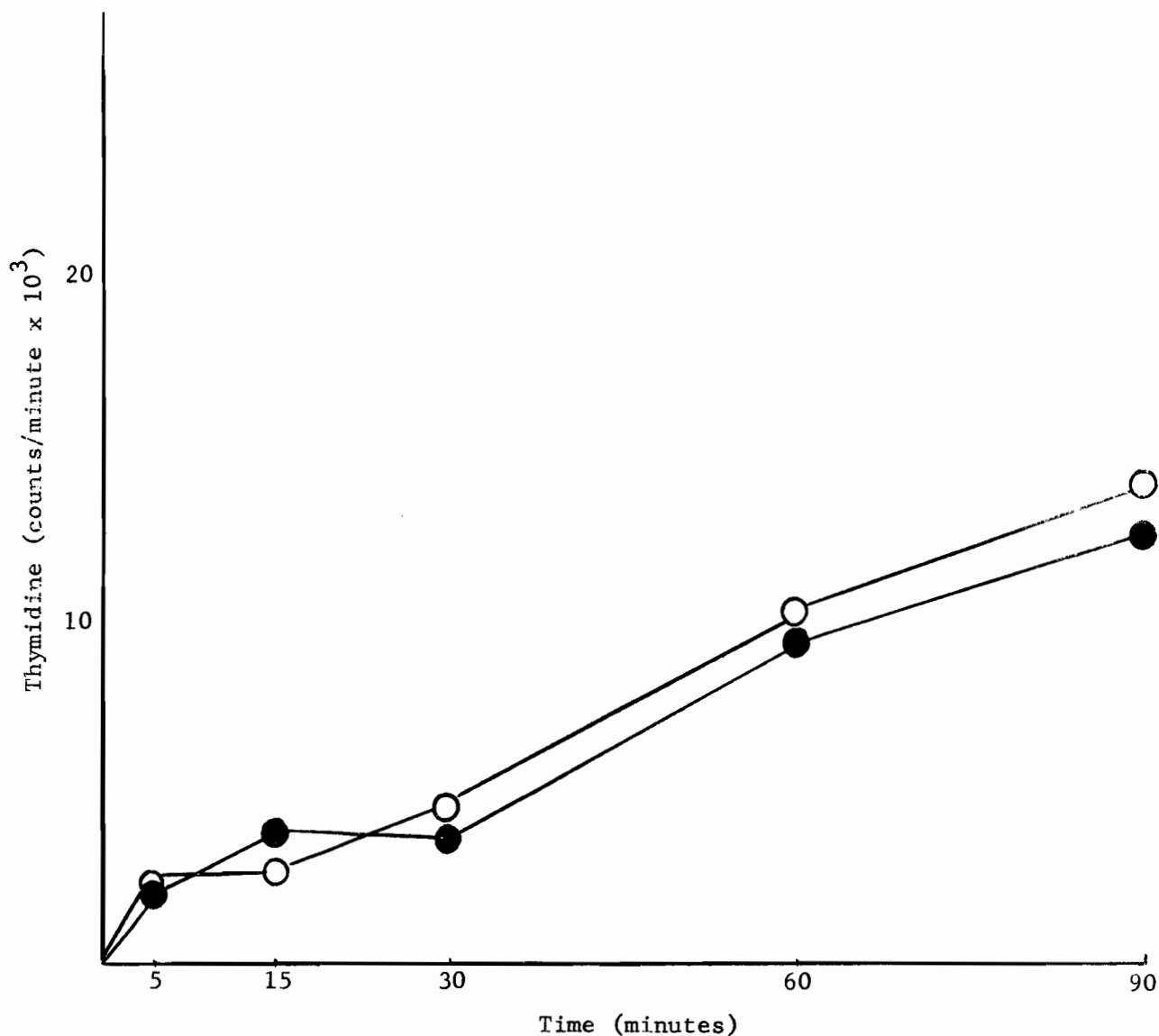
Subsequent studies in cell-free extracts have been carried out in the presence of 0.02 M phosphate buffer. The concentration of phosphate ions is less than optimal for maximum production of TMP, but at the same time is less inhibitory to the formation of thymidine from thymine.

4.3 Effect of Arsenate on the Formation of Thymidine from Thymine-2- C^{14}

These considerations have been further substantiated by experiments with arsenate. Time studies of thymidine formation from thymine have been carried out using arsenate in the presence or absence of ATP and MgCl_2 . In the presence of ATP and Mg^{++} , no inhibition with arsenate occurs (Figure 5). However, in their absence, a considerable inhibition of thymidine production

Figure 5

Effect of Sodium Arsenate on the Deoxyguanosine-stimulated Conversion of
Thymine-2-C¹⁴ to Labelled Thymidine in the Presence of ATP and MgCl₂ with
Increasing Time Intervals



Thymine-2-C¹⁴: 150,000 counts/minute/vessel/0.7 x 10⁻² M

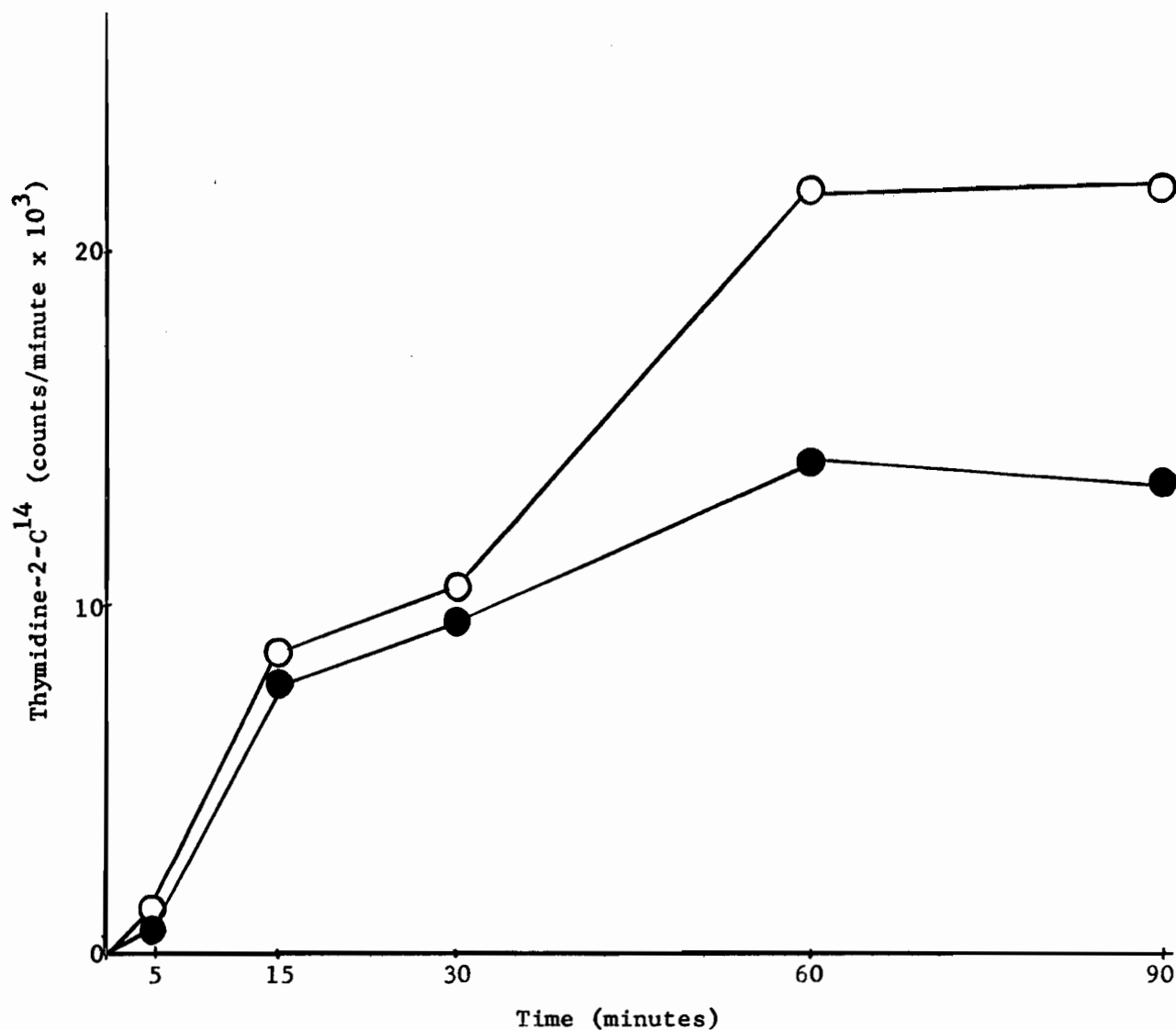
Extract prepared as in Table 11, with identical conditions to those described in Figure 4.

120 μ moles Na arsenate added 10 minutes after start of incubation.

- - control
- - arsenate

Figure 6

Effect of Sodium Arsenate on the Deoxyguanosine-stimulated Conversion of Thymine-2-C¹⁴ to Labelled Thymidine in the Absence of ATP and MgCl₂.



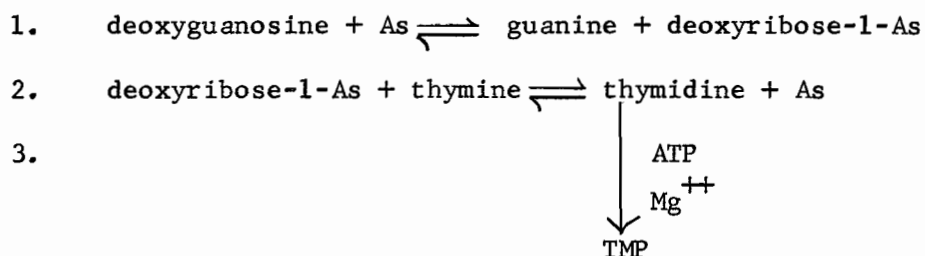
Thymine-2-C¹⁴: 150,000 counts/minute/vessel/ 0.7×10^{-2} M.

Extract prepared as described in Table 11 with identical conditions to those described in Figure 3.

Sodium arsenate (120 μ moles) added 10 minutes after start of incubation.

- - control
- - arsenate

develops (Figure 6). If the reactions studied are phosphorylytic in nature, the arsenate, which acts in a manner very similar to phosphate, would be involved in the following reaction:



Arsenate-bound compounds are in general much less stable than their phosphate-bound counterparts (186). It follows that the deoxyribose-1-arsenate intermediate would begin to break down with the result of progressively decreasing formation of thymidine. However, in the presence of ATP and Mg^{++} , any thymidine formed would be rapidly converted to TMP, which would lead to an increased rate of thymidine formation. The overall effect would be to shift reactions 1, 2 and 3 to the right.

In contrast to effects observed in the presence of phosphate, the addition of arsenate does not result in an increased rate of formation of TMP from thymidine. For a true evaluation of the effects of arsenate, and phosphate ions on these systems, detailed kinetic studies of all these reactions would be necessary. This, for the present, is outside of the scope of this research project.

4.4 The Effect of Various Deoxynucleosides on the Conversion of Thymine to Thymidine and to Thymidine Nucleotides

Several deoxy nucleosides, at concentrations of 1 mM, have been incubated with thymine-2- C^{14} in extracts of Ehrlich ascites tumour cells in the presence and absence of ATP and MgCl_2 . These experiments have been performed

in order to see which deoxynucleosides can donate a deoxyribose group to thymine.

From Table 11 it is evident that there is virtually no conversion of thymine-2-C¹⁴ to labelled thymidine or TMP without some source of deoxyribose. This corresponds to the lack of utilization of free thymine for DNA synthesis in intact cells.

Deoxyuridine is shown to increase considerably the formation of thymidine from thymine. In the presence of ATP and Mg⁺⁺ there is extensive conversion of the thymidine formed to thymidine nucleotides. This is in keeping with previous observations concerning the transfer of deoxyribose moiety of deoxyuridine to thymine (63, 167).

Thymidine itself will effectively exchange its deoxyribose group with thymine. However, there is apparently no thymidine nucleotide formation in the presence of ATP. This could be due to isotopic dilution. The active thymidine kinase systems present in Ehrlich ascites tumour cells would phosphorylate the unlabelled thymidine, and thus result in dilution of radioactivity from the labelled thymidine. Another possibility is that the unlabelled thymidine may successively form TMP and TTP so quickly that this end product would inhibit any further action of thymidine kinase by feedback mechanisms (69, 188). Thus the phosphorylation of labelled thymidine, which would by necessity occur later than the phosphorylation of unlabelled thymidine, would be inhibited.

Both deoxyadenosine and deoxycytidine exhibit some degree of transfer activity. The presence of deoxycytidine in this preparation results in the production of little thymidine, but on addition of ATP and MgCl₂, there is a large increase in the thymidine nucleotides. With deoxyadenosine,

TABLE 11

The Effects of Various Deoxynucleosides on the Conversion of Thymine-2-C¹⁴ to Labelled Thymidine and Thymidine Nucleotides in the Presence or Absence of ATP and MgCl₂ in an Extract of Ehrlich Ascites Tumour Cells

A d d i t i o n s	Per Cent of Total Thymine-2-C ¹⁴ Added to Vessel		
	Thymine	Thymidine	Thymidine Nucleotides
Nil	98.8	0.8	0.3
Nil, ATP, MgCl ₂	99.3	0.2	0.5
Deoxyuridine	67.3	31.7	0.9
Deoxyuridine, ATP, MgCl ₂	50.7	29.3	20.0
Thymidine	75.1	24.5	0.3
Thymidine, ATP, MgCl ₂	71.1	27.5	0.2
Deoxycytidine	96.3	2.7	0.9
Deoxycytidine, ATP, MgCl ₂	78.1	3.6	18.2
Deoxyadenosine	91.8	7.4	0.8
Deoxyadenosine, ATP, MgCl ₂	63.1	5.7	31.1
Deoxyguanosine	81.0	16.7	2.2
Deoxyguanosine, ATP, MgCl ₂	55.8	9.9	34.2
Deoxyinosine	80.7	16.7	2.4
Deoxynosine, ATP, MgCl ₂	54.8	11.6	33.4

Thymine-2-C¹⁴ : 150,000 counts/minute/vessel, 0.7×10^{-2} M, ATP 0.2 mM, MgCl₂ 0.2 mM, each deoxynucleoside 1.0 mM.

