Ph.D.

Biochemistry

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Nucleotide Metabolism in Ehrlich Ascites Cells

ABSTRACT

Studies have shown that about 95 per cent of the radioactivity incorporated from glucose-U-^{14}C into RNA nucleotides is found in the ribose moiety of each nucleotide.

The radiospecific activity of the ribose moieties of guanine and uracil were the highest; that of adenine was somewhat lower, while the specific activity of the ribose of cytosine was about ten times lower than the above three.

It has been shown that the specific activities of the various nucleotides of RNA are a reflection of the labelling of the corresponding nucleotides in the acid-soluble fraction.

These results are discussed in relation to the comparative utilization of purine and pyrimidine bases by radioactive ribose-phosphate for the formation of the labelled nucleotides of RNA.

NUCLEOTIDE METABOLISM IN EHRLICH ASCITES CELLS

by

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CHAPTER I

INTRODUCTION

The recognition of the biological significance of nucleic acids has led many investigators to study the way a living organism goes about assembling or biosynthesizing these polynucleotides. The nucleic acids are made up of a few relatively simple components. There are essentially five different nitrogenous bases, each joined with one sugar and one phosphate to form a nucleotide. The primary structures of deoxyribose nucleic acid and ribose nucleic acid are the same except for the absence of the hydroxyl group on carbon-2 of deoxyribose and the presence of thymine (5-methyl uracil) instead of uracil in DNA. Kornberg first demonstrated (1) that purine and pyrimidine nucleoside triphosphates are substrates for DNA synthesis. Many investigators (2) have confirmed this and shown that the above compounds are also precursors for the formation of RNA, as illustrated in schemes 1 and 2.

(1) $$n(dTTP + dCTP + dGTP + dATP) \xrightarrow[\text{DNA primer}]{\text{polymerase}} DNA - (dTMP\text{-}dCMP\text{-}dGMP\text{-}dAMP)_n + 4(n)\ PPi$$

(2) $$n(UTP + CTP + GTP + ATP) \xrightarrow[\text{Template DNA}]{\text{polymerase}} RNA - (UMP\text{-}CMP\text{-}GMP\text{-}AMP)_n + 4(n)\ PPi$$

Direct evidence for the importance of nucleotides was also obtained by Potter and his coworkers (3, 4) who identified the 5'-monophosphates, diphosphates and triphosphates of adenosine, guanosine, cytidine and uridine in acid-soluble extracts of rat tissues. Hurlbert and Potter (5) showed that orotic acid-6-^{14}C was converted to uridine-5'-phosphates (UMP, UDP, UTP) and was incorporated into RNA as UMP. The occurrence of the deoxyribonucleoside di- and triphosphates in the acid-soluble fractions of animal cells has also been established (6, 7). This suggested that the nucleoside-5'-phosphates constitute a metabolic pool for the immediate precursors of the nucleic acids.

In addition to this important function as precursors of nucleic acids, nucleoside triphosphates are also the precursors of many conjugated nucleotides such as nicotinamide adenine dinucleotide (NAD), coenzyme A (CoA), uridine diphosphate glucose (UDPG), cytidine diphosphate choline (CDPC), deoxycytidine diphosphate choline (dCDPC), deoxythymidine diphosphate rhamnose (dTDPR) etc., which are the co-factors or intermediates in various biochemical reactions involving electron transport and sugar or lipid metabolism (8).

In this introduction, the biosynthesis of the purine and pyrimidine nucleotides will be briefly described. Consideration will also be given to the metabolism of these nucleotides and of RNA in normal and neoplastic cells and to the regulatory functions of various nucleotides as positive or negative effectors in nucleotide biosynthesis.

1.1 Purine Biosynthesis de novo

The biosynthesis of purine nucleotide from small molecules such as glycine, formate, aspartic acid, glutamine and carbon dioxide has been elucidated chiefly by the studies of Buchanan, Greenberg and their coworkers (9-15). The reaction sequence leading to the formation of inosinic acid (IMP) is illustrated briefly in Fig. 1. In the de novo pathway, IMP is built up stepwise on the first carbon of ribose-5-phosphate. This compound is obtained from glucose via the transketolase-transaldolase or the hexose monophosphate shunt (16). The ribose-5-phosphate thus formed is then converted to phosphoribosylpyrophosphate (PRPP) by the donation of a pyrophosphate group by ATP to carbon one of ribose-5-phosphate through the action of a kinase (17, 18). PRPP is the activated form of ribose phosphate which reacts with free purines and pyrimidines to form nucleotides directly. In the present reaction sequence, it combines with glutamine to form 5-phosphoribosylamine. The successive addition of glycine (in the presence of ATP) and of formate (from N^{5-10}-anhydroformyltetrahydrofolic acid) yields formylglycinamide ribotide. Reaction with glutamine produces formylglycinamidine ribotide, which is converted by ATP to aminoimidazole ribotide. This compound then combines with CO_2 and with the amino group of aspartate to form 5-amino-4-imidazole-N-succinocarboxamide ribotide. The succino-compound is cleaved to fumaric acid and 5-amino-4-imidazole-carboxamide ribotide. The single carbon atom needed to complete the ring is contributed by N^{10}-formyltetrahydrofolic acid and the enzyme inosinicase catalyses the removal of water, closing the ring to form inosinic acid (IMP).

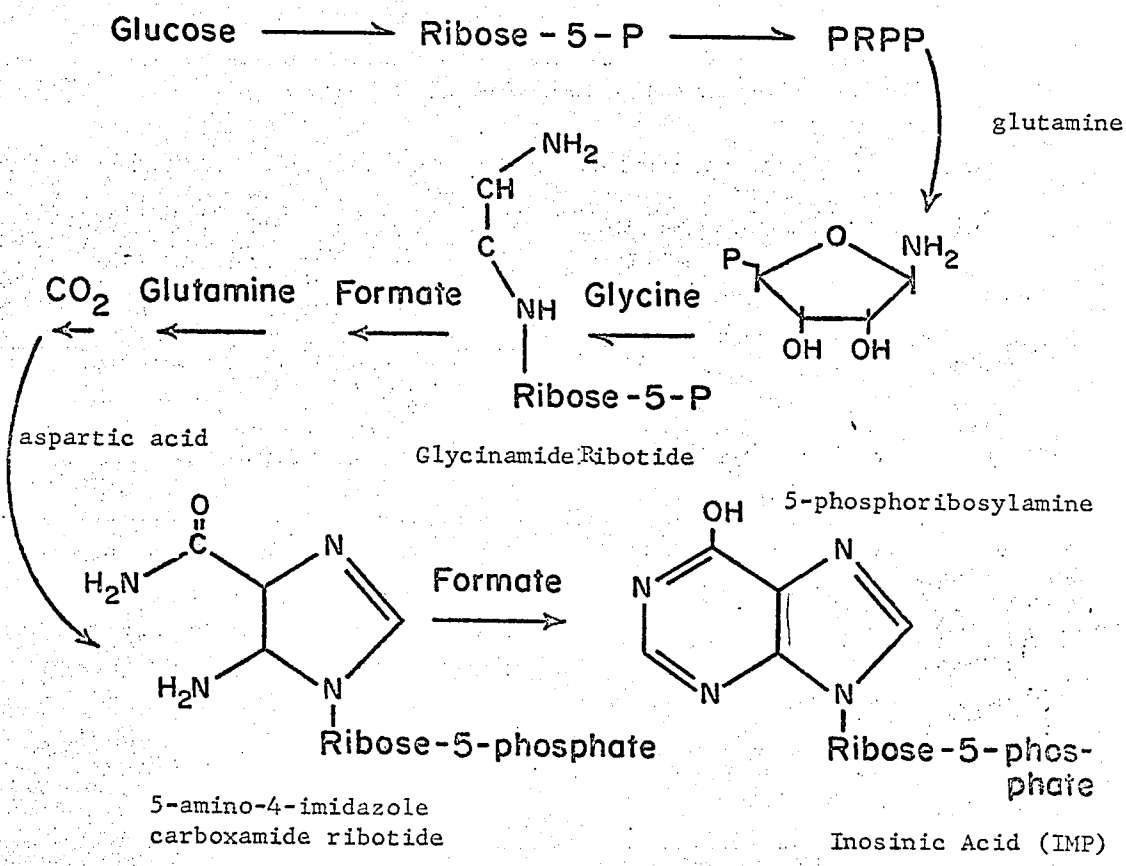


FIG. 1: Summary of purine nucleotides biosynthesis by the de novo pathway.

Since the several precursors of purine nucleotides are usually present in cells, major emphasis has been placed upon de novo synthesis.

1.2 Formation of Adenosine-5'-Phosphates and Guanosine-5'-Phosphates from Inosine-5'-Phosphates

Organisms supplying their purine requirements through de novo synthesis must convert IMP to AMP and GMP in order to provide nucleic acid adenine and guanine. The formation of these two compounds was, therefore, considered a part of the de novo biosynthetic route. The amination of IMP to AMP has been observed in animal (20, 21), and bacterial extracts (22). From the latter experiments Liebermann has shown that IMP is first converted to adenylosuccinic acid (SAMP) and then to AMP. In bone marrow, GMP is formed by the oxidation of inosinic acid to xanthylic acid (XMP) and amination to GMP (21). The aminating agent appears to be NH_4^+ in the bacterial system (23) and glutamine in the animal systems (20, 21). Deamination of AMP directly to IMP occurs in bacterial and mammalian extracts (24, 25) and a reductive deamination of GMP to IMP has been described in E.coli (26) and mammalian liver (27).

Magasanik et al. (26) have shown that in bacteria IMP occupies a central position in the metabolism of purine nucleotides (Fig.2). The pathways responsible for the biosynthesis of AMP and GMP, and for the interconversion of these nucleotides consist of irreversible reactions and pass through IMP. These features permit the cell to produce adenine and guanine nucleotides readily either from an exogenously supplied purine base or via de novo synthesis and to control the accumulation of these

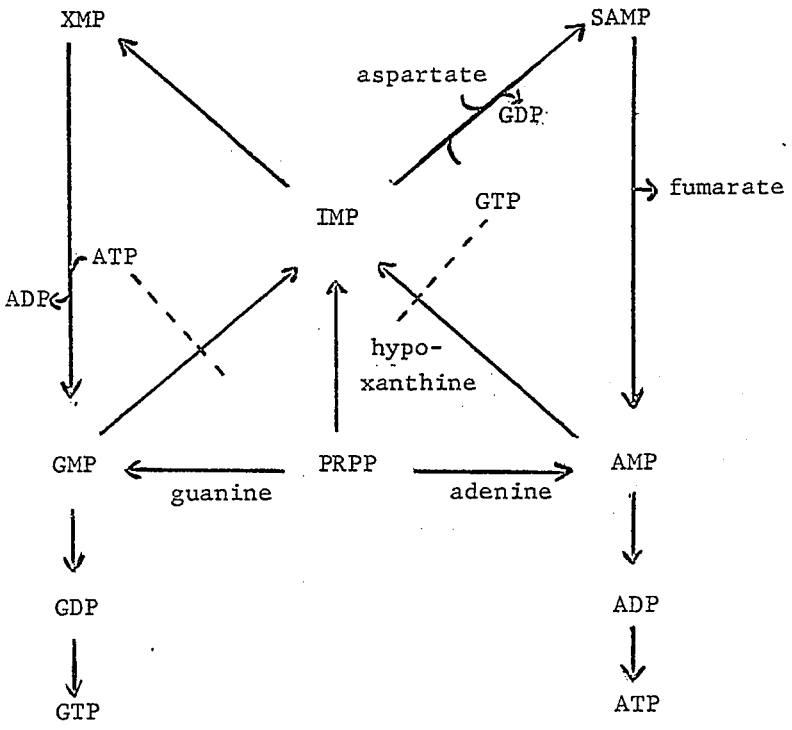


FIG.2: Formation of adenosine-5'-phosphates and guanosine-5'-phosphate from inosine-5'-phosphate and from preformed bases. (--------) inhibition.

nucleotides through feedback inhibition by the end products of these reactions, GTP and ATP. Although most of the enzymes for the operation of this cycle are widely distributed in animal tissues (25) a detailed analysis of the conversion of adenine nucleotides to guanine nucleotides has not been carried out in mammalian tissues.

1.3 Purine Biosynthesis by de novo and Salvage Pathways in Animal Cells

Experiments with both normal and malignant tissues from animals have shown that formate and glycine labelled with ^{14}C are incorporated into nucleic acid purines by the de novo route illustrated in Fig.1 (28-31). Of several tissues which have been studied, liver shows the greatest ability to carry out purine nucleotide synthesis de novo either in vivo or in vitro (31). Rabbit bone marrow (32), red blood cells (33) incorporate ^{14}C-formate very inefficiently into purine nucleotides in vitro, although formate-^{14}C is well incorporated into DNA thymine. Formate is used in the formation of the pyrimidine nucleotides at the level of transformation of dUMP into thymidylic acid.

Thomson, Smellie and Davidson (34) and Harrington (35) reported that addition of glucose to Ehrlich ascites cells greatly enhances the incorporation of formate-^{14}C into purine nucleotides of the acid-soluble and nucleic acid fractions. However, in bone marrow, there was no effect of glucose on formate-^{14}C incorporation into purine nucleotides. Henderson and LePage (36) and Herscovics and Johnstone (37) observed that maximum incorporation of glycine and formate-^{14}C into the purines of Ehrlich ascites

cells in vitro occurs when both L-glutamine and glucose are added to the incubation medium. This combination is much more effective than glucose or glutamine alone. The main function of glucose was shown to be a source of ribose phosphate (38). Glucose also stimulates the incorporation of glycine-2-^{14}C, uracil-2-^{14}C and adenine-8-^{14}C into the purine and pyrimidine nucleotides of various malignant tissues (38, 39). The requirements for L-glutamine in purine synthesis in Ehrlich ascites cells is in agreement with the observation that little, if any, free glutamine is present in these cells (40, 41). Selzman, Eagle and Sebring (42) have shown that the amide of glutamine is the source of two nitrogen atoms for nucleic acid adenine and of three atoms of nitrogen for nucleic acid guanine.

Lajtha and Vane (43) found that the nucleic acid purines of the bone marrow of rabbits receiving ^{14}C-formate were highly labelled. In hepatectomized animals, however, the utilization of formate-^{14}C for synthesis of DNA purines was much lower in the bone marrow, although the rate of ^{14}C-formate incorporation into DNA thymine was not altered. These workers concluded that the results seemed to indicate that in vivo the liver was the principal site of purine synthesis de novo and that it provides the tissues that have a low capacity for nucleotide synthesis de novo with partially formed purine precursors or possibly with a preformed purine. The findings that most tissues can utilize preformed purines for nucleotide synthesis (38, 44-48) are pertinent. The alternate pathways by-passing the de novo route have been termed "the salvage pathways" (40). These pathways have assumed special prominance in studies on

the mechanism of growth inhibition by purine and pyrimidine analogues. The main pathway by which purines are metabolized to ribonucleotides is via the nucleotide pyrophosphorylase enzyme reactions that combine the purine bases with PRPP to form the corresponding nucleotides. Kornberg and coworkers (50, 51) have shown that there are two phosphoribosyl transferase enzymes (called pyrophosphorylases) one for the conversion of adenine to AMP, and the other for the conversion of guanine and hypoxanthine to GMP and IMP, respectively (Fig.2).

The utilization of host purines by a transplanted mouse tumor was investigated by Henderson and LePage (52). Tumors of mice which had been injected with adenine-8-^{14}C three hours prior to transplantation of the tumor cells contained 2 per cent of the radioactivity found in the liver and 10 per cent of that in the blood cells. It was also shown that the transport of the host purine was probably mediated by the erythrocytes of the mouse (53). Siegel (54) has measured the incorporation of injected ^{14}C-adenine into RNA and DNA of several tissues of young mice. In rapidly metabolizing organs such as intestine, liver and spleen, the RNA was more highly labelled than in brain and carcass. DNA synthesis, an indicator of mitotic activity, was similarly determined and found to be high in the intestine and spleen but relatively low in the carcass, brain and liver. In liver the radioactivity of RNA adenine was much higher than that of DNA adenine.

High levels of purine nucleotide pyrophosphorylase activity have been found in rapidly dividing tissues such as the Ehrlich ascites tumor (55), human leukemic leucocytes (56) and rabbit and rat bone marrow

and spleen (55). The significance of active salvage pathways in rapidly growing tissues with respect to nucleotide and nucleic acid synthesis remains uncertain, but it certainly provides these cells with the ability of conserving extracellular purines derived from other organs and of reutilizing intracellular purine derived from the degradation of nucleic acids, nucleotides or nucleotide coenzymes.

1.4 de novo Synthesis of Pyrimidine Nucleotide

A. Synthesis of Uridine-5'-phosphate:

A sequence of enzyme reactions has now been discovered in mammalian and in microbial systems through which orotic acid is formed from small molecules and then transformed to UMP (Fig.3). The relationship of CO_2, NH_3 and aspartic acid to orotic acid was obtained mainly from in vivo (57, 58) and in vitro (59) isotope experiments. In rat liver slices CO_2 is specifically used as a source of carbon 2 of RNA pyrimidine and orotic acid (60). Furthermore, it was found that ^{15}N is introduced into orotic acid from ammonia and that the whole molecule of aspartate is a precursor of nitrogen 3 and carbons 4-7 of orotic acid in rat liver slices (60). Through the pioneering work of Cohen and Grisolia (61, 62) and Jones et al. (63) it was demonstrated that carbamyl phosphate is formed from CO_2, NH_3 and ATP in liver and microorganisms. Reichard (64, 65) was able to demonstrate that in mammalian liver the overall formation of carbamylaspartate from CO_2, NH_3 and aspartate require 2 molecules of ATP and an N-acetylglutamate derivative. The enzymic conversion of carbamyl aspartate to orotic acid was elucidated