The cell-free extract was prepared from cells disrupted by homogenization and centrifuged at 18,000 x g at 0°C.

Each vessel contained 0.2 ml of 0.1 M phosphate buffer, 0.5 ml supernatant from centrifuged homogenate, 0.1 ml thymine-2-C¹⁴ and water plus additions indicated above to a final volume of 1.0 cc. The extracts were incubated at 37°C for one hour.

the phosphorylation of thymidine is even greater, and, in fact, approaches the levels obtained with deoxyguanosine and deoxyinosine.

Deoxyguanosine and deoxyinosine produce practically identical increases in radioactivity in the thymidine and thymidine nucleotide portions. The stimulation of labelled thymidine formation with deoxyguanosine or deoxyinosine is 40 to 50% less than that obtained in the presence of thymidine and deoxyuridine. The conversion to thymidylic acid, however, is considerably greater with these purine deoxynucleosides. The inhibition of phosphorylation of thymidine by deoxyuridine has been reported in regenerating rat liver (189). This is presumably the result of competition with thymidine for thymidine kinase. It is possible that in Ehrlich ascites tumour cells there may be a similar inhibition by deoxyuridine of the production of TMP from thymidine. This might explain why there is less TMP formed with deoxyuridine than with deoxyguanosine or deoxyinosine.

The results of Table 11 indicate that these transformations are quite complex, and require further study in order to discover why there is greater formation of TMP in some cases, and greater production of thymidine in others, when ATP and MgCl_2 are present.

4.5 The Effect of Various Possible Sources of Deoxyribose on the Conversion of Thymine-2- C^{14} to Thymidine and Thymidine Nucleotides

The effects of (1) deoxyguanosine, (2) deoxyguanosine-5-monophosphate, (3) deoxyribose, and (4) deoxyribose-1-phosphate have been compared, in a study of the kinetics of thymine conversion to thymidine (Table 12). These experiments have been carried out in the presence and absence of ATP and MgCl_2 . Deoxyguanosine and deoxyguanosine-monophosphate are both effective. Most probably this is the result of the fairly active nucleotide phosphatase sys-

TABLE 12

The Effect of Various Possible Sources of Deoxyribose on the Conversion of Thymine-2-C¹⁴ to Labelled Thymidine and Thymidine Nucleotides in the Presence or Absence of ATP and MgCl₂ in an Extract of Ehrlich Ascites Tumour Cells

A d d i t i o n s	Per Cent of Total Thymine-2-C ¹⁴ Added to Vessel		
	Thymine	Thymidine	Thymidine Nucleotides
Nil	98.3	1.1	0.6
Nil, ATP, MgCl ₂	98.1	1.0	0.8
Deoxyguanosine	82.0	16.9	1.1
Deoxyguanosine plus ATP, MgCl ₂	52.1	15.0	32.8
dGMP	93.9	5.3	0.7
dGMP plus ATP, MgCl ₂	82.4	3.8	13.8
Deoxyribose	98.1	1.7	0.2
Deoxyribose plus ATP, MgCl ₂	98.9	0.6	0.4
Deoxyribose-1-phosphate	67.0	32.3	0.1
Deoxyribose-1-phosphate plus ATP, MgCl ₂	51.3	23.3	25.2

Thymine-2-C¹⁴ : 150,000 counts/minute/vessel, 0.7×10^{-2} mM.

ATP 0.2 mM.

MgCl₂ 0.2 mM.

each deoxyribose source: 1.0 mM.

Extracts prepared and incubated as described in Table 11.

tems present in Ehrlich ascites tumour cells (88), which would dephosphorylate dGMP to form deoxyguanosine. Conversely, deoxyribose has practically no effect at all. This result is similar to findings of experiments carried out with intact cells (not tabulated) where there is no stimulation of incorporation of thymine-2-C¹⁴ in the presence of deoxyribose.

Deoxyribose-1-phosphate is extremely effective in stimulating the conversion of thymine to thymidine. This demonstrates the existence of thymidine phosphorylase in this tissue. The presence of this enzyme in Ehrlich ascites tumour cells has been reported previously by Zimmerman (61) who claims, however, the absence of any 'transferase' activity in this tissue.

4.6 Effects of Various Possible Sources of Deoxyribose on the Conversion of Uracil-2-C¹⁴ to Deoxyuridine and to Related Deoxyuridine Nucleotide Derivatives

Very similar results have been obtained with (1) deoxyguanosine, (2) deoxyguanosine-5-monophosphate, (3) deoxyribose, (4) deoxyribose-1-phosphate on the conversion of labelled uracil to deoxyuridine, dUMP and related deoxynucleotide derivatives, in the presence and absence of ATP and MgCl₂ (Table 13).

Again deoxyguanosine is very effective in bringing this conversion about, while deoxyguanosine-monophosphate stimulates incorporation to a much lesser degree. Deoxyribose has no effect at all, but deoxyribose-1-phosphate is very active in stimulating the conversion of uracil to its higher derivatives.

The deoxyguanosine stimulation of labelled thymidine and deoxyuridine formation, with and without ATP and MgCl₂, is less than the increased

TABLE 13

The Effect of Various Possible Sources of Deoxyribose on the Conversion of Uracil-2-C¹⁴ to Labelled Deoxyuridine and Deoxyuridine Nucleotides in the Presence or Absence of ATP and MgCl₂ in an Extract of Ehrlich Ascites Tumour Cells

A d d i t i o n s	Per Cent of Total Uracil-2-C ¹⁴ Added to Vessel		
	Uracil	Deoxyuridine	Deoxyuridine Nucleotides
Nil	99.4	0.1	0.4
Nil plus ATP, MgCl ₂	98.7	0.4	1.1
Deoxyguanosine	87.5	11.7	0.8
Deoxyguanosine plus ATP, MgCl ₂	75.4	9.4	15.2
dGMP	95.0	4.5	0.4
dGMP plus ATP, MgCl ₂	83.7	6.1	9.0
Deoxyribose	99.7	0	0.2
Deoxyribose plus ATP, MgCl ₂	98.8	0.3	0.9
Deoxyribose-1-phosphate	74.9	24.4	0.6
Deoxyribose-1-phosphate plus ATP, MgCl ₂	69.2	19.7	11.1

Uracil-2-C¹⁴ : 500,000 counts/min./vessel $2.2 \times 10^{-2}M$

ATP 0.2 mM

MgCl₂ 0.2 mM

each deoxyribose source: 1.0 mM

Extracts prepared and incubated as described in Table 11.

TABLE 14

The Effect of Various Possible Sources of Ribose on the Conversion of Uracil- 2-C^{14} to Uridine and Uridine Nucleotides in the Presence and Absence of ATP and MgCl_2 in an Extract of Ehrlich Ascites Tumour Cells

A d d i t i o n s	Per Cent of Total Uracil- 2-C^{14} Added to Vessel		
	Uracil	Uridine	Uridine Nucleotides
Nil	99.1	0.5	0.8
Nil, ATP, MgCl_2	98.7	0.6	0.6
Guanosine	92.7	3.1	4.3
Guanosine, ATP, MgCl_2	63.7	1.5	34.8
Guanosine-5-monophosphate	98.4	0.8	0.7
Guanosine-5-monophosphate, ATP, MgCl_2	95.6	0.5	3.8
Ribose	98.7	0.8	0.5
Ribose, ATP, MgCl_2	98.5	0.7	0.8
Ribose-1-phosphate	95.3	0.8	3.8
Ribose-1-phosphate, ATP, MgCl_2	64.2	0.3	37.1

Uracil- 2-C^{14} : 500,000 counts/min./vessel $2.2 \times 10^{-2}\text{M}$

Extract prepared as described in Table 11.

ATP: 0.2 mM

MgCl_2 : 0.2 mM

Each ribose source 1.0 mM

conversion obtained in the presence of deoxyribose-1-phosphate. Conversely, deoxynucleotide formation (in the presence of ATP and MgCl_2) is somewhat greater with deoxyguanosine than with deoxyribose-1-phosphate.

4.7 The Effect of Possible Sources of Ribose on the Conversion of Uracil-2-C¹⁴ to labelled Uridine Nucleotides

Studies of possible ribose donors for uracil have shown that there is little or no conversion of uracil-2-C¹⁴ to uridine whatever the source of ribose, including ribose-1-phosphate. This is true both in the presence and absence of ATP. Possibly the 0.1M phosphate buffer exerts a greater inhibitory effect on the uridine phosphorylase, than on the corresponding systems described previously. It would thus seem that the enzyme(s) which brings about the conversion of thymine to thymidine, and uracil to deoxyuridine is different from that involved in the formation of uridine from uracil. Discrepancies in the phosphorylation of uridine and deoxyuridine to UMP and dUMP have previously been reported (68).

However, there is a considerable formation of UMP from the labelled uracil, when guanosine or ribose-1-phosphate are present together with ATP and MgCl_2 .

The lack of any appreciable formation of labelled uridine in the presence of ribose-1-phosphate could indicate that there is present an extremely active uridine kinase system. Skold (68) has reported high uridine kinase activity in the Ehrlich ascites tumour. This would result in the conversion of all uridine formed to UMP.

4.8 Studies with Tritiated Deoxyguanosine on the Conversion of Thymine to Thymidine in Extracts of Ehrlich Ascites Tumour Cells

TABLE 15

Effect of Tritiated Deoxyguanosine on the Conversion of Thymine to Thymidine
in an Extract of Ehrlich Ascites Tumour Cells

A d d i t i o n s	Thymine	Thymidine	Thymidine Nucleotides
Nil	332	401	4654
0.1 M thymine	449	964	3436
0.1 M thymine plus 0.1 M dG	472	1220	6357
1.0 M thymine	457	891	7166
1.0 M thymine plus 0.1 mM dG	414	952	6578

Deoxyguanosine -H³ : 1,000,000 counts/min./vessel 2.2 m umoles

Results expressed as counts/minute/50 mg wet weight extract

Extracts prepared and incubated as described in Table 11.

Studies of the effect of uniformly labelled tritiated deoxyguanosine on the conversion of thymine to thymidine, labelled in the deoxyribose group, have been carried out. The results of Table 15 indicate a definite increase in radioactivity in thymidine when unlabelled thymine is present. There is also a fair degree of labelling of the thymine moiety which at present is rather difficult to understand.

The extensive labelling in the nucleotide fraction undoubtedly also represents phosphorylated derivatives of the tritiated deoxyguanosine.

These results further support the assumption that deoxyguanosine can donate a deoxyribose group to thymine.

Summary of Chapter 4

Studies on cell-free extracts of the Ehrlich ascites carcinoma have indicated the existence of enzyme systems, phosphorylytic in nature, which are capable of carrying out the deoxyribose and ribose transfer reactions observed in Chapter 3.

1. Deoxyguanosine and deoxyinosine bring about the conversion of thymine to thymidine in these disrupted cells. When ATP and MgCl_2 are present, the thymidine thus formed is converted to TMP.

Deoxyuridine and thymidine are also capable of increasing thymidine formation from thymine in the presence and absence of ATP and MgCl_2 .

2. It has been found that most deoxynucleosides, in the presence of ATP and MgCl_2 , will stimulate the formation of TMP from labelled thymine. Only thymidine itself will not bring about this increase in TMP. This is probably due either to isotopic dilution, or to feedback inhibition.

3. Several results obtained in these cell-free preparations indicate the presence of thymidine phosphorylase in Ehrlich ascites tumour cells:

(a) the formation of thymidine from thymine in the presence of deoxyguanosine is inhibited by increasing concentrations of phosphate buffer. This suggests the cleavage of any thymidine formed to thymine and deoxy-ribose-1-phosphate,

(b) this inhibition of thymidine formation from thymine is also observed when the extracts are incubated with arsenate in the absence of ATP. When ATP and MgCl_2 are present, however, there is no inhibition of this conversion of thymine to thymidine with arsenate. This is explained by the fact that in the presence of ATP and MgCl_2 , thymidine kinase is very active, and converts all thymidine formed to TMP before it can be cleaved to thymine,

(c) in these preparations labelled thymine will readily combine with deoxyribose-1-phosphate with the resulting formation of thymidine. This is direct evidence of the existence of thymidine phosphorylase.

4. Other similar phosphorylase systems exist in these tissues as well.

(a) Uracil will combine with deoxyribose-1-phosphate forming deoxyuridine, in the absence of ATP and Mg^{++} , and dUMP, as well as deoxyuridine, when ATP and Mg^{++} are present. Thus the presence of deoxyuridine phosphorylase is indicated,

(b) When uracil is incubated with ribose-1-phosphate, there is no formation of uridine. However, in the presence of ATP and MgCl_2 , there is considerable formation of UMP. This would suggest the existence of uridine phosphorylase in these tissues. The highly active uridine kinase of Ehrlich ascites tumour cells could conceivably convert all the uridine formed to UMP.

5. Deoxyguanosine can be enzymatically cleaved to deoxyribose in the presence of phosphate buffer in these tissues.

6. Experiments with uniformly tritiated deoxyguanosine incubated in the presence of unlabelled thymine, indicate the formation of tritiated thymidine (presumably labelled in the deoxyribose moiety) from thymine. This is further evidence that deoxyguanosine transfers its deoxyribose group to thymine.

7. The results from Chapter 4 indicate that one or more systems exist in Ehrlich ascites tumour cells which can i) break down deoxyguanosine to deoxyribose-1-phosphate and guanine ii) synthesize thymidine and deoxyuridine from the available deoxyribose-1-phosphate plus thymine and uracil, respectively.

Thus these results further substantiate the experimental evidence obtained in Chapter 3 and give greater support to the concept of active salvage pathways in tumour cells.

CHAPTER 5

STUDIES WITH COLCHICINE ON THE BIOSYNTHESIS OF NUCLEOTIDES AND NUCLEIC ACIDS OF EHRLICH ASCITES TUMOUR CELLS 'IN VITRO'

Several investigations have been carried out on the effects of colchicine on the metabolism of nucleic acids in many tissues. The results obtained have made it of interest to observe the effects of this mitotic inhibitor on the incorporation of various precursors into the acid-soluble, RNA, and DNA fractions of Ehrlich ascites tumour cells. As part of this study of the biosynthesis of nucleotides and nucleic acids in the Ehrlich ascites carcinoma, we have observed the effects of colchicine on some of the systems studied previously.

There have been many investigations recently on this drug, dealing with its effect on the synthesis of RNA and RNA precursors. As mentioned in Chapter 1, Creasey and Markiw (134) in 'in vivo' studies on Ehrlich ascites cells, have obtained an inhibition of uridine- H^3 incorporation into soluble RNA. Weinfeld and Sandburg (137) have demonstrated an increase in utilization of RNA precursors in rat liver, 'in vivo'. A decrease in ATP and UTP concentrations in rat liver in the presence of the drug has also been reported (136).

An inhibition of DNA biosynthesis by colchicine in insect and mammalian tissues has been shown by Ilan and Quastel (148). Further studies of the effect of colchicine on nucleic acid metabolism have led to the realization of a fairly complex mode of action of this drug.

5.1 The Effect of Colchicine on the Incorporation of Various Precursors into Nucleotides and Nucleic Acids of Ehrlich Ascites Tumour Cells

TABLE 16

Effect of 1 mM Colchicine on Incorporation of Various Precursors Into Nucleotide
and Nucleic Acid Portions of Ehrlich Ascites Tumour Cells

P r e c u r s o r	P e r c e n t e f f e c t		
	Acid soluble nucleotides %	R N A %	D N A %
Adenine-8-C ¹⁴	- 11	- 6	- 41
Thymidine-2-C ¹⁴	+ 10	-	- 37
Guanine-8-C ¹⁴	- 56	- 42	- 57
Uridine-2-C ¹⁴	- 58	- 52	- 64

Cells incubated as described in Table 1, with 10 mMolar glucose omitted in the case of thymidine-2-C¹⁴.

Table 16 is a compilation of the effects of colchicine on the incorporation of several preformed precursors of nucleic acids into Ehrlich ascites tumour cells. At once it can be seen that the drug, at a concentration of 1 mM, has apparently more than one site of action. In every case (including thymine-2-C¹⁴ and deoxyguanosine, which have not been tabulated here), there is a definite decrease in incorporation of radioactivity into DNA in the presence of colchicine. The least inhibition is with adenine-8-C¹⁴ or thymidine-2-C¹⁴. With these two precursors there is little or no corresponding inhibition of incorporation into the RNA and acid-soluble portions.

However, the incorporations of guanine-8-C¹⁴ and uridine-2-C¹⁴ into all three fractions are inhibited in the presence of the drug. In addition, a significantly greater inhibition of incorporation of these precursors into the DNA is obtained. The colchicine-produced inhibition of adenine and thymidine incorporation into the DNA is constantly of the order of 40%, while the drug produces closer to a 60% inhibition of utilization of uridine and guanine for DNA synthesis. One can assume that there are at least two discreet sites of action of colchicine.

Contrary to our results, which indicate no effect of colchicine on the formation of nucleotides from adenine, a decrease in the rate of formation of ATP in rat liver has been reported (136). A partial reversal of the antimitotic effect of the drug, in the presence of ATP, has also been reported (119, 120). However, if colchicine does inhibit the formation of any nucleotide of adenine higher than AMP, it would not be detected by this method of measuring total acid-soluble radioactivity. The only indication that there is no such inhibition in Ehrlich ascites tumour cells is the fact that the drug

TABLE 17

The Effect of Varying Concentrations of Colchicine on the Incorporation of Uridine-2-C¹⁴ Into the Acid-Soluble Nucleotides of Ehrlich Ascites Tumour Cells (a) Directly After Removal from Peritoneal Cavity, (b) After Preincubation at 0°C for 24 Hours

Colchicine Concentration, mM	% e f f e c t	
	Direct Incubation	Preincubation at 0°C for 24 hrs
0.00	-	-
0.05	- 9	+ 5
0.10	- 11	+ 1
0.50	- 51	- 25
1.00	- 63	- 29
5.00*	- 78	- 36

Uridine-2-C¹⁴ : 500,000 counts/minute/vessel/24 ugm

Control values: direct incubation: 34,925 counts/minute/50 mgm wet weight
preincubation at 0°C: 17,350 counts/min./50 mgm wet weight

* O₂' consumption inhibited 30% with 5.0 mM colchicine

The cells were incubated as described in Table 1, in both cases, with 30 mM tris hydrochloride buffer (pH 7.4).

produces no inhibition of incorporation of label into the RNA, which would occur if the amount of available ADP or ATP were decreased.

With regard to thymidine metabolism, any effect of colchicine on the incorporation of labelled thymidine into deoxynucleotides would be reflected in the incorporation of radioactivity into the acid-soluble fraction, because this compound forms only deoxynucleotides. If the inhibition of synthesis of DNA were the result of a blockage at some stage of deoxynucleotide formation, it would be detected by this method. However, no appreciable effect of colchicine on this portion has been obtained.

The colchicine inhibition of incorporation of uridine-2-C¹⁴ and guanine-8-C¹⁴ is very possibly concerned with nucleotide formation. Any decrease in the availability of acid-soluble precursors would result in decreased incorporation into RNA and DNA.

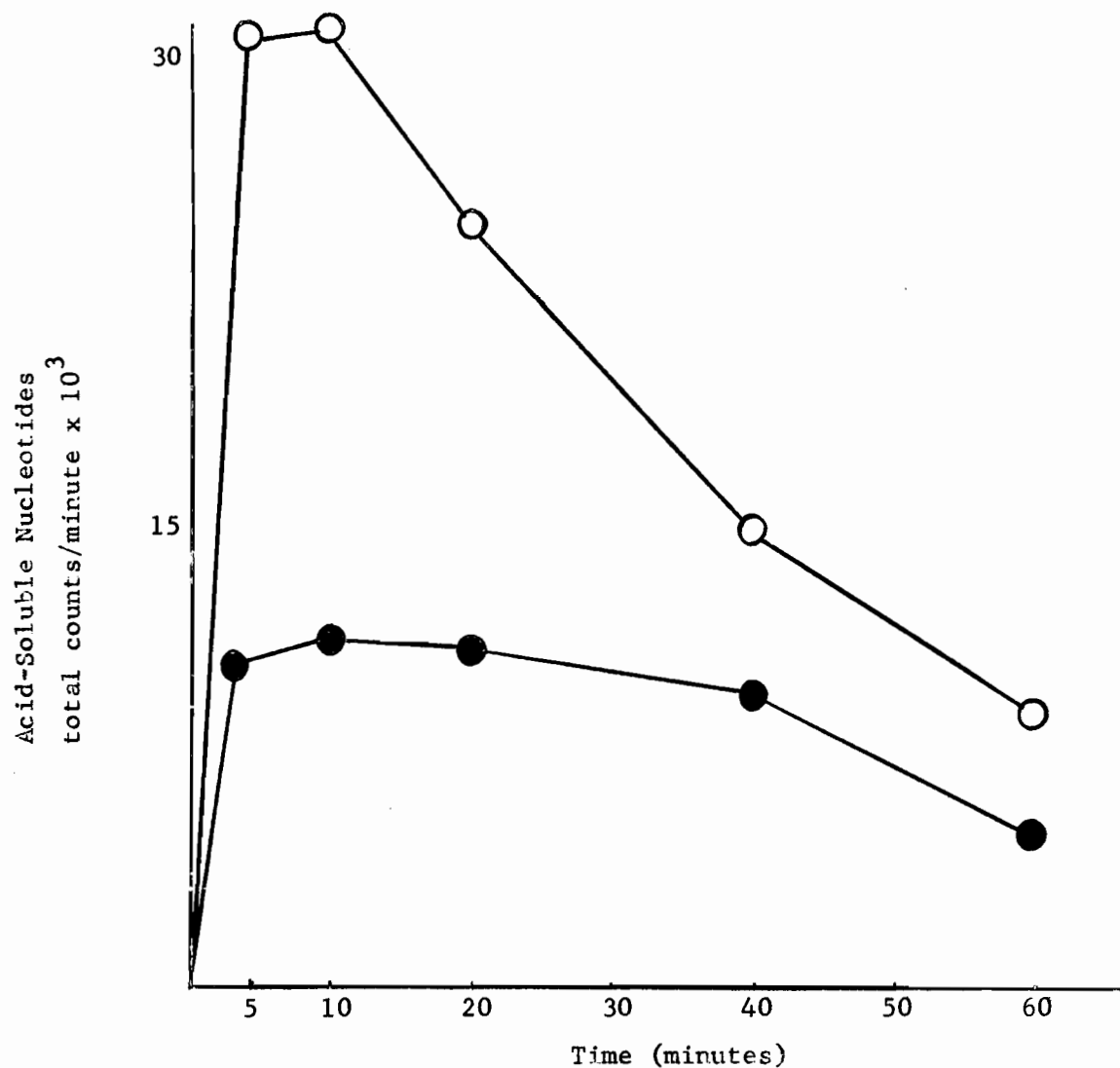
Further studies have been carried out only on uridine-2-C¹⁴ incorporation. Guanine is virtually insoluble in water, and therefore is unsuitable for procedures involving chromatography. Uridine is easily separated from its derivatives, however, and studies on uridine are more closely related to the data presented in Chapter 3.

5.2 The Effect of Varying Concentrations of Colchicine on the Incorporation of Uridine-2-C¹⁴ into the Acid-Soluble Nucleotides of Ehrlich Ascites Tumour Cells (1) Directly After Removal from the Peritoneal Cavity, (2) After Incubation at 0°C for 24 Hours

Table 17 is, in part, a concentration curve of the colchicine inhibition of uridine incorporation into uridine nucleotides. The maximum effect of colchicine is obtained at a concentration of 1.0 mM. Above this concentra-

Figure 7

Effect of Colchicine on the Incorporation of Uridine-2-C¹⁴ into Acid-Soluble Nucleotides of Ehrlich Ascites Cells with Increasing Time Intervals.



Uridine-2-C¹⁴: 250,000 counts/minute/vessel/12ug.

Cells incubated as described in Table 1.

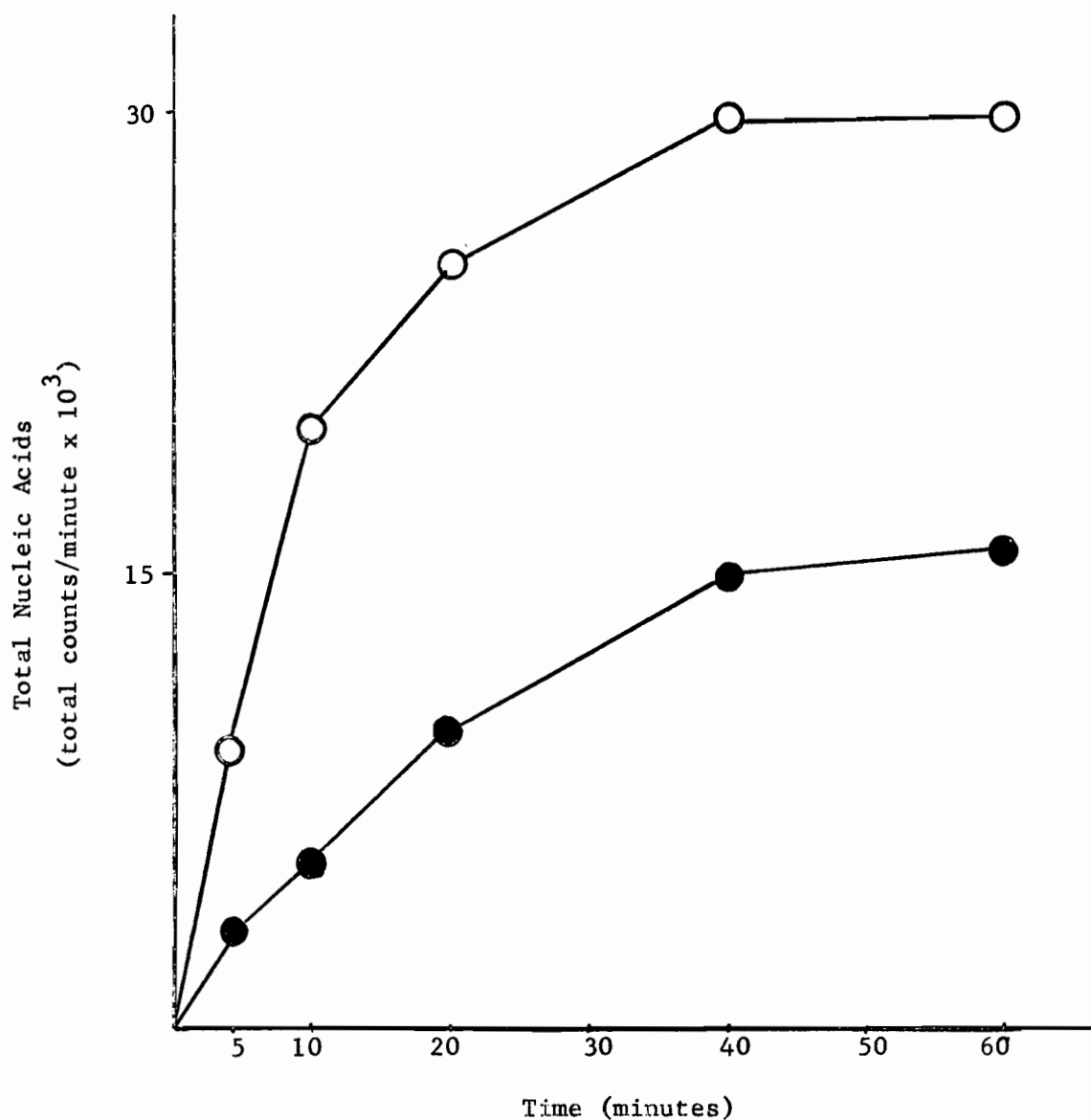
Colchicine: 1 mM.