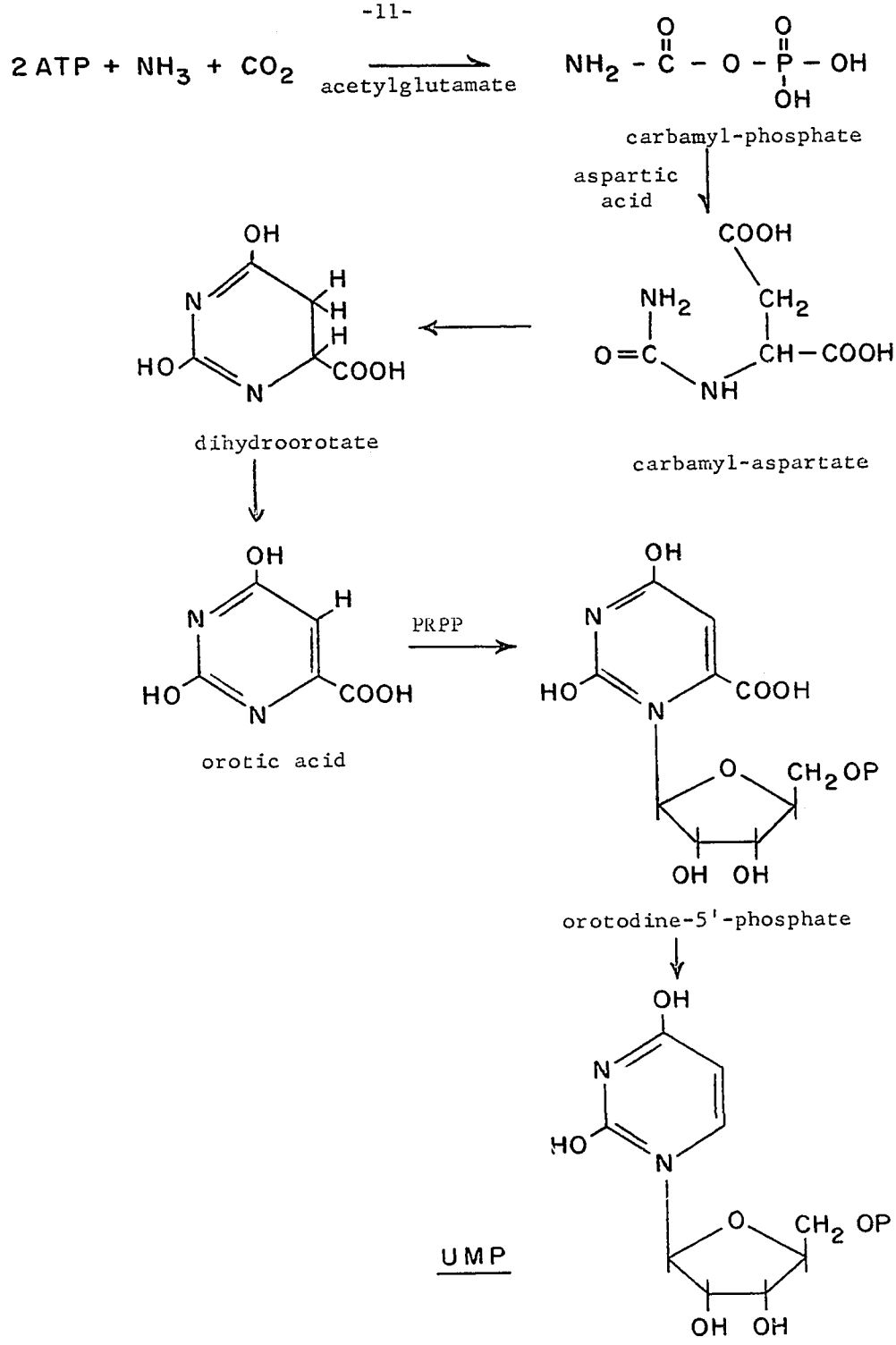


FIG.3: The de novo pathway for pyrimidine biosynthesis in animal cells.

by Lieberman and Kornberg (66, 67) who isolated two enzymes from microorganisms. Dihydroorotase effects the ring closure of carbamylaspartate to dihydroorotic acid and dihydroorotic dehydrogenase removes two hydrogen atoms at C-5 and 6 of dihydroorotic acid to yield orotic acid.

The formation of orotic acid as described above represents the actual de novo synthesis of the pyrimidine ring. There remains a series of transformation by which orotic acid is built up to uridine nucleotides to serve as building blocks for nucleic acid synthesis. The work of Hurlbert and Potter (68) and Weed and Wilson (69) showed extensive incorporation of orotic acid-6-^{14}C into acid-soluble and RNA uridine nucleotides and implicated uridine phosphates as products of orotic acid metabolism. It was the investigation of this problem that led Lieberman and Kornberg (70) to discover PRPP and the pyrophosphorylase enzyme reactions mentioned previously. They isolated two enzymes from yeast (orotidine-5'-phosphate pyrophosphorylase and orotidylic decarboxylase) and showed that the first enzyme which catalyzes the reaction between orotic acid and PRPP to give orotidine-5'-phosphate is specific for orotic acid; uracil, cytosine, adenine and dihydroorotic acid are not used as substrates. The other enzyme catalyzes the decarboxylation of orotidine-5'-phosphate to UMP. The presence of these enzymes and their specificity has been demonstrated in different mammalian tissues (71).

B. Synthesis of Cytidine Nucleotide:

A connection between uridine and cytidine nucleotides was obtained through the finding that injection of isotopic orotic acid into

animals labelled both the RNA uracil and cytosine nucleotides (68, 69). However, the uridine nucleotides of the acid-soluble fraction were labelled before the cytidine nucleotides (72).

The first demonstration of an enzyme catalysing the conversion of uridine nucleotide to cytidine nucleotide was obtained by Lieberman (73, 74) using a purified enzyme from E.coli. The conversion was found to occur at the triphosphate level by a reaction dependent upon ammonia and ATP. UMP, uridine or uracil did not serve as substrates for the enzyme but UDP was converted to a cytidine derivative at half the reaction rate obtained with UTP. It was found however that the enzyme preparation contained a nucleoside diphosphate kinase, which suggests that the UDP may have been converted to UTP prior to its conversion to CTP. Kammen and Hurlbert (75) obtained evidence for conversion of orotic-6-^{14}C and uridine-5'-phosphate-6-^{14}C to cytidine nucleotide with a partially purified enzyme from Novikoff hepatoma and rat liver. Optimal incorporation of these labelled precursors into cytidine nucleotides was obtained after addition of ATP, GTP and glutamine to the incubation medium. The experiments did not establish the nature of the acceptor for the amino group from glutamine but the involvement of UTP was considered likely.

Recently, Long and Pardee (76) reported that the cytidine triphosphate synthetase in E.coli is capable of using glutamine or ammonia as the amino donor. The enzyme has been purified 300 fold and shown to require UTP as substrate, UDP was converted to cytidine nucleotide at only 20 per cent the rate obtained with UTP.

1.5 Pyrimidine Biosynthesis by the de novo and Salvage Pathways in Animal Cells

Orotic acid serves as a precursor of uridine-5-phosphate in animal and cancer cells (71, 77). However, of all mammalian organs examined only liver seems to contain significant amounts of carbamyl phosphate (78, 79). Even tissues, such as the Ehrlich ascites tumor cells and the Novikoff hepatoma cells, which have a high pyrimidine nucleotide requirement, do not contain carbamylphosphate synthetase, although the other enzymes of the orotic pathway are present (80). Hertzfeld et al. (81) showed that no significant amount of carbamyl-phosphate is transported by the blood, indicating that the liver cannot supply this compound to extra-hepatic tissues. Hager and Jones (80) have, however, in recent experiments demonstrated that ^{14}C-bicarbonate is incorporated in vitro into carbon-2 of the uracil of the acid-soluble fraction of Ehrlich ascites cells. When orotic acid was added to the incubation medium a depression in the radioactivity of the uracil and an increase in the labelling of the carbamylaspartate were observed indicating that carbamylphosphate and orotic acid are probably intermediates in the biosynthesis of uridine nucleotides in these cancer cells. The above workers further observed that much lower concentrations of L-glutamine than ammonia were required to effect maximal incorporation of ^{14}C-bicarbonate into the uracil of the nucleotide fraction of the Ehrlich tumor cells. Mayfield et al. (82) working with Novikoff hepatome also found maximal incorporation of ^{14}C-bicarbonate into uracil of RNA in the presence of L-glutamine. This has led to the supposition that

extra-hepatic tissues probably contain a glutamine-dependent carbamyl-phosphate synthetase (80). This type of enzyme activity has been found in mushroom (83) and in E.coli (84).

The ability of rapidly growing tissues, such as tumors, to incorporate uracil and uridine into the nucleic acids has raised the possibility that in these cells the salvage pathways are of major significance for pyrimidine nucleotide synthesis. Rutman et al. (72) have shown that in vitro uracil-2-^{14}C is incorporated to a greater extent into the liver RNA of rats treated with the carcinogen 2-acetylamino-fluorene, than into RNA of normal liver. Heidelberger et al. (85) have demonstrated a similar precursor role of uracil-2-^{14}C in rat intestinal mucosa and in the Flexner-Jobling carcinoma. Experiments with uracil, uridine and uridine-5'-phosphate have indicated that both the nucleoside and the nucleotide are incorporated to a greater extent into the RNA of normal rat liver than is uracil-2-^{14}C (86). Liebman and Heidelberger (87) have shown that the nucleotide undergoes dephosphorylation prior to its entry into the cell.

Work by Fink et al. (88, 89), Rutman et al. (90) and Canellakis (92) indicated that uracil-2-^{14}C incubated with rat liver slices was rapidly catabolized with the formation of β-amino acids and CO_2. Subsequent work on the isolated enzymes showed that uracil was degraded to β-alanine, CO_2 and ammonia via 4,5-dihydrouracil and β-ureidopropionic acid. Despite the demonstrated reversibility of the enzymatic reactions resulting in the conversion of uracil to β-alanine, it is generally held that this pathway is used for the degradation of uracil not its

synthesis (93).

In contrast to previous studies, Canellakis (94) demonstrated that rat liver slices, incubated with uracil-2-^{14}C at high extracellular concentrations, incorporated this compound into RNA as effectively as uridine or orotic acid.

In subsequent study, he showed the presence of a uridine phosphorylase and uridine kinase in rat liver which together defined a pathway for the utilization of uracil by mammalian liver (95).

$$\text{Uracil} + \text{Ribose-1-phosphate} \xrightarrow[\text{phosphorylase}]{\text{uridine}} \text{uridine} + \text{Pi}$$

$$\text{Uridine} + \text{ATP} \xrightarrow[\text{kinase}]{\text{uridine}} \text{UMP}$$

Paege and Schlenk (96) have isolated uridine phosphorylase from E.coli and shown that uridine phosphorylase splits uridine but is inactive towards cytidine, thymidine or the purine nucleosides (adenosine, guanosine, inosine). At physiological pH, nucleoside formation is the favoured reaction.