○ - control

● - colchicine

Figure 8

Effect of Colchicine on the Incorporation of Radioactivity from Uridine-2-C¹⁴
into Nucleic Acids of Ehrlich Ascites Cells with Increasing Time Intervals.



Uridine-2-C¹⁴: 250,000 counts/minute/vessel/12 μ g.

Cells incubated as described in Table I.

Colchicine: 1 mM.

○ - control

● - colchicine

tion, there is some inhibitory effect of the drug on respiration which becomes quite pronounced at 5 mM, where the oxygen consumption is reduced by 30%. At a concentration of 0.1 mM, colchicine exerts an almost negligible inhibition of uridine incorporation which rises sharply in proportion to the concentration until 0.5 mM, after which increasing concentrations of the drug exert relatively smaller inhibitions.

These studies have also been carried out on cells which have been removed from the peritoneum and kept under identical conditions at 0°C for 24 hours. This has been done to see if prolonged exposure to colchicine will result in greater sensitivity to the drug. Maintenance of the ascites cells at 37°C would be more ideal, but this results in extensive loss of viability. In 'in vivo' experiments with the drug, several hours of exposure to colchicine are necessary before the maximum effects are obtained. The incorporation of uridine-2-C¹⁴ into uridine nucleotides is decreased by 50% after incubation at 0°C for 24 hours. However, the effect of colchicine is also diminished by approximately 50%, resulting in half the inhibition obtained with directly incubated cells. Whatever systems are sensitive to colchicine in normally prepared cells, become less sensitive after the cells have been preincubated at 0°C. Thus, prolonged exposure to the drug 'in vitro' does not seem to increase its effect.

5.3 The Effect of Colchicine on Incorporation of Radioactivity into the Nucleotides and Nucleic Acids with Increasing Intervals of Time

Figures 7 and 8 are time studies on the colchicine inhibition of incorporation of radioactivity from uridine-2-C¹⁴ into acid-soluble nucleotides (Figure 7), and total nucleic acids (Figure 8). Incorporation of label-

TABLE 18

Effect of Increasing Concentrations of Carrier Uridine on the Colchicine In-
hibition of Uridine-2-C¹⁴ Incorporation Into the Acid-Soluble, RNA and DNA
Fractions of Ehrlich Ascites Tumour Cells

Amount of carrier uridine per vessel micrograms	Amount of Uridine Incorporated Microgrs.		
	Acid-Soluble Nucleotides	R N A	D N A
*24 μ g	4.651	2.871	0.107
24 μ g plus Colchicine	2.249	1.244	0.061
68 μ g	13.364	4.060	0.148
68 μ g plus Colchicine	6.631	2.561	0.078
140 μ g	18.743	3.465	0.157
140 μ g plus Colchicine	9.637	2.004	0.071
236 μ g	14.266	3.580	0.190
236 μ g plus Colchicine	10.321	1.632	0.086
476 μ g	28.068	2.834	0.201
476 μ g plus Colchicine	12.052	1.551	0.130
624 μ g	37.414	3.001	0.157
624 μ g plus Colchicine	22.501	1.741	0.098

Uridine-2-C¹⁴: * 1,000,000 counts/minute/vessel/24 ugms

Colchicine: 1 mM

The cells were incubated as described in Table 1, with 30 mM tris-hydrochloride buffer (pH 7.4).

led uridine into the acid-soluble pool rises sharply, reaches a peak at 10 minutes, and subsequently declines. The effect of colchicine seems to be instantaneous, and, in fact, is greater at 10 minutes (causing a 65% inhibition), than at 60 minutes (where the inhibition is more of the order of 40-50%).

Uridine-2-C¹⁴ incorporation into the total nucleic acids increases sharply and reaches a constant rate. The degree of colchicine inhibition remains approximately the same throughout. The curves in Figures 7 and 8 suggest that the decrease in acid-soluble radioactivity is due to increased conversion of acid-soluble nucleotides and deoxynucleotides into nucleic acids. The inhibition of nucleic acid synthesis observed with colchicine may be a reflection of decreased availability of acid-soluble precursors. If the drug were also to have a specific effect on RNA synthesis, this would result in some accumulation of nucleotides, which would diminish the inhibition observed at the nucleotide level. Most likely the decrease in nucleic acid synthesis (specifically RNA) is due to decreased availability of precursors when the cells are incubated in the presence of colchicine.

5.4 The Effect of Increasing Concentrations of Carrier Uridine on the Colchicine Inhibition of Incorporation of Uridine-2-C¹⁴ into the Acid-Soluble, RNA, and DNA Fractions of Ehrlich Ascites Tumour Cells

The results in Figure 7 indicate that the largest effect of colchicine occurs at the time of the greatest rate of incorporation of uridine. In view of this, it has been decided to study the effects of the drug with increasing concentrations of carrier uridine, on uridine-2-C¹⁴ incorporation into the Ehrlich ascites cells (Table 18). Thus increasing the concentration of unlabelled uridine might stimulate the general phosphorylation of uridine in the cells.

The rate of incorporation of uridine-2-C¹⁴ into acid-soluble nucleotides has not attained its maximum level, even at concentrations of uridine approaching 1 mM (in Table 18, 624 ug represent a concentration of 0.83 mM). However, the rate of formation of RNA appears to reach a maximum almost immediately. Tripling the concentration of uridine over that of 'carrier-free' uridine-2-C¹⁴, results in an increased rate of incorporation of uridine into RNA. With further increases in the concentration of carrier uridine, the incorporation is decreased, suggesting that the excess uridine or its derivatives are acting as inhibitors of RNA synthesis. Conversely, the rate of incorporation of labelled uridine derivatives into DNA continues to increase in the presence of much greater concentrations of carrier uridine. It too reaches a maximum level at 0.8 mM uridine, and at higher concentrations, the incorporation seems to begin to decline.

Contrary, perhaps, to expectations from the results of Figure 7, greater incorporation of uridine obtained by increasing the concentration of carrier does not result in increased effects of the drug. In fact, at 0.83 mM uridine, the inhibition drops from 50% (obtained with all lesser concentrations of uridine) to 38%. This appears to be the case with RNA and DNA as well, again a possible reflection of the effect of the drug on acid-soluble nucleotides.

5.5 The Effect of Colchicine on the Incorporation of Uridine-2-C¹⁴ into Acid-Soluble Nucleotides in Intact Cells, and Cell-Free Extracts of Ehrlich Ascites Tumour Cells

In view of the notable effect of colchicine on the formation of acid-soluble nucleotides from uridine, this fraction has been investigated using chromatographic procedures, in order to detect any specific effect

TABLE 19

Effect of 1 mM Colchicine on Incorporation of Uridine-2-C¹⁴ Into Acid-Soluble Nucleotides in: (a) Whole Cell Preparations, (b) Cell-Free Extracts of Ehrlich Ascites Tumour Cells

P r e p a r a t i o n	N u c l e o t i d e f r a c t i o n			% Effect
	UMP counts/min.	UDP/UTP combined counts/min.	Total counts/min.	
Intact cells contr.	888	5699	6587	-
+ colchicine	333	3808	4141	- 37
Cell extract contr.	940	695	1635	-
+ colchicine	1088	665	1753	+ 5

Input: Uridine-2-C¹⁴ : 500,000 counts/min./vessel/24 µgms

Results expressed as counts/min./50 mg wet weight tissue

Solvent system used to separate acid-soluble derivatives: ethanol/1.0 M ammonium acetate (pH 7.5)//75/30//V/V

Intact cells incubated as described in Table 1.

Cell extracts prepared as described in Chapter 2 and incubated with the additions given in Table 11.

of the drug. This study has been carried out with intact cell suspensions and cell-free extracts, prepared as described in Chapter 2.

In Table 19 it can be seen that in whole-cell systems, the greatest effect of colchicine is exerted directly at the first stage of uridine-2-C¹⁴ incorporation, namely the phosphorylation of uridine to UMP. This would also produce the decrease apparent in the higher uridine nucleotides.

No such effect is obtained when colchicine is incubated with a cell-free extract of the ascites cells. The drug apparently has no effect on the synthesis of uridine nucleotides in disrupted cells.

It has been reported that the colchicine inhibition of xanthine oxidase, which takes place in rat liver slices, is not obtained in a rat liver homogenate (124). However, when these investigators have highly purified the enzyme, colchicine again seems to be an effective inhibitor, even at low concentrations. These observations have led to the following experiments.

5.6 The Effect of Native and Denatured Cell-Free Extracts on the Colchicine Inhibition of Incorporation of Uridine-2-C¹⁴ into Nucleotides and Nucleic Acids of Intact Ehrlich Ascites Tumour Cells

The results of the experiments depicted in Table 20, give at least some explanation of the lack of effect of colchicine on uridine incorporation into a cell-free extract. When a cell-free extract (prepared as described in Chapter 2) is added to suspensions of intact tumour cells and incubated with uridine-2-C¹⁴, the inhibition produced by colchicine is greatly decreased.

As can be seen from Table 20, the addition of extract results in a considerable decrease in incorporation of radioactivity from uridine-2-C¹⁴ into the acid-soluble nucleotides, when compared to the control value. This

TABLE 20

Effect of Native and Denatured Cell-Free Extracts on Colchicine Inhibition of Incorporation of Uridine-2-C¹⁴
Into Nucleotide and Nucleic Acid Portions of Intact Ehrlich Ascites Tumour Cells

A d d i t i o n s	Acid-Soluble Nucleotides		R N A		D N A	
	Counts/Min.	% Inhibition	Counts/Min.	% Inhibition	Counts/Min.	% Inhibition
Control	36637	-	57995	-	2105	-
Contr.+ Colchicine	15112	60	21319	63	428	80
Extract	10500	-	12434	-	213	-
Extr.+ Colchicine	8487	19	10441	15	177	17
Boiled extract	38125	-	73842	-	1409	-
" " + Colch.	14500	62	27284	63	507	64

Input: Uridine-2-C¹⁴ : 500,000 counts/min./vessel/24 ugms.

Results expressed as counts/min./50 mg wet weight tissue.

Colchicine concentration: 1 mM

Intact cells: incubated as described in Table 1.

Cell-free extract: prepared as described in Chapter 2, 0.5 ml added to vessels.

Boiled extract: prepared by placing cell-free extract in boiling water bath for 10 minutes, 0.5 ml boiled extract added to vessels.

can be explained by assuming that the native extract which is added directly to the buffered Krebs-Ringer medium, which contains labelled uridine, and is incubated along with the intact cells at 37°C, is also capable of phosphorylating the uridine-2-C¹⁴. The results of the several experiments presented in Chapter 4 indicate that such extracts are capable of converting as much as 30% of uracil-2-C¹⁴ to UMP when suitable sources of ribose are present. Thus, it can be reasonably assumed that labelled uridine would be phosphorylated with equal or greater facility. The intact cells present which are unable to incorporate phosphorylated compounds would have less uridine-2-C¹⁴ to take up, and consequently less could be converted to nucleotides inside the cell.

When the added extract is previously boiled, the systems for phosphorylating uridine would be inactivated. This results in no significant difference in the extent of incorporation of labelled uridine into the acid-soluble portion of intact cells. Colchicine, in the presence of heated extract, does not exhibit any decrease in its usual inhibition.

These results imply the combination of the drug with some heat-labile component of the disrupted Ehrlich ascites tumour cells, which renders it inactive. Taylor (113) has reported the binding of radioactive colchicine to unidentified cellular sites, in cultures of human cells. When the constituents of the extract are boiled, they are apparently no longer in a form capable of combining with colchicine, and the drug retains its effectiveness as an inhibitor.

The results obtained in the acid-soluble pool are reflected in the RNA and DNA portions, adding support to the suggestion that the primary effect of the drug is at the nucleotide level.