Canellakis also showed that uridine phosphorylase did not react with ribose-5-phosphate nor did rat liver contain any pyrophosphorylase activity towards uracil. This enzyme catalyzes the formation of uridylic acid from uracil and PRPP and has been shown in extracts of bacteria (97). The isolation of the uridine di- and triphosphates was also reported in this study. Canellakis concluded that the existence of two enzymic pathways in the rat liver, one anabolic the other catabolic, may constitute part of a homeostatic mechanism governing the rate of RNA synthesis.

Reichard and Skold (98) demonstrated the occurrence of uridine phosphorylase and uridine kinase in acetone powder extracts of Ehrlich ascites cells. The extracts also possessed a very feeble uridine-5'-phosphate pyrophosphorylase activity capable of condensing uracil with PRPP.

In a study of alternate pathways for uridine nucleotide synthesis from uracil, Reichard and Skold (99) showed that there was a considerable increase in the activity of uridine phosphorylase and especially uridine kinase in Ehrlich ascites cells and regenerating liver over that found in either normal rat or mouse liver. In addition, rapid incorporation of uracil-2-^{14}C into RNA was obtained by the former two tissues whereas normal liver catabolized uracil to $^{14}CO_2$ extensively. Skold (100) has also found that uridine kinase increases first during liver regeneration. In addition the specific activity of this enzyme is 10-20 times lower than the phosphorylase enzyme. The author (100) concluded that uridine kinase was a rate limiting enzyme and that some degree of correlation exist between the rate of proliferation of rapidly growing tissues, such as regenerating liver and Ehrlich ascites carcinoma cells, and the efficiency of this alternate pathway.

In a more detailed study on uridine kinase (101) the enzyme has been purified 400 fold from the Ehrlich ascites cells. The enzyme requires ATP and Mg^{++} for optimal activity. It can react with cytidine and with 5 fluorouracil, a pyrimidine analogue which has found extensive use in cancer chemotherapy.

1.6 Metabolic Stability of Polynucleotides

The work of Schoenheimer and his coworkers (102) supported the concept that macromolecules and proteins undergo a continuous process of synthesis and degradation. With respect to nucleic acids, this view was called into question by Hershey (103) who observed that ^{32}P-orthophosphate incorporated into RNA and DNA of E.coli was conserved through several generations. Siminovitch and Graham (104, 105) working with bacteria and animal cells demonstrated that there was no significant loss of ^{32}P-RNA after numerous periods of cell multiplication. Watts and Harris (106) showed that loss of label from the nucleic acids depended on the nature of the radioactive precursor used. If cells were labelled with ^{32}P-orthophosphate and then transferred to medium containing unlabelled orthophosphate, there was no loss of radioactivity of RNA. However, cells labelled with adenine-^{14}C and transferred to medium containing unlabelled adenine showed an appreciable loss of radioactivity from the RNA. In studies carried out for 12-hour periods, these authors found no evidence for renewal of DNA, but for the RNA there was extensive renewal and this renewal was associated with reutilisation of breakdown products.

Similarly, Hecht and Potter (107) studied the specific activities of liver nuclear and cytoplasmic RNA as well as DNA at various times after injection of orotic acid-6-^{14}C into rats which had been partially hepatectomized 24 hours earlier. After 64 days, the specific activities of the RNA's were very low, but the specific activity of DNA was still at a high level. The results of Kihara, Amano and Sibatani (108) and of Swick,

Koch and Handa (109) indicate that in the liver of young animals there is also considerable renewal of RNA but not of DNA. Scott et al. (110, 111) studied the stability of nucleic acids in tumors. They labelled Ehrlich ascites tumor cells with orotic acid-6-^{14}C and adenine-8-^{14}C transplanted the cells into other mice and observed that the labelling in the RNA and DNA was conserved even after several generations.

Bennet et al. (112) in studying adenine-^{14}C metabolism in various tumors found a loss of label from the RNA but a decreased capacity to degrade the breakdown products of nucleic acid and an increased ability to reutilize these compounds for nucleic acid synthesis. These and other studies of conservation of nucleic acids have led Bennet et al. (112) to conclude that RNA and DNA are conserved in rapidly growing cells. The conservation of RNA is associated with some return of the acid-soluble precursors to the biosynthetic pathway, whereas the DNA is metabolically stable as long as the cell is viable. In resting cells or slowly dividing cells, DNA is conserved and is metabolically inert but RNA is extensively renewed.

1.7 Enzymes Involved in the Synthesis and Breakdown of RNA

A. Synthesis:

The nucleotide sequences of most of the cellular RNA require a directed biosynthesis and experiments carried out in many laboratories have shown that most of the RNA in the cell is probably synthesized with DNA as a template. Weiss et al. (113) isolated from rat liver a particulate

enzyme (RNA polymerase) which required the presence of all four ribonucleoside triphosphates for the formation of polyribonucleotides in the presence of DNA. Preincubation of the enzyme with small quantities of DNAase inactivated the system, suggesting that DNA is involved in this RNA synthesis. Alkaline hydrolysis of the RNA formed in this reaction after incubation with α-^{32}P-phosphate in any one of the nucleoside triphosphate precursors resulted in the liberation of 2'-3' nucleoside monophosphates that were all labelled. This indicated that the incorporated nucleotide residues were adjacent to all of the other three nucleotides. Either native or single stranded DNA (phage Ø X-174 DNA or heated DNA preparations) will act as template in the reaction, directing the assembly of ribonucleotides into polynucleotide chains.

Studies of nucleotide incorporation in cell-free preparations have disclosed the existence of a variety of enzyme fractions that catalyze the incorporation of simple nucleotides into polynucleotide material. Edmonds and Abrams (114, 115) have isolated an enzyme from extracts of calf thymus nuclei which catalyses the formation of poly A with ATP as substrate. Its activity is dependent on a polynucleotide which appears to contain an adenylate sequence. End group analysis of the newly formed poly A has shown that all of the incorporated adenylate units are in internucleotide linkages.

An enzyme or enzyme system has been partially purified from the cytoplasm of rat liver and from E.coli (116, 117, 118), which incorporates AMP and CMP from ATP and CTP in the order of 2 CMP residues

followed by one AMP residue into the terminal position of RNA. Hecht et al. (119) have shown that the product formed is probably RNA-CCA. Studies by Canellakis and Herbert (120), Hecht et al. (121), Harbers and Heidelberger (122) and others (123-126) have shown that enzymes exist in tissues that not only incorporate CMP and AMP, but also GMP and UMP to pre-existing RNA chains. Amino acid acceptor RNA contains the terminal nucleotide sequence pCpCpA but the significance of RNA terminated by other nucleotide sequences is unknown.

B. Breakdown:

Three enzymes which are capable of depolymerizing ribonucleic acid are known. One is the polynucleotide phosphorylase first isolated by Grunberg-Manago and Ochoa from E.coli (127). This enzyme is believed to be responsible for the rapid breakdown of messenger RNA in bacteria. Cohen et al. (128) observed that rapidly labelled RNA formed in phage infected E.coli after a pulse of uracil-^{14}C (defined as messenger RNA (129)) is converted to ribonucleoside-5'-diphosphates, while the bulk of ribosomal RNA is left intact. Another enzyme is the ribosomal ribonuclease (RNAase III) which is an endonuclease isolated from ribosomes of E.coli and yields nucleoside-3'-monophosphates (130). When released from the ribosomes it becomes active and attacks RNA to yield nucleoside-3'-phosphates via a 2'-3' cyclic phosphate intermediate (131, 132). According to Laskowski (133) an enzyme that catalyzes the successive removal of mononucleotides from an oligonucleotide in a stepwise manner is classified as an exonuclease; an endonuclease catalyzes the hydrolysis of phosphodiester bonds at many points within the chain.

The third enzyme is an exonuclease (RNAase I) which has been isolated from mouse leukemic cells (134), E.coli (135), Ehrlich ascites carcinoma cells and mouse liver (136). Sekiguchi and Cohen (135) have shown that this enzyme is similar to polynucleotide phosphorylase in that it can degrade the rapidly labelled RNA in phage infected E.coli but yields ribonucleotide-5'-phosphates. The properties of this enzyme are similar to those of snake venom phosphodiesterase.

Tissieres and Watson (137) have shown that ribosomes containing ^{14}C-uracil labelled messenger, incubated with a fraction containing the phosphodiester enzyme leads to breakdown of the labelled RNA to acid soluble fragments.

The work of many investigators (138-142) has demonstrated that a large proportion of the RNA synthesized in the nucleus after a short pulse of labelled precursor appears to be broken down to acid-soluble fragments. Harris (138) and Lazarus (136) suggest that the presence of this exoribonuclease in the nucleus of mammalian tissues indicates that the enzyme may be the principal agent responsible for the breakdown of nuclear RNA in mammalian cells.

2.8 End-Product Inhibition

A living cell consist in large part of a concentrated mixture of hundreds of different enzymes, each of which is a highly effective catalyst for one or more chemical reactions involving various components of the cells. The paradox of intense and highly diverse chemical activity

on the one hand and delicately poised biochemical stability (biological homeostasis) on the other is one of the most fundamental problems of biology. In the normal cell there is obviously control of the tendency to grow and to divide. The general concept that cancer might be the result of loss of these controls has been considered by many workers (112,143).

In recent years, it has become apparent that the end-product of a series of reactions may inhibit an early step in the sequence even though the end-product bears no simple structural relation to the original reactant. The first demonstration of metabolic regulation at the molecular level was discovered by Umbarger (144, 145) to operate in the sequence leading from threonine to L-isoleucine. In these reactions, L-isoleucine, the end-product, strongly inhibited the activity of L-threonine deaminase, which catalyzes the first reaction in which L-threonine is converted to isoleucine.

A case which has been particularly thoroughly studied is that of the inhibition of aspartate transcarbamylase, the first step in the pathway of pyrimidine synthesis (146). The inhibition of aspartate transcarbamylase is of considerable interest because of the dissimilarity between the structure of CTP, the inhibitor, and that of the substrate, aspartate, with which it apparently competes. Gerhart and Pardee (147, 148) presented evidence that the inhibition of aspartate transcarbamylase by CTP was not due to steric competition at the active site between the substrate and the inhibitor, but that there must be at least two different, mutually interacting types of sites. Comparison of the properties of this system with those of a number of other "regulatory enzymes" (149-153)

led to the definition of the concept of allosteric interaction (154). Allosteric proteins are assumed to possess two, or at least two, distinct non-overlapping receptor sites. One of these the active site binds the substrate and is responsible for the biological activity of the protein. The other, or allosteric site, is complementary to the structure of the allosteric effector, with which it can bind. The formation of the enzyme-allosteric effector complex is assumed to bring about a reversible transition of the protein. This transition modifies the properties of the active site, so that the kinetic parameters which characterize the biological activity of the protein are altered (e.g. the V_{max}, the affinity of the substrate or even the specificity.).

Numerous other examples are now available (155) which suggest that metabolic control in animal cells commonly involves regulation of the affinity of an enzyme for its substrate, particularly when the enzyme catalyzes the first reaction of a metabolic sequence. In addition, such changes in affinity may alter the selective effectiveness with which the enzyme competes with other enzymes that act on the same metabolite. As a result, the partitioning of that metabolite among various alternative sequences may be precisely controlled without involving the production of new metabolites or new enzyme activities.

1.9 Qualitative and Quantitative Differences between Normal and Neoplastic Cells

There has been numerous studies of the metabolism of normal

and cancer cells in the hope of finding some biochemical differences of a qualitative or quantitative nature which may be exploited for therapeutic purposes. In searches for such differences much attention has been devoted to the study of the metabolism of nucleotides *in vivo* and *in vitro* of normal and cancer cells. The reason for selecting nucleotide metabolism stems largely from two considerations:

1) Nucleic acids are essential components of heritable biological units such as genes and viruses, which are thought to be at the basis of the transformation of normal cells to cancer cells.

2) Compounds that are effective anti-cancer agents often exert their effect on nucleic acid metabolism and are for the most part structural analogues of the nucleotides that are used for nucleic acid synthesis.

The results, however, from many studies have not been conclusive. To date, no qualitative differences have been found between normal and cancer cells. Studies with various labelled precursors of purine and pyrimidine nucleotides have shown that these compounds are incorporated in the nucleic acids of tumor cells presumably by the same pathways as the normal cells. The isotopic compounds that have been used to study nucleotide metabolism in normal and cancer cells have been mainly formate labelled with ^{14}C, glycine, orotic acid, purine bases and pyrimidine bases and nucleosides labelled with ^{14}C and ^{15}N. This has led to a great deal of information as to the quantitative differences of the various pathways between normal and neoplastic cells.

However, since each of these radioactive tracers is incorporated into nucleotides by only one pathway, as shown in Fig.4, these studies give no information as to the relative contribution of the de novo and salvage pathways for nucleotide synthesis in the neoplastic cell.

Studies carried out in this laboratory on the metabolism of glucose in Ehrlich ascites tumor cells have shown that it is extensively used for nucleotide and nucleic acid synthesis (38, 45). Since ribose-phosphate is an intermediate in the synthesis of both purine and pyrimidine nucleotides de novo or by utilization of preformed bases, as shown in Fig.4, ^{14}C-labelled glucose was used in the present study to assess the significance of some of these alternate pathways in the Ehrlich ascites carcinoma cells.

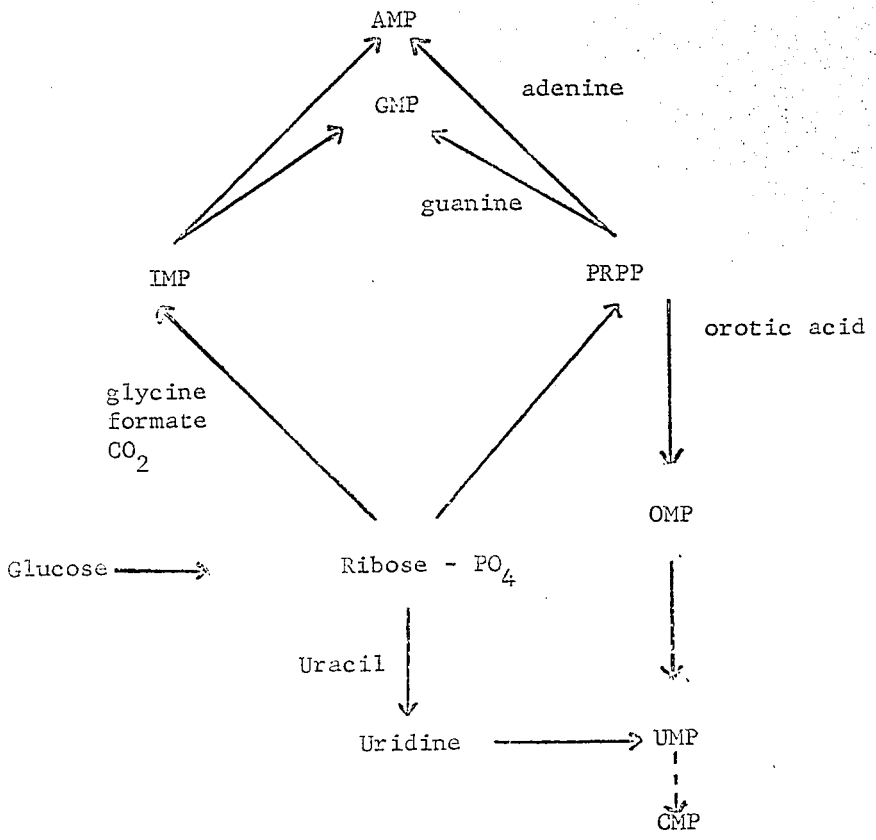


FIG.4: Compounds that can function as precursors of the nucleotides for nucleic acids.

Pointed arrow indicates that the level of phosphorylation has not been taken into account.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

A. Source of Chemicals

All common chemicals were of "Reagent grade" and were used without further purification. All bases, nucleosides and nucleotides were obtained from Sigma Chemical Co., St.Louis, Missouri, U.S.A. Actinomycin D was a gift from Dr. S.C. Sung, of the Unit of Cell Metabolism, McGill University. Azaserine was obtained from Dr. H.B. Wood, Jr., of the Cancer Chemotherapy National Service Center, National Institute of Health, Bethesda, U.S.A.

Snake venom phosphodiesterase was obtained at first from Calbiochem, Los Angeles, Calif., later from Worthington Biochemical Corp., Freehold, New Jersey. RNAase was a product of the above company.

The radioactive chemicals: glucose-U-^{14}C, glucose-1-^{14}C, glycine-1-^{14}C, phenylalanine-1-^{14}C, thymidine-2-^{14}C, adenine-8-^{14}C, uracil-2-^{14}C, cytosine-2-^{14}C, uridine-2-^{14}C, cytidine-2-^{14}C, were obtained from the Radiochemical Center, Amersham, England. (^{32}P)-orthophosphate was obtained from Charles E. Frosst Co., Montreal.

B. Purification of Norit

Norit activated charcoal, obtained from Fisher Scientific Ltd., was purified by the following procedure (156). The charcoal was suspended

in pyridine for 2-3 hours and then washed with N HCl. It was boiled for 30 minutes in HCl, filtered and washed with distilled water until all the pyridine was removed and the filtrate was neutral. After drying the charcoal was ready for use.

C. Preparation of Solutions

All solutions were made up with distilled water. Solutions of organic compounds were stored at -20°C. Solutions containing labelled compounds were made up as stock solutions without addition of carrier. For the experiments, an aliquot of the stock solution was diluted with a solution of the corresponding non-radioactive compound.

2.2 Maintenance of Tumor Cells

The Ehrlich ascites carcinoma was grown in Swiss white mice weighing 20-25 grams. The tumor was transplanted into the peritoneal cavity by injection of a suspension of cells usually obtained from the first liquid transplantation. The first liquid transplantation was routinely isolated from the subcutaneous solid tumor and stored in dry ice (157). Tumors grown for 6-8 days in the animals were used for the experiments.

2.3 Preparation of Tumor Cells

The animals were killed by cervical dislocation and the tumor cells were removed from the peritoneal cavity with a Pasteur pipette. The ascitic fluid containing the cells was diluted to 12 ml with ice cold

isotonic saline and the suspension was centrifuged at 800 x g in an International Clinical Centrifuge for 20 seconds. This procedure of differential centrifugation separated the tumor cells from the blood cells. The cells were washed at least four times with cold isotonic saline . The cells were then centrifuged at 800 x g for 2 minutes to obtain the packed cell volume. Finally, they were diluted with five times their volume of calcium-free Krebs-Ringer solution. One ml of this suspension (equivalent to 20-22 mg dry weight of tissue), unless otherwise stated, was used per three ml of incubation medium for the experiments.

2.4 Preparation of Cell-free Extracts

The tumor cells obtained after washing with cold saline were suspended in ice-cold tris buffer (0.2M), pH 8.0 to give a 30% suspension. This cell suspension was sonicated for 90 seconds in 30 second bursts and kept on ice during sonication. The ruptured cells were then centrifuged at 100,000 x g (40,000 rpm) in a Spinco Model L ultracentrifuge, Beckman rotor Ti 50, for 60 minutes. The supernatant was removed carefully taking care to exclude the lipid layer floating at the surface.