5.7 The Effect of Colchicine on Actinomycin-D Inhibition of DNA-Dependent RNA Synthesis in Ehrlich Ascites Tumour Cells

TABLE 21

The Effect of Colchicine on the Inhibition of DNA-Dependent RNA Synthesis by Actinomycin-D. The Effect of both Drugs on the Incorporation of Uridine-2-¹⁴C Into the Acid-Soluble, RNA and DNA Fractions of Ehrlich Ascites Tumour

Cells

A d d i t i o n s	Acid-sol. nucleot.		R N A		D N A	
	c/min.	% eff. of colchicine	c/min.	% eff. of colchicine	c/min.	% eff. of colchicine
Nil	43,550	-	41,231	-	1,706	-
Colchicine	26,100	- 40	18,031	- 56	501	- 70
Actinomycin-D (1 microgram)	78,950	-	12,646	-	1,933	-
Actinomycin-D (1 ug) + colch.	46,525	- 48	8,249	- 35	770	- 60
Actinomycin-D (5 micrograms)	85,750	-	4,465	-	1,560	-
Actinomycin-D (5 ug) + colchic.	48,200	- 44	2,071	- 53	555	- 65

Uridine-2-C¹⁴ : 500,000 counts/min./vessel/24 ugms uridine

Results expressed as counts/min./50 mg wet weight tissue

Colchicine: 1 mM

Cells incubated as described in Table 1 with 30 mM tris hydrochloride buffer pH 7.4.

Actinomycin-D has been extensively studied as an antibiotic with a profound influence on nucleic acid metabolism. It acts by inhibiting the DNA-dependent synthesis of RNA (190). It has been shown to combine with the guanine moiety in the shallow groove of helical DNA (191), and to prevent the alignment of mRNA for further RNA synthesis. These effects are obtained with extremely small quantities of the drug.

The effect of colchicine on this process has been studied and is presented in Table 21. Here the actinomycin-D exerts its characteristic effect on RNA biosynthesis, while the values for incorporation of label from uridine-2-C¹⁴ into DNA do not change appreciably. A corresponding increase in acid-soluble radioactivity occurs when actinomycin-D is present. This is the result of accumulation of nucleotides meant for RNA synthesis which is blocked by the actinomycin.

These changes, the decrease in RNA and the increase in the nucleotides, do not have any significant effect on the inhibition by colchicine. Whatever the deviation from normal produced by actinomycin, its value is still approximately halved in the presence of colchicine. This would further strengthen the suggestion that the colchicine effect is exerted at the level of uridine incorporation to UMP. There is thus no appreciable interaction of the two drugs, on the Ehrlich ascites tumour cells, which can be detected by these methods.

5.8 Comparison of the Effects of the Vinca Alkaloids with those of Colchicine on the Incorporation of Uridine-2-C¹⁴ into Ehrlich Ascites Tumour Cells

Recently attention has been focused on the vinca alkaloids, whose antitumour properties have been the subject of many investigations (149, 192).

TABLE 22

Comparison of the Effects of Colchicine, Vinblastine and Vincristine on the Incorporation of Radioactivity from Uridine-2-C¹⁴ Into the Acid-Soluble Nucleotides, RNA and DNA Portions of Ehrlich Ascites Tumour Cells

A d d i t i o n s	P e r C e n t E f f e c t		
	Acid-Soluble Nucleotides	RNA	DNA
Nil	-	-	-
Colchicine 1.0 mM	- 44	- 53	- 65
Vinblastine 0.25 mM	- 5	+ 30	- 19
Vinblastine 0.50 mM	- 50	- 20	- 54
Vinblastine 1.0 mM	- 60	- 57	- 68
Vincristine 0.25 mM	- 4	+ 20	- 20
Vincristine 0.50 mM	- 14	+ 3	- 17

Uridine-2-C¹⁴ : 500,000 counts/vessel/minute/24 μ g

Control values: acid soluble nucleotides: 38,730 counts/min./50 mg wet weight

RNA : 21,145 counts/min./50 mg wet weight tissue

DNA : 1,791 counts/min./50 mg wet weight tissue

Cells incubated as described in Table 1.

Their mode of action is fairly similar to that of colchicine (151), while they produce less toxic side effects. Like colchicine they produce mitotic arrest presumably by interfering with spindle formation (151). In a comparative study of the effects of vincristine and vinblastine to those of colchicine, on the incorporation of uridine- H^3 into Ehrlich ascites cells 'in vivo', Creasey and Markiw (134) have observed inhibitory effects exhibited by all three compounds, differing in time and quantity of effect, but with the same general tendencies. Extending these studies to 'in vitro' work (154), they have shown a decreased incorporation of uridine- H^3 into soluble RNA with drug levels of the same order as presented in this thesis. A comparative study of the effects of vinblastine and vincristine to those of colchicine has been undertaken. The results are shown in Table 22. In experimental conditions identical to the other 'in vitro' studies with colchicine, described in this chapter, these two vinca alkaloids have been incubated with the Ehrlich ascites tumour cells. (A limited supply of vincristine has not permitted experimentation with concentrations greater than 0.5 mM). The results indicate patterns of effects of vinblastine markedly similar to those of colchicine at corresponding concentrations. Vincristine, however, does not seem to be as effective, although there might be a considerable increase in degree of inhibition at a concentration of 1 mM. Studies of the 'in vivo' effects of these drugs presented in Chapter 6 indicate that this is probably not the case, and that the incorporation of radioactivity into separate fractions of nucleic acids is not as profoundly affected by vincristine as by vinblastine and colchicine.

Vinblastine, as suggested from Table 22, would also seem to have a primary effect on the acid-soluble fraction. The activity of this drug is

also diminished at low concentrations. The remarkably similar patterns of activity of colchicine and vinblastine lead one to suggest that their similar mitotic effects might be related to their biochemical actions.

Summary of Chapter 5

1. As part of a study of the biosynthesis of nucleotides and nucleic acids of Ehrlich ascites tumour cells, the effects of colchicine, a well-known mitotic inhibitor, on these systems have been investigated. These studies have shown that colchicine, at a concentration of 1 mM, inhibits the incorporation of several labelled precursors into DNA 'in vitro'. These include adenine-8-C¹⁴, guanine-8-C¹⁴, thymidine-2-C¹⁴ and uridine-2-C¹⁴.

2. In addition, the drug decreases the incorporation of guanine-8-C¹⁴ and uridine-2-C¹⁴ into the acid-soluble nucleotides and RNA of the Ehrlich ascites cells. There is no effect of the drug on the incorporation of adenine or thymidine into the acid-soluble and RNA fractions. Further studies on the 'in vitro' effect of colchicine have been carried out, using uridine-2-C¹⁴.

3. The concentration of colchicine required for the maximum inhibition of incorporation of uridine-2-C¹⁴ into acid-soluble nucleotides and nucleic acids is 1 mM. Above this the O₂ consumption is decreased. At concentrations of 0.1 mM there is little or no effect of the drug on incorporation of uridine into these fractions.

4. Time studies of the inhibition by colchicine on uridine incorporation, reveal that the effect of the drug is practically instantaneous. The drug inhibits the incorporation of uridine into acid-soluble nucleotides to a greater extent at 10 minutes, than at one hour. The inhibition by colchicine of the incorporation of labelled uridine into the nucleic acids is also

immediate. These results suggest that the inhibition of incorporation of radioactivity from uridine into nucleic acids is a result of the inhibition of synthesis of acid-soluble nucleotides by the drug.

5. When the acid-soluble nucleotides of intact cells are chromatographed after incubation with labelled uridine in the presence and absence of colchicine, there is a considerable decrease in the formation of UMP when the drug is present. This indicates that the primary effect of the drug is on the conversion of uridine to UMP. Decreased availability of UMP would result in a decreased amount of nucleotides available for RNA and DNA synthesis.

6. In contrast to results obtained with intact cells, when a cell-free extract of the Ehrlich ascites carcinoma is incubated with colchicine, there is no effect of the drug on the phosphorylation of uridine to UMP or on the further conversion of UMP to UDP and UTP.

7. These results are partially explained by experiments where intact cells have been incubated in the presence of labelled uridine, colchicine, and cell-free extracts of these tumour cells. In such conditions the colchicine inhibition is greatly diminished. Colchicine presumably combines with some protein which is liberated upon cell disruption and the effective concentration of the drug is thereby diminished.

Incubation of the intact cells with a cell-free extract which has been boiled prior to the incubation, does not decrease the inhibition by colchicine. Thus the combination of the drug with the substance(s) in a cell-free extract does not occur following heat denaturation of the components of the extract.

8. Some attempts have been made to see if the extent of inhibition

by colchicine can be increased. These, in general, have proved to be fruitless:

a) When the concentration of carrier uridine is increased, the amount of uridine incorporated into acid-soluble nucleotides also increases. Even at concentrations of unlabelled uridine approaching 1 mM, the systems which incorporate uridine into uridine nucleotides are not saturated. The inhibitory effect of colchicine on uridine-2-C¹⁴ incorporation into acid-soluble nucleotides is not increased when these high quantities of carrier uridine are present. In fact, when the concentration of this nucleotide approaches 1 mM, the inhibition by the drug is diminished.

b) Preincubation of the tumour cells with colchicine for 24 hours in a buffered Krebs-Ringer medium, at 0°C, does not increase the sensitivity of the systems studied to the drug. Conversely, there is also decreased inhibition by colchicine of the incorporation of labelled uridine into acid-soluble nucleotides when the cells have been kept in contact with the drug for 24 hours at 0°C.

c) The colchicine inhibition of incorporation of uridine-2-C¹⁴ into the Ehrlich ascites cells is not affected by the addition of Actinomycin-D, which, although it exerts its characteristic inhibition of RNA synthesis, does not produce any potentiative effects in the presence of colchicine.

9. Similar investigations of the effects of vinblastine and vincristine have been carried out at corresponding concentrations to those of colchicine, on the incorporation of uridine-2-C¹⁴ into nucleotides and nucleic acids. It has been found that vinblastine exerts very similar effects to those of colchicine. At 1 mM this substance also inhibits the incorporation

of labelled uridine into the acid-soluble, RNA and DNA fractions of the Ehrlich ascites tumour cells, by about 60%.

Vincristine, however, at a concentration of 0.5 mM, has no effect at all on the incorporation of labelled uridine into acid-soluble nucleotides and RNA, and exerts only a slight inhibition on incorporation of radioactivity into DNA.

CHAPTER 6

STUDIES OF INCORPORATION OF RADIOACTIVE PRECURSORS INTO EHRLICH ASCITES TUMOUR CELLS WHICH HAVE BEEN PREVIOUSLY TREATED WITH COLCHICINE 'IN VIVO'

The results obtained from 'in vitro' studies with colchicine have led us to investigate the effects of this drug on similar aspects of nucleic acid metabolism in tumour cells which have been treated with colchicine 'in vivo'. These experiments have involved preinjection of the drug into the peritoneal cavity of mice bearing the Ehrlich ascites tumour, prior to 'in vitro' studies on incorporation of various labelled precursors into the nucleic acids of the tumour cells. These experiments have indicated several 'in vivo' effects of the drug. Several of the results so obtained differ from those described in Chapter 5.

A notable decrease in the actual quantity of tumour cells obtained from the peritoneum after administration of colchicine has been found. The amount of 'liquid' tumour cell suspension obtained is often reduced by as much as 50% after injection of the drug, when compared to mice pretreated with normal saline. However, administration of colchicine in this manner, has tended to produce unpleasant side effects in tumour-bearing mice. These include diarrhea, listlessness (as opposed to the normal hyperactivity observed in these mice) and roughening of the coats of the animals. If the mice are not sacrificed prior to 48 hours after injection of colchicine, some die, and by 72 hours all the mice have expired. Thus the evident anti-tumour activity of colchicine is made useless by the extreme toxicity of the drug.

All experiments, except that described in Table 24, have been

TABLE 23

Preinjection of Colchicine Into Ehrlich Ascites Tumour-bearing Mice, 16 hours prior to 'in vitro' Incorporation of Various Labelled Precursors Into the Acid-Soluble, RNA and DNA Fractions

P r e c u r s o r	Acid-Soluble Nucleotid.		R N A		D N A	
	counts/min.	s.e.m.	counts/min.	s.e.m.	counts/min.	s.e.m.
Uridine-2-C ¹⁴ contr. (6)	57254	$\pm$ 2409	47323	$\pm$ 3324	1061	$\pm$ 124
colchicine (6)	48546	$\pm$ 2121	59163	$\pm$ 2928	341	$\pm$ 49.5
% effect	- 15	P < 0.05	+ 25	P < 0.05	- 68	
Adenine-8-C ¹⁴ contr. (3)	49288	$\pm$ 688	3248	$\pm$ 15	158	$\pm$ 28
colchicine (4)	41638	$\pm$ 6163	2481	$\pm$ 241	74	$\pm$ 3
% effect	- 15	P > 0.05	- 23	P < 0.05	- 53	
Thymidine-2-C ¹⁴ contr. (4)	6025	$\pm$ 576	-	-	9735	$\pm$ 1657
colchicine (4)	2258	$\pm$ 404	-	-	2404	$\pm$ 731
% effect	- 63				- 76	

Uridine-2-C¹⁴ : 500,000 counts/min./vessel/24 ug
 Adenine-2-C¹⁴ : 400,000 " "
 Thymidine-2-C¹⁴ : 220,000 " "

Results expressed as means of values of the number of mice indicated, in terms of counts/min./50 mg. wet weight tissue. S.e.m.: standard error of mean. P: probability.

Colchicine: 1 μ g/gm body weight injected intraperitoneally.

Cells incubated as described in Table 1 with 10 mM glucose omitted in the case of thymidine.

carried out by preinjection of colchicine, followed by a fixed length of time prior to sacrificing the mice and removing the cells. These pre-treated cells have been incubated as normally, in buffered Krebs-Ringer medium with labelled precursors. Thus, although the 'in vivo' effects of the drug have been the object of these studies, the actual incubations have been carried out 'in vitro'.