2.5 Incubation Methods

Incubation of cell suspensions was carried out in calcium-free Krebs-Ringer solution containing 145 mM NaCl, 5.8 mM KCl, 5.8 mM KH_2PO_4 and 1.5 mM $MgSO_4$ usually in a total volume of 3 ml. The pH was maintained at 7.4 by phosphate buffer (10 mM). All incubations were carried out at

37°C in 25 ml Erlenmeyer flasks containing center wells. When glucose was added to the reaction mixture 0.2 ml 20% KOH or Hyamine hydroxide was added to the center well to trap the carbon dioxide evolved during the oxidation of glucose. After incubation, the flasks were rapidly put in crushed ice to stop the reaction.

For the incubation of cell-free extracts the reaction medium contained tris buffer pH 8.0, 60 mM phosphoglyceric acid 17.8 mM ATP 6.7 mM, $MgCl_2$, in a total volume of 0.3 ml.

2.6 Measurements of Radioactivity

Radioactivity was determined using a series 3000 Packard Instrument Company liquid scintillation counter. Portions (50-200 λ) of the nucleotide solutions were counted in plastic or glass vials containing 10 ml volumes of a scintillation mixture consisting of 5.0 g. 2,5-diphenyloxazole (PPO), 50 mg 1,4 bis-2-(4 methyl-5-phenyloxazolyl)-benz[illegible] (dimethyl-PoPoP), and 80 g naphtalene in 1 litre 1:1:1 (by vol.) toluene:dioxane:ethanol.

In some experiments radioactive spots cut out from chromatograms were placed in counting vials containing 15 ml of scintillation fluid consisting of 4 gm PPO, 100 mg dimethyl-PoPoP in 1 l toluene.

2.7 Determination of the Radioactivity Incorporated into the Acid-soluble, RNA, DNA and Protein Fractions

The following separation procedures were employed.

The incubation mixture was cooled and the cells plus media transferred to centrifuge tubes containing 7 ml ice-cold Krebs-Ringer solution. The mixture was centrifuged at 800 x g for 2 minutes. The supernatant was discarded and the last drops removed with a Pasteur pipette. Subsequently 3 ml 2.5% ice cold perchloric acid (PCA) was added to precipitate the acid-insoluble components and to extract the acid-soluble compounds. After standing with occasional stirring for approximately 30 minutes, in a bath of crushed ice the mixture was centrifuged for three minutes at 800 x g. The supernatant was kept for analysis of the acid-soluble nucleotides as described in the section "Separation of the Nucleotides of the Acid-soluble Fraction".

The PCA precipitate was washed with 5 ml cold 5% trichloroacetic acid (TCA). After centrifugation the lipids were extracted from the residue by washing twice each with 5 ml 95% ethanol, ethanol-chloroform (3:1 by vol.), ethanol-ether (1:1 by vol.) as described by Hutchison et al. (158). The initial extraction with ethanol was carried out in the cold in order to prevent loss of nucleic acid material from the acid-wet residue (158).

The lipid-free residue was then washed once with 3 ml ether or with 3 ml 2% cold PCA. Residues washed with ether were allowed to stand at room temperature for about one hour to evaporate the ether. The air dried powder was suspended in 1.0 ml 0.3 N KOH and the RNA extracted according to the method of Schmidt and Thannhauser (159). When the precipitate was washed with PCA the RNA was extracted according to

a modification of the method of Ogur and Rosen (160). The washed precipitate was suspended in 1.5 ml ice cold 1.2 N PCA and allowed to stand overnight at 0-4°C. After centrifugation in the cold at 3,000 rpm for 5 minutes the supernatant, containing the RNA fraction, was decanted. The precipitate was suspended in 1.5 ml cold PCA for 30 minutes, centrifuged and the supernatant pooled with the first acid extracts. This solution was neutralized with KOH and the $KClO_4$ allowed to precipitate in the cold for at least 2 hours. The mixture was centrifuged in the cold as described above and the supernatant was taken to dryness under reduced pressure. The residue was dissolved in 0.2 ml distilled water and centrifuged at 800 x g for 3 minutes to pack the residual $KClO_4$. An aliquot of the supernatant was removed for determination of radioactivity. Further separation of the individual components of the RNA fraction was carried out by paper electrophoresis.

The precipitate obtained after extraction of RNA was washed twice with 5 ml cold 5% TCA to remove residual RNA. The DNA was then removed from this precipitate by hydrolysis at 90°C for 20-25 minutes with 1.5 ml 5% TCA. The mixture was centrifuged and the extraction was repeated with 1 ml of 5% TCA. The tube was centrifuged and the supernatant pooled with the first extract. An aliquot of this supernatant was removed for determination of radioactivity in the DNA fraction.

The precipitate, containing only proteins, was washed with 3 ml 5% TCA and suspended in 0.2 ml Hyamine hydroxide for estimation of radioactivity.

2.8 Isolation of RNA by the Phenol Method

When (^{32}P)-orthophosphate was added to the medium the RNA was extracted from the Ehrlich ascites cells with hot freshly distilled phenol according to the technique of Scherrer and Darnell (161). The tumor cells obtained after washing with cold saline were suspended in 6 ml 0.01 M acetate buffer containing sodium dodecyl sulfate, final concentration 0.25 per cent, and polyvinyl sulfate, final concentration 0.05 per cent. An equal volume of hot (60°) acetate-saturated phenol was then mixed with this solution and shaken vigorously for 10 minutes in a 60°C water bath. At the end of the extraction the temperature of the solution was reduced to 4-5°C by rapid chilling in a -20°C ice bath. The emulsion was broken by centrifugation at 20,000 x g (13,000 r.p.m.) in the rotor 870 of an International B-20 centrifuge for 5 minutes. The aqueous phase containing the RNA was removed and the extraction repeated once more for 5 minutes with hot phenol. After centrifugation as described above the aqueous phase was removed. The RNA was precipitated from the aqueous layer by the addition of 2 volumes of cold ethanol overnight at -10°C. After centrifugation, in a PR-2 International centrifuge, the RNA was washed twice with cold ethanol and dissolved in distilled water. This solution was made acid and the precipitated RNA washed once more with ethanol. It was then dissolved in 0.7 ml of distilled water.

2.9 Separation of the Nucleotides of the Acid-Soluble Fraction

To separate the nucleotides of the acid-soluble fraction

from other radioactive components (amino acids, sugar phosphates, etc.) 30 mg of activated charcoal was added to the PCA extract to absorb the nucleotides. This mixture was allowed to stand at room temperature for 30 minutes with occasional shaking, 0.2 ml of 95% ethanol layered on top of the tube to favour sedimentation of the charcoal and centrifuged at 800 x g for 5 minutes. The supernatant was discarded and the charcoal was washed twice with 3 ml distilled water, each time layering the top with 0.2 ml ethanol.

2.10 Elution of the Nucleotides from the Charcoal (221)

5 ml 5% pyridine in 50% ethanol was added to the charcoal and the mixture transferred quantitatively into 25 ml Ehlenmeyer flasks and shaken at 37°C for at least 1 hour. The mixture was decanted into a tube and centrifuged. The supernatant, containing 70-85% of the nucleotides, was taken to dryness as described previously and the residue dissolved in 0.1 ml of distilled water. An aliquot of this solution was removed for estimation of radioactivity. Further separation of the nucleotides was carried out by two dimensional paper chromatography.

2.11 Separation of Nucleotides from Nucleosides and Bases

When labelled bases (uracil-^{14}C, adenine-^{14}C, cytosine-^{14}C) were used as precursors, the incubation mixture was precipitated with 2 ml cold 5% TCA. The acid soluble fraction was extracted 6 times with 3 ml portions of ether to remove TCA and the free bases and nucleosides were separated from the nucleotides by extracting the mixture 5 to 6

times with n-butanol saturated with water (45). This procedure removes free bases and nucleosides but leaves the nucleotides in the aqueous phase.

2.12 Hydrolysis of RNA and Nucleotides to the Bases

Hydrolysis of the nucleotides in the acid soluble fraction and of RNA was carried out as described by Marshak and Vogel (162). The nucleotides and RNA fraction were dissolved in 0.1 ml 70% PCA and the tubes placed in a boiling water bath for one hour with occasional stirring. After cooling the mixture was diluted to 1.0 ml with distilled water, centrifuged, and decanted into tubes containing activated charcoal. The charcoal was spun down, washed once with water and the bases eluted as described previously. The eluate was taken to dryness, the residue dissolved in 0.1 ml water and spotted on paper. The individual purine and pyrimidine bases were then separated by descending paper chromatography.

2.13 Hydrolysis of Nucleic Acids to Mononucleotides

A. Hydrolysis of DNA to Deoxynucleotide:

In order to hydrolyze DNA into individual deoxynucleotides, the precipitates obtained after removal of the acid soluble fraction and RNA was dissolved with 1 ml 0.01 N NH_4OH (final pH, 7.0) $MgCl_2$ (final concentration 0.01 M) and DNAase (about 0.1 mg) were added and the solution incubated at 37°C for about 6 hours. The pH was adjusted to 8.5 with 1 N NH_4OH, snake venon Phosphodiesterase was added (about

2 units/mg DNA) and the solution kept at 37°C for another five hours. At the end of the incubation, the solution was made acid and kept in ice for about 30 minutes to precipitate proteins and undigested DNA. This solution was centrifuged and the supernatant taken to dryness. The residue was dissolved in a small volume of distilled water and an aliquot applied on chromatography paper for separation of the nucleotides by electrophoresis.