6.1 Effects of Preinjection of Colchicine on the Incorporation of Various Radioactive Precursors into the Acid-Soluble, RNA and DNA Fractions

The effects of treatment of tumour-bearing mice with colchicine 16 hours prior to subsequent incorporation of uridine-2-C¹⁴, adenine-8-C¹⁴, and thymidine-2-C¹⁴ into the various nucleic acid fractions of Ehrlich ascites tumour cells 'in vitro', have been investigated. The results of these experiments are presented in Table 23. These studies indicate a complex mode of action of the drug.

As is evident from Table 23, the incorporation of radioactivity from uridine-2-C¹⁴ is most greatly affected at the DNA site, where it is considerably inhibited. There is also a slight decrease in incorporation of this precursor into the acid-soluble nucleotides. However, the amount of labelled uridine utilized for RNA synthesis is somewhat greater than control values.

The incorporation of adenine-8-C¹⁴ into DNA of tumour cells which have been pretreated with colchicine is also inhibited to a considerable extent. A slight inhibition of incorporation of adenine into RNA is also evident in colchicine-treated cells. However, there is no significant effect of this drug on the incorporation of labelled adenine into the acid-soluble nucleotides.

TABLE 24

Incorporation of Radioactivity from Uridine-2-C¹⁴ into the Acid-Soluble, RNA and DNA Fractions of Ehrlich Ascites Tumour Cells 'in vivo', 16 hours after Intraperitoneal Injection of Colchicine

P r e c u r s o r	Acid-Soluble Nucleotid.		R N A		D N A	
	counts/min.	s.e.m.	counts/min.	s.e.m.	counts/min.	s.e.m.
Control (6)	44.4	± 5.7	28.4	± 4.2	1.92	± 0.17
Colchicine (5)	29.0	± 4.5	42.4	± 7.2	0.92	± 0.3
		$P > 0.05$		$P > 0.05$		$P < 0.05$

Colchicine (1 ug/gm weight animal) was injected into the peritoneal cavity of mice bearing the Ehrlich ascites carcinoma, 16 hrs. prior to subsequent injection of uridine-2-C¹⁴ (2,000,000 counts/min./96 ug).

After one hour the mice were sacrificed and the cells removed, washed and packed as described in Chapter 2.3. The cells were then suspended in 5 ml ice-cold 5% trichloroacetic acid per 50 mg wet weight tissue, and treated exactly as in 'in vitro' studies.

Results expressed as counts/minute/mgm tissue. s.e.m.: standard error of mean. P: probability.

Colchicine inhibits greatly the incorporation of thymidine into acid-soluble nucleotides and into DNA. The inhibition of incorporation of labelled thymidine into DNA is greater than that observed with any other precursor.

From these studies, it is evident that the most pronounced effect of colchicine, when administered in this way, is an extensive inhibition of incorporation of radioactivity from labelled precursors into the DNA.

6.2 'In Vivo' Incorporation of Radioactivity from Uridine-2-C¹⁴ into Acid-Soluble, RNA, and DNA Fractions of Ehrlich Ascites Tumour Cells, 16 Hours after Intraperitoneal Injection of Colchicine

The effects of colchicine on the 'in vivo' incorporation of radioactivity from uridine-2-C¹⁴ into the Ehrlich ascites tumour cells are shown in Table 24. Extensive variation in values obtained from different tumour-bearing mice has made it difficult to assess the effects of this drug 'in vivo'. The differences between the means of control and experimental animals cannot be considered significant.

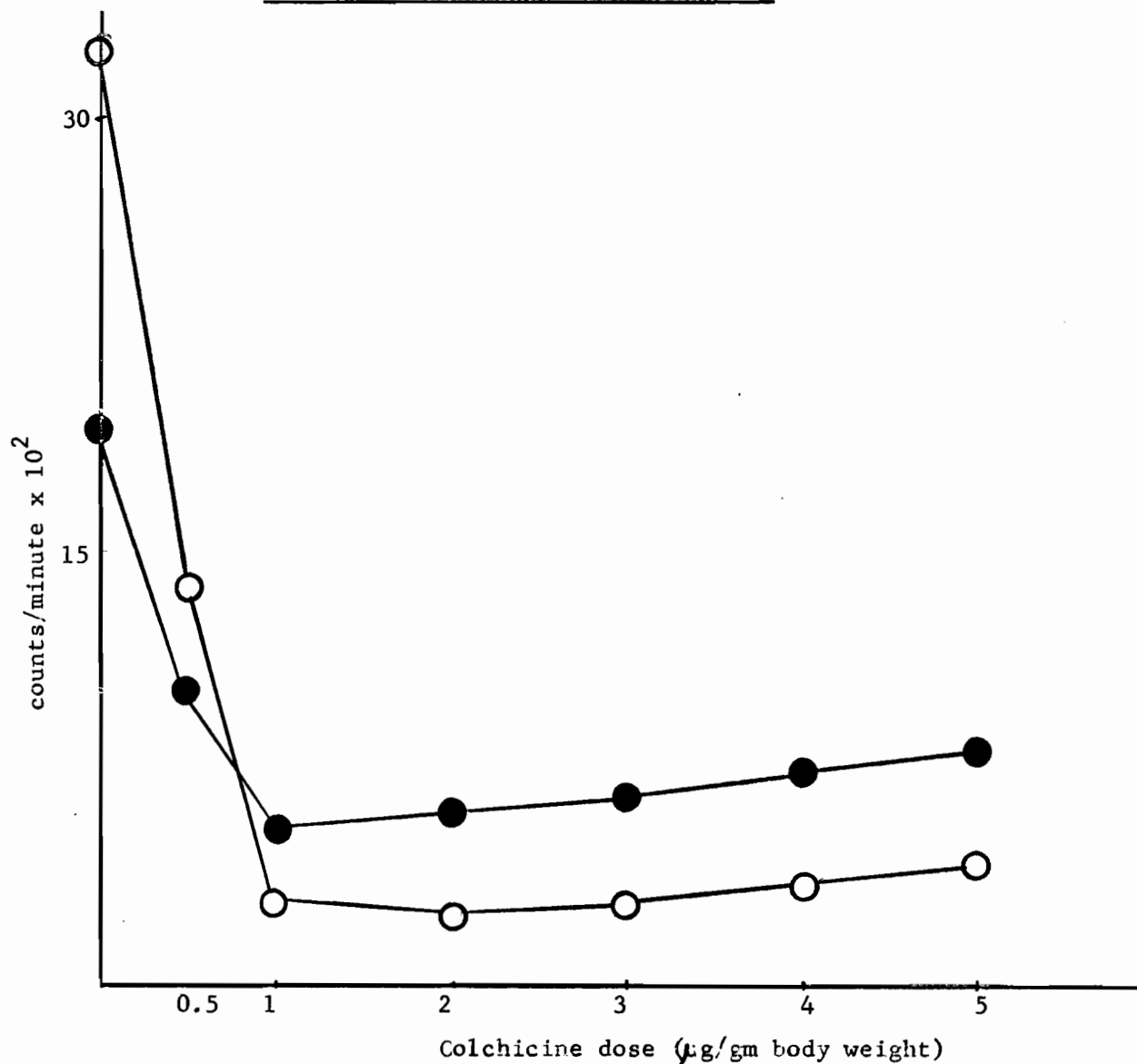
As in the experiments presented in Table 24, there is a tendency for colchicine to slightly inhibit the incorporation of uridine into acid-soluble nucleotides, and to increase its incorporation into RNA. However, the only statistically significant result is the decreased incorporation of radioactivity from uridine-2-C¹⁴ into DNA, in pretreated animals.

6.3 The Effects of Varying Amounts of Colchicine Preinjected into Ehrlich Ascites Tumour-Bearing Mice, 16 Hours prior to Incorporation of Thymidine-2-C¹⁴ into the Acid-Soluble Nucleotides, RNA and DNA

Figure 9 is a study of the effects of different doses of colchi-

Figure 9

Effect of Preinjection of Varying Amounts of Colchicine on the In Vitro
Incorporation of Thymidine-2-C¹⁴ into the Acid-Soluble Nucleotides and
DNA of Ehrlich Ascites Tumour Cells



Thymidine -2-C¹⁴: 150,000 counts/minute/vessel.

Cells incubated as in Table I with glucose omitted.

Colchicine injected 16 hours prior to incubation.

Number of animals/trial: 4.

○ - DNA

● - acid-soluble nucleotides

cine on the incorporation of thymidine-2-C¹⁴ into acid-soluble nucleotides and DNA of Ehrlich ascites tumour cells. The most pronounced inhibitory effects of this drug are obtained with quantities of 1 or 2 micrograms/gram body weight.

As is evident from Figure 9, the acid-soluble nucleotides and DNA, exhibit parallel patterns of inhibition by increasing amounts of colchicine. Doses in excess of 5 ug colchicine/gram body weight prove to be lethal. All subsequent experiments have been carried out in mice preinjected with 1 ug/gram body weight.

6.4 Administration of Colchicine into Tumour-Bearing Mice for Varying Periods of Time prior to Incorporation of Radioactivity from Uridine-2-C¹⁴ into the Acid-Soluble, RNA and DNA Fractions

Table 25 is a study of the 'in vitro' incorporation of uridine-2-C¹⁴ into the acid-soluble nucleotides, RNA and DNA of Ehrlich ascites tumour cells, at various times after intraperitoneal injection of colchicine.

There does not seem to be any effect of colchicine on the incorporation of labelled uridine into the acid-soluble fraction, up to 48 hours following the administration of the drug.

These results do indicate, however, a slight stimulation by colchicine, of the incorporation of this precursor into the RNA fraction. Creasey and Markiw (134) have reported stimulated incorporation of uridine-H³ into soluble RNA in Ehrlich ascites tumour cells 'in vivo', 12-16 hours after administration of colchicine. However, they have demonstrated that 24 hours after treatment with the drug there is an inhibition of incorporation of uridine into this fraction.

TABLE 25

Effect of Preinjection of Colchicine Into Ehrlich Ascites Tumour-Bearing Mice for Varying Periods of Time prior to 'in vitro' Incorporation of Radioactivity from Uridine-2-C¹⁴ Into the Acid-Soluble, RNA and DNA Fractions.

Time (hours)	Acid - Soluble			R N A			D N A		
	counts/ min.	s.e.m.	% eff.	counts/ min.	s.e.m.	% eff.	counts/ min.	s.e.m.	% eff.
1 saline	106,088	± 7375	-	21,119	± 1360	-	2394	± 167	-
1 colchicine	90,938	± 16000	- 13	21,684	± 2851	+ 2	1636	± 250	- 32
							P = 0.06		
7 saline	114,038	± 1364	-	26,336	± 1747	-	2504	± 53	-
7 colchicine	96,158	± 3648	- 16	30,392	± 1727	+ 19	1196	± 124	- 52
16 saline	105,508	± 7601	-	23,604	± 1662	-	2122	± 148	-
16 colchicine	107,092	± 4361	+ 2	29,582	± 1464	+ 25	682	± 99	- 68
24 saline	107,625	± 3627	-	21,784	± 1451	-	1820	± 153	-
24 colchicine	113,310	± 4011	+ 5	26,640	± 1550	+ 22	673	± 124	- 63
						P > 0.05			
48 saline	90,865	-		19,622	-		1230	-	-
48 colchicine	92,409	-	+ 2	18,914	-	+ 3	371	-	- 70

Uridine-2-C¹⁴ : 1,000,000 counts/min./vessel/48 ugm. Results expressed as counts/min./50 mg wet weight tissue. S.e.m.: standard error of mean. At all times except 48 hrs., the number of animals for each value was 4. At 48 hrs. the number of mice was 2. Colchicine: 1 ug/gm body weight. Cells incubated as described in Table 1.

The method employed in these experiments (Table 25) measures the incorporation of radioactivity into total RNA. This would not reveal the effects of the drug on separate RNA fractions, and a decrease in synthesis of soluble RNA would therefore not be detected.

The inhibition of incorporation of radioactivity from labelled uridine into the DNA fraction is evident soon after injection of colchicine. The results obtained one hour after treatment with colchicine indicate a tendency of decreased incorporation of label from uridine into the DNA. Although the difference in the means of the control and experimental groups after one hour does not fall between the 95% confidence limits necessary for the difference to be absolutely significant, it is safe to say that an inhibitory effect of the drug is indicated. The P value obtained from the difference of these means is 0.06 which is not too far from the accepted value of $P = 0.05$ to disregard the significance of these results.

The short time duration necessary for colchicine to inhibit the incorporation of radioactivity from uridine-2-C¹⁴ into DNA makes it difficult to explain the difference in the amounts of drug required to produce such effects 'in vivo' and 'in vitro'. We had assumed that the only possible way that such small quantities of colchicine could so greatly inhibit DNA synthesis 'in vivo', would be by the requirement of the drug for a considerable length of time in order to exert its effects. However, these results indicate that the effects of colchicine are produced only one hour after administration.

Subsequent experiments have been carried out with a period of 16 hours between the injection of colchicine, and the incubation of the tumour cells. After this length of time maximum effects of the drug are obtained.

TABLE 26

A Comparison of the Effects of Preinjected Colchicine, Vinblastine, Vincristine and Demecolcin (*) on the 'in vitro' Incorporation of Radioactivity from Uridine-2-C¹⁴ Into the Acid-Soluble, RNA and DNA Portions of Ehrlich Ascites Tumour Cells

D r u g	Number of Animals	Acid-Soluble Nucleotides			R N A			D N A		
		counts/ min.	s.e.m.	% eff.	counts/ min.	s.e.m.	% eff.	counts/ min.	s.e.m.	% eff.
Nil	(4)	48,100	± 6259	-	65738	± 1593	-	1718	± 190	-
Colchicine	(4)	63,733	± 5439	+ 30	75783	± 6422	+ 15	897	± 105	- 48
				P > 0.05						
Vinblastine	(4)	55,263	± 5303	+ 14	63306	± 3404	- 4	796	± 143	- 54
				P > 0.05						
Vincristine	(4)	57,950	± 4702	+ 20	49292	± 7597	- 25	1563	± 134	- 9
				P > 0.05			P < 0.05			
Demecolcin*	(2)	59246	-	+ 23	64680	-	- 2	1755	-	+ 2

Uridine-2-C¹⁴ : 500,000 counts/min./vessel/24 ug

Results expressed as means of number of animals indicated: - counts/min./50 mg wet weight tissue,
s.e.m.: standard error of mean
P : probability

Doses: colchicine 1 µg/gm weight animal
vinblastine 1 µg/gm " "
vincristine 2 µg/gm " "
demecolcin 1 µg/gm " "

* demecolcin: N, N-dimethyl-colchicine

Cells incubated as described in Table 1.

6.5 Comparison of the Effects of Preinjected Colchicine, Vinblastine, Vincristine, and Demecolcin, on the Incorporation of Labelled Uridine into the Ehrlich Ascites Tumour Cells

In accordance with studies of the vinca alkaloids carried out 'in vitro' (Chapter 5), it has been considered important to investigate the effects of these compounds 'in vivo'. Vinblastine and vincristine, at dose levels comparable to those of colchicine, have been injected intraperitoneally into Ehrlich ascites tumour-bearing mice, 16 hours prior to incubation of the tumour cells with uridine-2-C¹⁴. In addition, a colchicine derivative, demecolcin (N'N-dimethyl colchicine) has been similarly investigated.

As can be seen from Table 26, vinblastine is the only compound except for colchicine, which inhibits the incorporation of radioactivity from labelled uridine into DNA. As with colchicine, vinblastine has very little effect on the incorporation of uridine into acid-soluble nucleotides or RNA.

When vincristine is preinjected into the tumour-bearing mice, there is no subsequent effect on the incorporation of uridine into any fraction of the nucleic acids except for a slight decrease into RNA. This corresponds to studies carried out 'in vitro' (Chapter 5) where vincristine has been shown to have no effect on uridine-2-C¹⁴ incorporation into the cells.

Vincristine had been administered in doses of 1 µg/gram body weight, in earlier experiments. However, for the results shown in Table 26, the amount has been doubled in order to see if any greater effect could be obtained. Even at this doubled dose there is only a very slight inhibition of DNA synthesis.

Demecolcine, at the dose indicated in Table 26, is completely ineffective. This study reveals that, as in the previous 'in vitro' studies, the Ehrlich ascites carcinoma is sensitive only to colchicine and vinblastine, of the drugs tested.

6.6 The Incorporation of Thymidine-2-C¹⁴ and Thymidine-2-C¹⁴ Nucleotides into DNA in Cell-Free Extracts of Tumour Cells Pretreated with Colchicine

Because the effects of colchicine in these studies have been most marked on the incorporation of thymidine-2-C¹⁴ into the acid-soluble nucleotides and DNA, we have decided to investigate more closely the effects of the drug on the incorporation of this precursor.

It is demonstrated in Table 27 that extracts of Ehrlich ascites cells from animals pretreated with colchicine incorporate much less thymidine-2-C¹⁴ into DNA than do extracts of cells from mice treated only with normal saline. In fact, the extent of incorporation of thymidine into the DNA of colchicine-treated cells is almost negligible.

Although the mechanism of DNA synthesis in cell-free extracts is not identical to that in intact cells, the fact that there is a decrease in thymidine incorporation in both cases after treatment with colchicine, indicates that the inhibition takes place at some step common to both systems. This would involve either the phosphorylation of thymidine or the polymerization of the deoxynucleotide triphosphates.

A subsequent experiment, also described in Table 27, has shown that colchicine exerts its effect at some stage of thymidine phosphorylation to thymidine triphosphate. In this study, extracts of control and

TABLE 27

Effect of Preinjected Colchicine (1) on 'in vitro' Incorporation of Thymidine-2-C¹⁴ into DNA in Cell-Free Extracts Prepared from Control and Pretreated cells, (2) on 'in vitro' Incorporation of Thymidine-2-C¹⁴ Nucleotides into DNA of Cell-Free Extracts Prepared from the Control and Pretreated Cells

P r e c u r s o r	DNA counts/min./50 mg wet weight extract	
	Control	Colchicine
Thymidine-2-C ¹⁴	658	84
	600	68
	740	80
Thymidine-2-C ¹⁴	448	733
Nucleotides	476	430
	742	413

Thymidine-2-C¹⁴ : 220,000 counts/min./vessel

Extracts of the tumour cells were prepared as described in Chapter 2 and incubated with 0.1 ml of 0.1 M phosphate buffer, 0.1 ml calf thymus DNA (0.2 mg/ml), 2 umoles ATP, 2 umoles MgCl₂, thymidine-2-C¹⁴ and water to 2 ml.

To obtain labelled thymidine nucleotides, an extract of tumour cells was prepared as in Chapter 2, and incubated with the same additions as above except for the DNA. After one hour, the reaction was stopped with trichloroacetic acid (5%) at 0°C.

Chromatography of an aliquot of this acid-soluble fraction revealed a 70% conversion of labelled thymidine to thymidine derivatives.

The supernatant containing labelled thymidine nucleotides was added to extracts of cells pretreated with saline or with colchicine.

pretreated cell have been incubated with C^{14} -labelled thymidine nucleotides, which have been obtained as follows: an extract of the Ehrlich ascites tumour cells has been prepared as described in Chapter 2.4, and incubated with phosphate buffer, ATP, $MgCl_2$ and thymidine-2- C^{14} (Table 26). After one hour's incubation, the reaction has been terminated, and the acid-soluble fraction obtained. Chromatography of this fraction has revealed that 70% of the labelled thymidine is converted to thymidine nucleotides, of which 85% are in the form of C^{14} -TTP. Extracts from colchicine-treated cells incubated with these labelled nucleotides do not synthesize any less DNA than do saline-treated tumour cells. Thus the effect of colchicine is not on DNA polymerase.

Studies on the incorporation of uridine-2- C^{14} into DNA of colchicine-treated mice suggest that the inhibition of incorporation of labelled thymidine is at some stage other than the phosphorylation of thymidine to TMP. When uridine is incorporated into DNA, it must be first converted to TMP. This does not involve thymidine kinase, for the uridine must first be phosphorylated to UMP.

The reaction sequence is as follows: uridine $\rightarrow$ UMP $\rightarrow$ UDP $\rightarrow$ dUDP $\rightarrow$ dUMP $\rightarrow$ TMP. There is no formation of thymidine itself in this series of reactions. Yet there is an inhibition of incorporation of radioactivity from uridine-2- C^{14} into DNA in colchicine-treated tumour cells. Assuming that this inhibition is due to the decreased incorporation of the labelled TMP formed from uridine-2- C^{14} , the effect of the drug would, by necessity, occur at some stage later than the phosphorylation of thymidine to TMP.

As the majority of the labelled thymidine nucleotides in Table 27 are in the form of thymidine triphosphate, the exact stage of inhibition

of phosphorylation of thymidine derivatives would not be detected, and no inhibition of DNA is observed. Further studies involving the separation of thymidine nucleotides in cell-free extracts of colchicine-treated tumour cells are necessary in order to clarify the exact site of action of colchicine.

Summary of Chapter 6

1. When Ehrlich ascites tumour-bearing mice are injected with colchicine and sacrificed 16 hours later, there is a marked decrease in the synthesis of DNA, as measured by 'in vitro' incubation of the harvested tumour cells with labelled nucleic acid precursors. The incorporation of radioactivity from thymidine-2-C¹⁴, adenine-8-C¹⁴ and uridine-2-C¹⁴ is greatly diminished when the cells have been thus pretreated with colchicine.

Only in the case of thymidine-2-C¹⁴ is there a concurrent inhibition of incorporation of radioactivity into the acid-soluble nucleotides in cells which have been administered the drug.

2. Time studies have revealed a tendency of colchicine to inhibit DNA synthesis as early as one hour after preinjection of the tumour cells with the drug. Sixteen hours after administration of colchicine, the incorporation of radioactivity from uridine-2-C¹⁴ into DNA is maximally decreased.

3. The amount of colchicine required to produce the maximum inhibition of DNA synthesis is 1 or 2 µg/gram body weight. Doses of the drug in excess of 5 µg/gram weight animal have proved to be lethal.

4. The effects of preinjected colchicine on the 'in vivo' incorporation of uridine-2-C¹⁴ into the nucleotide and nucleic acid fractions of

Ehrlich ascites tumour cells have been studied. These indicate that there is also a marked decrease in DNA synthesis. As in the experiments where the colchicine-treated cells have been incubated 'in vitro', there are no significant effects on the incorporation of this nucleoside into the acid-soluble fraction, or RNA 'in vivo', after administration of the drug.

5. Similar investigations have been carried out on tumour cells pretreated with vinblastine, vincristine and demecolcin. As in the studies described in the previous chapter, only vinblastine exerts a similar inhibitory effect on DNA synthesis to that of colchicine. Vincristine and demecolcin at the doses indicated have no marked effect on uridine-2-C¹⁴ incorporation into nucleotides or nucleic acids.

6. Cell-free extracts prepared from Ehrlich ascites tumour cells which have been pretreated with colchicine 'in vivo' incorporate much less thymidine-2-C¹⁴ into DNA, than do extracts from cells pretreated with normal saline.

However, when extracts of colchicine-treated tumour cells are incubated with C¹⁴-labelled thymidine nucleotides, there is no inhibition of DNA synthesis when compared to control cells. Thus the effect of colchicine on the incorporation of thymidine-2-C¹⁴ into DNA is due to decreased conversion of labelled thymidine, at some stage, to labelled TTP.

CHAPTER 7

DISCUSSION

The results of Chapters 1 and 2 demonstrate the existence of active deoxynucleoside phosphorylases in the Ehrlich ascites carcinoma. These studies have been carried out both in intact cells and in crude cell-free extracts, in order to see how these systems function in relation to the other enzymes of nucleic acid biosynthesis. The exact nature of any of these enzymes could not be discerned with these methods.

However, their functioning in the intact cells is directly influenced by the other existing enzymes. For example, thymidine kinase in these tumour cells will phosphorylate any thymidine formed from the reaction of thymine and deoxyribose-1-phosphate. This would tend to consistently "pull" the reaction towards more thymidine formation. Even if the results of studies on purified extracts of thymidine phosphorylase were to reveal that the isolated reaction favours the cleavage of thymidine to thymine and deoxyribose, this is not a true picture of what occurs 'in vivo'.

As discussed in Chapter 1, rapidly proliferating tissues have a tendency to utilize scavenger mechanisms for the synthesis of DNA. Certain of these alternate pathways are active in the Ehrlich ascites carcinoma. These include the phosphorylases and the kinases, which exist mainly for the utilization of preformed precursors.

Ehrlich ascites cells possess active systems for the 'de novo' synthesis of purines (193, 194). However, evidence has been presented that this tissue does not utilize certain precursors which are involved in the 'de novo' biosynthetic pathway of pyrimidine nucleotides.

Kusama and Roberts (195) have demonstrated the inability of Ehrlich ascites tumour cells to incorporate labelled CO_2 into pyrimidines. Thus the cells are incapable of fixating CO_2 to form carbamyl phosphate, and would depend on other host tissues for a supply of this pyrimidine precursor. Scholefield (196) has also reported a lack of incorporation of C^{14} -labelled aspartic acid into pyrimidine nucleotides in the Ehrlich ascites carcinoma. These investigations emphasize the importance of the utilization of alternate pathways for the synthesis of pyrimidine nucleotides in this tissue. If the cells are unable to synthesize pyrimidines 'de novo' and are dependent on the supply of carbamyl phosphate from other sources, it is possible that they might "choose" the alternative of utilizing completed pyrimidines for DNA synthesis.

However, the fact cannot be ignored that the levels of free bases and deoxynucleosides in these tissues are exceedingly low. Experimental results from our laboratory, which are not tabulated in this thesis, indicate that when labelled pyrimidines or deoxynucleosides are incubated with intact Ehrlich ascites cells, there is very little free radioactive precursor detected in the acid-soluble fraction after incubation. As much as 90% of the radioactivity is located in the nucleotide portion of the cells. Chang and Looney (197) have reported that tritiated thymidine disappears 30 minutes after injection into the circulatory system of the rat. Any thymidine which is not phosphorylated in regenerating rat liver is degraded to β -aminoisobutyric acid within two minutes.

The problem, then, lies in the source of thymine or thymidine as substrates for the active phosphorylase and kinase systems of the Ehrlich ascites tumour cells. One report which may be of some use is that

starvation of chickens reduces the number of cells undergoing DNA synthesis (198). The possibility exists that some nucleic acid precursors may be nutritionally supplied to Ehrlich ascites tumour cells and are incorporated to nucleotides and nucleic acids as soon as they have entered the cells.

The data presented in this thesis has been fairly broad in scope and does not indicate the exact nature of the transformations studied. For example, it is not possible to discern from these experiments whether the transfer of deoxyribose from deoxyguanosine to thymine is the result of coupled phosphorylase systems, or of the action of a single enzyme. It has been shown that systems involving the transfer of ribose from nucleoside to base, which are very similar to the reactions described in this thesis, are the result of two separate enzymes (55, 199).