B. Hydrolysis of RNA to 5'-mononucleotides:

The RNA obtained by the phenol extraction was hydrolyzed to 5'-mononucleotide by snake venon phosphodiesterase. The RNA precipitated in the cold by ethanol was dissolved in 0.7 ml H_2O, 0.3 ml magnesium (final concentration 0.01 M) was added. The solution was brought to pH 8.8 by addition of 2 ml Tris-acetate buffer (final concentration 0.05 M). The phosphodiesterase enzyme was then added (0.3 mg) and the mixture incubated at 37°C. The pH of the solution was maintained at pH 8.8 and after 15 hours, another 0.2 mg of enzyme was added. After a total incubation time of 24 hours, the digestion mixture was put in ice and the solution made acid to precipitate undigested RNA and enzyme protein. After centrifugation, the supernatant was taken to dryness, the residue dissolved in 0.2 ml water and the 5'-mononucleotides separated by paper electrophoresis.

2.14 Solvents for Paper Chromatography

A. Separation of Acid-soluble Nucleotides:

Two dimensional descending paper chromatography was carried

out to separate the individual nucleotides of the acid-soluble fraction. The sample (100 µl) was spotted on a 18 x 24" sheet of Whatman No. 3 MM paper. The paper was placed in a tank containing isobutyric acid, concentrated NH_4OH, water (57:4:30, by vol.), (163) for about 20 hours. The paper was then allowed to dry and placed in a second chromatography tank containing isopropanol and HCl-water 130:33:37 by vol. (.164) for 24 hours.

B. Separation of Free Bases:

One dimensional descending paper chromatography of the free purine and pyrimidine bases was performed in isopropanol-HCl-water (130:33:37 by vol.) for 24 hours using Whatman No. 3 MM paper.

2.15 Paper Electrophoresis

High voltage paper electrophoresis was employed to separate the RNA and DNA nucleotides. The buffer chosen was ammonium acetate, 0.2 M and pH 3.7. A paper strip, 25 cm wide cut from a 18 x 24" sheet of Whatman No. 3 MM, was soaked in the buffer solution and blotted between two pieces of chromatography paper and then placed on an aluminium plate that was cooled by circulating tap water. An aliquot (50 µl) of the RNA or DNA samples was streaked on a line about 8 cm from one end of the paper with a 10 µl micropipette. The paper was then covered with a plastic sheet and compressed by a glass plate. The ends of the paper strip were allowed to dip into the troughs

containing the buffer solution. The power supply was adjusted to provide a constant voltage of 3,000 volts and an initial current density of 30-40 mA. The buffer solution was changed after every second run.

2.16 Determination of Specific Activity of the Nucleotides Eluted from the Chromatograms

After separation of the nucleotides of the acid-soluble fraction and of the nucleic acids the chromatography paper were allowed to dry in air at room temperature. The nucleotides were located on the paper with the aid of a mineralight ultraviolet lamp and marked with a pencil. The area corresponding to each nucleotide was then cut out and stapled on both sides to strips of chromatography paper. These papers were placed in 25 cm troughs and the nucleotides were eluted from the paper by descending chromatography with distilled water or 0.05 N HCl. This method of elution gave very consistent results and recovered about 85-90% of the nucleotide material spotted on a disk of Whatmen No. 3 MM paper. 3 to 4 ml of the eluates were collected, the solutions taken to dryness and the residues dissolved in a small amount of distilled water. To allow for ultraviolet absorbing substances in the paper, a blank equal in area to the spots was cut out from each chromatography paper and eluted as described above.

Aliquots of the above elutions were removed for counting the radioactivity. In order to estimate the concentration of each nucleotide,

the remainder of the solutions was transferred to quartz cuvette and the absorbance measured in a Beckman DU spectrophotometer at 260 mu. The blank was used to adjust the instrument to zero absorbance the molar extinction coefficient reported in Pabst catalogue No. OR-10 was used to estimate the concentration.

The identity of each nucleotide was determined by comparing the R_f values obtained after chromatography with maps prepared with known compounds. The purity of each nucleotide was determined by comparing the 250/260 and 280/260 ratio of the nucleotides isolated with those compounds chromatographed under the same conditions.

CHAPTER III

EVALUATION OF EXPERIMENTAL TECHNIQUES

The procedure of Schmidt and Thannhauser (159) has been used by many workers for the extraction of nucleic acids from animal tissues. The advantage of the method is its convenience for handling small amounts of material and the relative simplicity in isolating RNA and DNA from the same sample. Several authors (165-167) however, have indicated that the nucleic acid fractions obtained by this procedure are not pure and may be contaminated by protein. Since glucose is incorporated into proteins as well as nucleic acids (38) it was necessary to insure that radioactive purity was obtained in the nucleic acid fractions isolated after incubation of Ehrlich ascites cells with labelled glucose.

3.1 Extraction of Nucleic Acids from Ehrlich Ascites Cells

The experiment reported in Table I was carried out to test whether extraction with 1.2 N cold PCA as described in Chapter II was adequate for removing RNA and DNA from Ehrlich ascites cells. In these experiments, the Schmidt-Thannhauser method was used as a reference because satisfactory recovery of RNA and DNA can generally be obtained by using alkaline digestion (166). The amount of RNA and DNA obtained by the PCA method is very similar to that obtained by the Schmidt-Thannhauser procedure. In both procedures, RNA was estimated by the orcinol reaction (168) and DNA by the diphenylamine method (169).

TABLE I

Comparison of Schmidt-Thannhauser and the Perchloric Acid Procedure for Extraction of Nucleic Acids from Ehrlich Ascites Cells

Nucleic acid	Schmidt-Thannhauser method	Perchloric acid method
RNA	89.5*	87.0
DNA	37.0	38.4

The lipid-free residue was suspended in cold PCA or 0.3 N KOH and the RNA and DNA extracted as described in "Materials and Methods".

* The results are the mean data obtained from two experiments and refer to µg nucleic/mg dry weight of cells.

3.2 Incorporation of Phenylalanine-1-^{14}C

In order to test for radioactive contamination from protein in the RNA and DNA fractions, Ehrlich ascites cells were incubated with carrier free phenylalanine-1-^{14}C. This radioactive amino acid cannot label the nucleic acids but is a precursor of protein Table II shows the amount of radioactivity found in the RNA, DNA and protein fractions obtained by the Schmidt-Thannhauser procedure and the PCA extraction method. It is seen that by alkaline digestion of the acid insoluble residue, about 20 per cent of the radioactivity incorporated from phenylalanine-1-^{14}C was found in the RNA fraction. No radioactivity was found in the RNA fraction obtained by the cold PCA extraction method. The DNA fraction obtained after PCA extraction contained fewer counts (6-7 per cent) than the DNA from the Schmidt-Thannhauser method which contained about 18 to 20 per cent of the total counts. During the course of these experiments, it was observed that the extent of radioactive contamination of the DNA fraction after extraction of RNA by the cold PCA, is dependent to some extent to the amount of stirring of the suspension during extraction with hot TCA. Vigorous stirring, however, is required to recover 85-90 per cent of the DNA.

3.3 Incorporation of Thymidine-2-^{14}C

The results obtained thus far indicated that protein did not contaminate the nucleic acid fractions obtained by cold PCA extraction.

TABLE II

Incorporation of Phenylalanine-1-^{14}C into RNA, DNA and Protein Fractions Obtained by Various Procedures

Fraction	Schmidt-Thannhauser procedure		Perchloric acid procedure	
	Expt.1	Expt.2	Expt.1	Expt.2
RNA	113*	136	0	0
DNA	106	120	27	33
Protein	355	386	500	636

Cells were incubated for 60 minutes at 37°C in Krebs-Ringer phosphate buffer as described in "Materials and Methods". The medium contained 0.02 µC phenylalanine-1-^{14}C (29,500 counts/min.) and 5 mM glucose.

* The results are expressed as counts/min/mg dry weight of cells.

In order to test further the purity of the RNA fraction obtained by this method, Ehrlich ascites cells were incubated with carrier free thymidine-2-^{14}C, a specific and close precursor of DNA (171). Table III demonstrates the amount of radioactivity found in RNA, DNA and protein fractions. It is clear that no DNA breakdown occurs during the extraction of the RNA by the cold PCA. These results were confirmed by estimating the DNA content of the RNA fraction. The results also show that about 10-15 per cent of the thymidine-2-^{14}C incorporated into DNA remains in the protein fraction even after vigorous stirring during 25 minutes with hot TCA.