Zimmerman and Seidenberg (61) have stated that although the Ehrlich ascites tumour contains thymidine phosphorylase, there is no evidence of deoxyribose transferase activity in this tissue. By "transferase" they define an enzyme which can directly transfer deoxyribose from a deoxynucleoside to a base. However, our results indicate that several deoxynucleosides can donate deoxyribose to thymine or uracil in the Ehrlich ascites carcinoma. The system referred to by these authors is one where the transfer reaction is carried out by a single enzyme, a "deoxyribosyl transferase". If this is so, then what we have observed in the Ehrlich ascites tumour cells must be a coupled system involving two enzymes. The only known systems which can transfer deoxyribose in one step, are found in bacteria (64, 177). Undoubtedly, studies on more highly purified extracts are necessary to establish the exact mechanism.

Several results from the experimental work carried out for this thesis indicate differences in the systems which incorporate uracil to UMP in the presence of guanosine, and to dUMP in the presence of deoxyguanosine. Studies of intact cells have shown that there is much greater conversion of uracil into acid-soluble nucleotides in the presence of guanosine, than in the presence of deoxyguanosine. Thus either the transfer of ribose from guanosine to uracil is more active than the donation of deoxyribose from deoxyguanosine to this base, or the phosphorylation of uridine to UMP is more rapid than the phosphorylation of deoxyuridine to dUMP. Skold (68) has found that highly purified uridine kinase will not convert deoxyuridine to dUMP. It has also been shown that thymidine kinase will phosphorylate deoxyuridine (69).

In cell-free extracts, there is no uridine formed from uracil and guanosine in the absence of ATP and $MgCl_2$. However, there is considerable formation of deoxyuridine from uracil and deoxyguanosine when incubated under identical conditions. This is also true when uracil is incubated with ribose-1-phosphate, or with deoxyribose-1-phosphate. No uridine is formed from uracil when it is in the presence of ribose-1-phosphate when ATP and $MgCl_2$ are absent. Yet deoxyuridine accumulates when uracil is incubated with deoxyribose-1-phosphate in the identical manner. De Verdier and Potter (63) have demonstrated that the transfers of ribose and deoxyribose to thymine from uridine and deoxyuridine are carried out by two separate enzymes. This would be in keeping with our results. It is therefore possible that uridine and deoxyuridine phosphorylase are two separate enzymes, as has been postulated for uridine and deoxyuridine kinase.

Thus the results of Chapters 3 and 4 indicate that the Ehrlich ascites tumour cells possess several mechanisms for the utilization of thymine and uracil for DNA biosynthesis. This further supports the theories discussed in Chapter 1, of increased incorporation of preformed precursors into rapidly proliferating tissues.

With regard to colchicine, the treatment of the Ehrlich ascites tumour cells with this drug has produced rather baffling results. The complexity of its effects would seem to indicate that it has several sites of action. There is a considerable discrepancy in the effects of the drug 'in vivo' and 'in vitro'.

'In vitro', colchicine inhibits the incorporation of labelled uridine into nucleotides, RNA, and DNA to a considerable extent. The effect of the drug on this precursor seems to be at the stage of phosphorylation of uridine to UMP. However, when the tumour cells have been treated with colchicine 'in vivo', the incorporation of labelled uridine into the total acid-soluble nucleotides and RNA is unaffected. Only the incorporation of radioactivity from this precursor into DNA is inhibited.

Conversely, thymidine-2-C¹⁴ incorporation into DNA is inhibited in the presence of colchicine 'in vitro' but the drug has no effect on the incorporation of this precursor into acid-soluble nucleotides. However, when colchicine is administered to the Ehrlich ascites tumour cells 'in vivo' there is a profound inhibition of incorporation of labelled thymidine into both the acid-soluble and DNA fractions. The effect of colchicine 'in vivo' has been therefore located tentatively at some stage of phosphorylation of thymidine to TTP. From the experiments we have carried out, the 'in vivo' effects of colchicine seem to be more clearly defined and

to fall, as it were, into a neater pattern.

The discrepancy in the amounts of colchicine necessary to produce effects 'in vivo' and 'in vitro' are quite difficult to explain at present. One might suggest some sort of previous activation of the drug which would occur with greater facility in the host than in suspensions of cells. In keeping with this idea, experiments have been carried out where the effects of colchicine have been studied in suspensions of tumour cells incubated in the presence of mouse liver and kidney slices (not tabulated). These have not revealed any increased activity of the drug, and there does not therefore seem to be any apparent metabolism of colchicine to a more active form by these host tissues. Nor is there an enhancement of the inhibition by colchicine when the cells are incubated in their own ascitic fluid.

One of the main differences between Ehrlich ascites tumour cells 'in vitro' and 'in vivo' is the fact that the cells divide only when they are inside the host. 'In vitro' preparations of cells exhibit no growth whatever under the conditions studied. Since the known effect of colchicine is on mitosis, the 'in vitro' studies with the drug are possibly totally unrelated to the action of the drug on cell division.

This leads to the question of the relation of colchicine as an anti-mitotic agent, to its effects on DNA synthesis 'in vivo', which have been described in Chapter 6. Several reports have described the temporal relation of DNA synthesis to mitosis. These indicate that the phase of DNA duplication takes place only in interphase (109, 111). It is known that cells which are undergoing mitosis do not synthesize DNA (112).

Since colchicine produces a blockage of metaphase (117), the number of cells in mitosis would increase after treatment with the drug.

This has been demonstrated in Ehrlich ascites tumour cells where, twelve hours after administration of colchicine, the percentage of cells undergoing mitosis increases from 0.82 to 5.9 (202). However, DNA synthesis, as measured by the incorporation of labelled adenine, thymidine or uridine, is inhibited by 50% or more by colchicine at 16 hours after administration. This inhibition is too great to be accounted for by the increased percentage of cells in mitosis.

Conversely, it is not possible to state with certainty that the blockage of DNA synthesis accounts for the anti-mitotic effects of the drug. If this were so, the effects of colchicine would more likely appear at the prophase stage (105). However, cytological studies with 5-fluorodeoxyuridine (5-FUdR) have revealed that this analogue produces identical effects to those observed in colchicine-treated cells (200, 201). These include arrested mitotic division, large, fused nucleoli and masses of ribonuclear protein in the nucleoplasm. This compound inhibits DNA synthesis and cell division in tissue culture, and its effects can be reversed by the addition of thymidine.

These cytological observations indicate that there may be some connection between DNA synthesis and metaphase arrest. Perhaps the DNA whose synthesis is inhibited is responsible for coding the proteins which form the spindle.

In relation to these studies on 5-FUdR and mitotic inhibition, our results on the specific effect of colchicine on the incorporation of thymidine into acid-soluble nucleotides are especially significant. Both colchicine and 5-FUdR limit the availability of thymidine triphosphate for DNA synthesis, and both produce mitotic arrest in the same manner.

These preliminary studies on the effect of colchicine on DNA synthesis 'in vivo' have been very interesting to the present investigator and most certainly will be pursued in the future.

CLAIMS TO ORIGINAL RESEARCH

1. Deoxyguanosine, deoxyinosine and, to a lesser extent, deoxyuridine, at concentrations of 1 mM, stimulate the incorporation of thymine-2-C¹⁴ into DNA of Ehrlich ascites tumour cells. Other deoxynucleosides have no such effect in intact cells.

2. When deoxyguanosine is incubated with thymine-2-C¹⁴ in these cells, there is an increase in labelled thymidine in the acid-soluble fraction, and a marked increase in radioactive thymidine nucleotides. This indicates that deoxyguanosine may function as a source of deoxyribose forming thymidine from thymine.

3. Deoxyguanosine inhibits the incorporation of radioactivity from thymidine-2-C¹⁴ and uridine-2-C¹⁴ into DNA of Ehrlich ascites cells. This is accompanied by an increase in the labelling of the acid-soluble fraction, presumably due to accumulation of DNA precursors which have been blocked from incorporation into DNA.

4. Deoxyguanosine and deoxyinosine increase the incorporation of radioactivity from uracil-2-C¹⁴ into acid-soluble nucleotides and into DNA. Unlike thymine-2-C¹⁴, the incorporation of uracil into these fractions is also stimulated by guanosine, inosine and adenosine.

5. Glucose (10 mMolar) inhibits the incorporation of thymine-2-C¹⁴ into DNA in the presence of deoxyguanosine. Glucose also slightly inhibits the incorporation of radioactivity from uridine-2-C¹⁴ into DNA.

6. When uracil-2-C¹⁴ is incubated in the presence of deoxyguanosine, there is an increase in labelled thymine from the hydrolysed DNA. This indicates that, as with thymine-2-C¹⁴, there is a transfer of deoxyribose to

uracil forming deoxyuridine. The deoxyuridine can form TMP and be incorporated into DNA, by a series of known transformations.

7. Deoxyguanosine, deoxyinosine, deoxyuridine and thymidine, at concentrations of 1 mM, stimulate the conversion of thymine to thymidine in cell-free extracts of Ehrlich ascites tumour cells.

8. In the presence of ATP and $MgCl_2$, deoxyguanosine, deoxyinosine, deoxyuridine, deoxycytidine, and deoxyadenosine (all 1 mM), stimulate the formation of labelled thymidine nucleotides from thymine-2-C¹⁴. There is no such increase in radioactive thymidine nucleotides when thymine-2-C¹⁴ is incubated with thymidine in identical conditions. This is presumably due to feedback inhibition of thymidine kinase by unlabelled TTP.

9. The presence of thymidine phosphorylase in extracts of these tissues is indicated by

- (a) decreased formation of thymidine from thymine plus deoxyguanosine with increased concentrations of phosphate ions. This indicates phosphorylytic cleavage of the thymidine formed.
- (b) arsenate brings about a similar inhibition of thymidine formed from thymine and deoxyguanosine when incubated in the absence of ATP and $MgCl_2$ (in the presence of ATP and $MgCl_2$, there is no inhibition by arsenate).
- (c) labelled thymine combines readily with ~~ribose-1-phosphate~~ ^{deoxyribose-1-phosphate} in extracts of Ehrlich ascites tumour cells, with the resulting formation of thymidine-C¹⁴.

10. Labelled uracil combines with deoxyribose-1-phosphate or deoxyguanosine forming deoxyuridine. However, when uracil-2-C¹⁴ is incubated with ribose-1-phosphate or guanosine, there is no formation of radioactive uridine, in identical conditions. These findings indicate that there are two separate systems for forming uridine and deoxyuridine from uracil.

11. Tritiated deoxyguanosine incubated with unlabelled thymine produces labelled thymidine which is further evidence of deoxyribose transfer from deoxyguanosine to thymine.

12. Colchicine, at a concentration of 1 mM inhibits the incorporation of radioactivity from adenine-8-C¹⁴, guanine-8-C¹⁴, thymidine-2-C¹⁴ and uridine-2-C¹⁴ into DNA of Ehrlich ascites tumour cells 'in vitro'. In addition, the drug markedly inhibits the incorporation of labelled uridine and guanine into acid-soluble nucleotides.

13. When intact Ehrlich ascites tumour cells are incubated with uridine-2-C¹⁴ in the presence of 1 mM colchicine, the drug inhibits the conversion of labelled uridine to UMP. However, when a cell-free extract is incubated with uridine-2-C¹⁴ there is no inhibition of its phosphorylation to UMP by colchicine.

14. When a cell-free extract is added to intact tumour cells, the inhibition of incorporation of uridine-2-C¹⁴ into the nucleotides and nucleic acids by colchicine is considerably diminished. This indicates that the drug combines with some cell component, liberated upon disruption of the cells, which renders it inactive.

If the extract is boiled prior to incubation with the whole cell suspension, there is no decrease in the usual inhibition caused by colchicine.

15. Two vinca alkaloids, at concentrations corresponding to those of colchicine, have been incubated with uridine-2-C¹⁴ in Ehrlich ascites tumour cells 'in vitro'. Vinblastine inhibits the incorporation of labelled uridine into nucleotides and nucleic acids to the same extent as colchicine (a 60% inhibition is obtained). Vincristine, however, is apparently less effective an inhibitor of uridine incorporation into these cells.

16. Preinjection of Ehrlich ascites tumour-bearing mice with colchicine (1 μ g/gm body weight) results in a subsequent decrease of incorporation of radioactivity from C^{14} -labelled adenine, uridine and thymidine into DNA of the tumour cells 'in vitro'. There is a concurrent decrease in incorporation of thymidine-2- C^{14} into the acid-soluble nucleotides of pretreated cells. The time required for colchicine to exert its maximum inhibitory effects 'in vivo' is 16 hours.

17. When colchicine is administered to Ehrlich ascites tumour cells, there is also a subsequent decrease of incorporation of radioactivity from uridine-2- C^{14} into DNA 'in vivo'.

18. The incorporation of thymidine-2- C^{14} into DNA in cell-free extracts of tumours from mice which have been previously treated with colchicine is greatly inhibited. However, pretreated cell-free extracts do not incorporate any less labelled thymidine nucleotides into DNA than to extracts from control animals. Thus the inhibition of incorporation of thymidine-2- C^{14} into DNA of colchicine-treated cells is at some stage of thymidine phosphorylation to TTP.

19. When vinblastine, at doses corresponding to those of colchicine, is preinjected into Ehrlich ascites tumour cells, there is a subsequent inhibition of incorporation of radioactivity from uridine-2- C^{14} into DNA. However, vincristine and demecolcin are ineffective at these doses.

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