3.4 Electrophoretic Separation of Nucleotides

One objective was to measure the radioactivity incorporated from glucose into adenosine, guanosine, cytidine and uridine nucleotides of RNA. It was, therefore, necessary to determine the nature of the fragments obtained after digestion of the RNA with cold PCA. For this purpose, high voltage paper electrophoresis of the cold acid hydrolyzate was carried out with 0.1 M ammonium formate buffer (172). The results, illustrated in Fig.5, demonstrate the presence of four separate ultraviolet absorbing bands migrating towards the anode. This indicates that extraction of RNA by PCA results in the liberation of nucleotides, since nucleosides or bases would move towards the cathode. The spots containing the nucleotides were eluted from the paper and identified as UMP, GMP, AMP and CMP by the methods described in Chapter II. In further experiments, 0.2 M

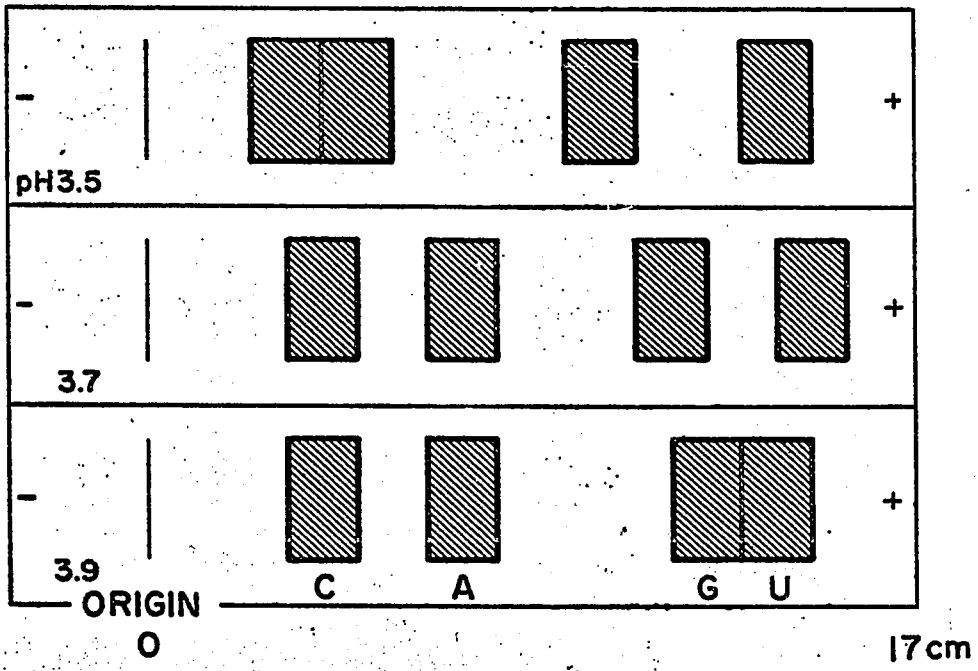
TABLE III

Incorporation of Thymidine-2-^{14}C into RNA, DNA and Protein Fractions Obtained by Perchloric Acid Extraction

Fraction	Expt.1	Expt.2
RNA	0	0
DNA	504	682
Protein	55	82

The experimental conditions were as described for Table II. The medium contained 0.06 μC thymidine-2-^{14}C. The results are expressed in counts/min/mg dry weight of cells.

FIG.5: Separation of the RNA Nucleotides by High Voltage Paper Electrophoresis



The nucleotides were separated by electrophoresis for 2.5 hrs at 3,000 V as described in "Materials and Methods".

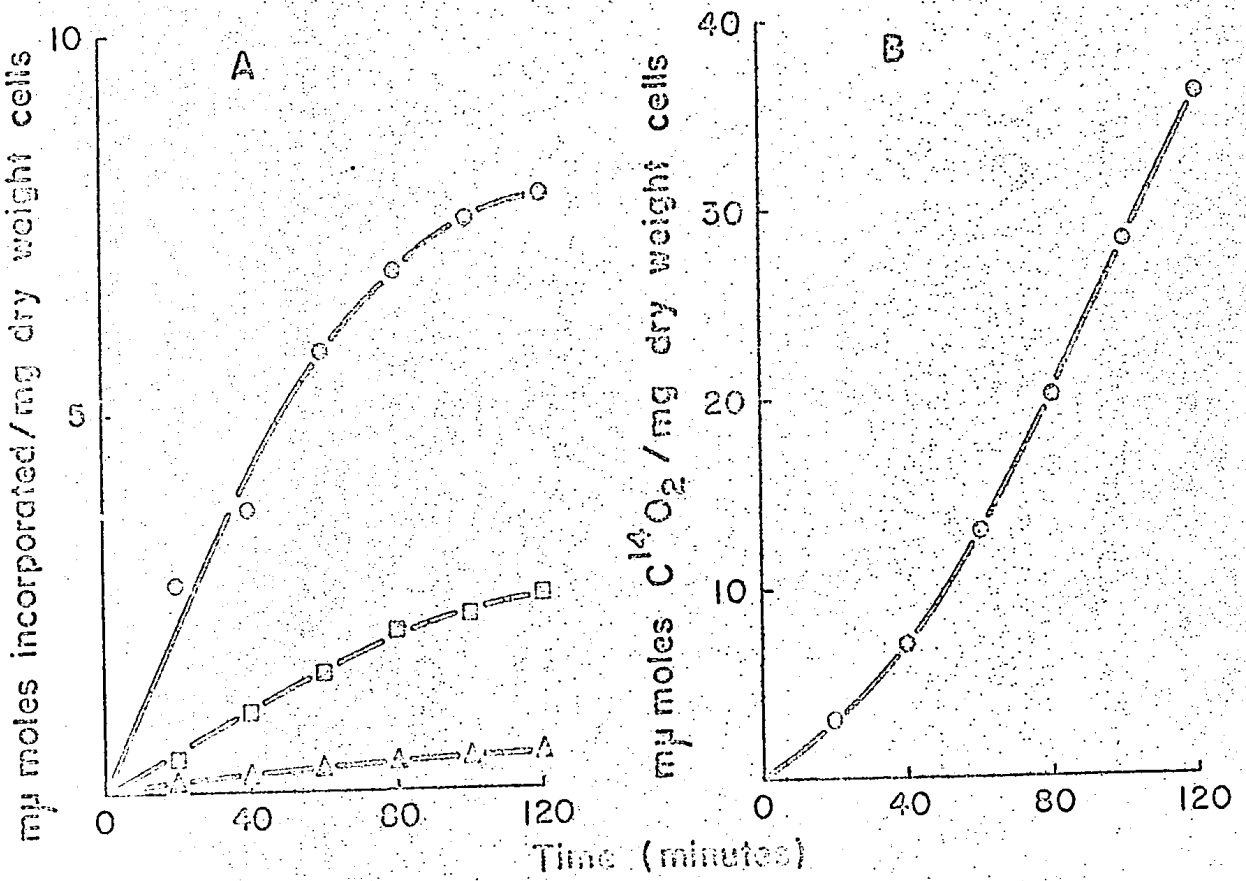
These separations were carried out in 0.2 M ammonium acetate (the pH of the buffer is indicated in each frame). U, G, A, C refer to uridylic, guanylic, adenylic, cytidylic acids.

ammonium acetate buffer was chosen over ammonium formate because the amperage was significantly lower during the separation thereby decreasing the heating of the paper during the electrophoresis. It was important to adjust the pH of the system very carefully to pH 3.7 since CMP and AMP did not separate at lower values and it was difficult to separate GMP from UMP at higher values.

3.5 Time Course of Glucose Metabolism

Preliminary experiments on glucose-U-^{14}C incorporation into the acid-soluble nucleotides, RNA and DNA fractions of Ehrlich ascites tumor cells were carried out to determine optimal conditions for labelling these various fractions. The incorporation of glucose into the various cellular fractions was measured at different time intervals (Fig.6A). The incorporations in all cases increased during the two hours of incubation. To determine at what point exogenous glucose was being depleted from the cells, glucose oxidation to $^{14}CO_2$ was measured throughout the course of the experiment (Fig.6B). After an initial lag the evolution of $^{14}CO_2$ from glucose was very rapid and continued linearly for 2 hours. After this time, there was a rapid fall in the $^{14}CO_2$ evolved, with a subsequent decrease in the labelling of the nucleotide and the nucleic acid fractions. This rapid disappearance of glucose from the medium is in agreement with reports of many other workers (38, 173-175) that glucose is metabolized very rapidly to lactic acid and $^{14}CO_2$ in tumor cells. In subsequent experiments the cells were incubated for less than 2 hours.

FIG.6: Glucose-U-^{14}C Oxidation to $^{14}CO_2$ and Incorporation into Acid-Soluble Nucleotides, RNA and DNA.



The experimental conditions were as described in Table II. The incubation medium contained 5 mM glucose-U-^{14}C (1 μC). ○, acid-soluble nucleotides; □, RNA; △, DNA.

3.6 Effect of Iodoacetate on Glucose-1-^{14}C Metabolism

Because the incorporation of labelled glucose into the nucleic acids was relatively low, an attempt was made to find conditions under which the incorporation of glucose into nucleotides and nucleic acids might be increased. Based on the findings of Ellis and Scholefield (45) that iodoacetate (IAA) inhibits glycolysis to a greater extent than nucleotide synthesis, cells were incubated with glucose-1-^{14}C in the presence of various concentrations of IAA. At the end of the incubation, the amount of radioactive carbon dioxide evolved was estimated, as well as the extent of incorporation of glucose into lactic acid and acid-soluble nucleotides. Since $C^{14}O_2$ evolved from glucose-1-^{14}C is obtained mainly via the hexose monophosphate shunt (38), the results presented in Table IV indicate that at very low concentrations of IAA (0.005 mM) there is no inhibition of the hexose monophosphate shunt while a 50% decrease in glycolysis is still obtained, as measured by the amount of lactate formed (176). However, the amount of glucose incorporated into acid-soluble nucleotides remained the same as in the control, 7.1 mmoles/mg dry weight of cells as compared to 6.8 mmoles/mg/dry weight of cells.

TABLE IV

Effect of Iodoacetate on Glucose-1-^{14}C Oxidation and Incorporation into Lactate and Acid Soluble Nucleotides

Iodoacetate mM	mμmoles $C^{14}O_2$ produced/mg dry wt.of cells	mμmoles lactate produced/mg dry wt.of cells	mμmoles labelled glucose incorporated into acid-soluble nucleotides/mg dry weight of cells
0.5	11.4	180	2.5
0.3	16.0	175	2.5
0.01	26.7	400	4.8
0.005	43.5	530	7.1
0.0	43.5	1080	6.8

Incubations were carried out as described in Table II. The medium contained 5 mM glucose-1-^{14}C (1 μC) and further additions as noted. The time of incubation was 2 hours.

SUMMARY OF CHAPTER III

The work presented in this Chapter has dealt mainly with an estimation of two of the procedures available for extraction of nucleic acids from animal tissues. The Schmidt-Thannhauser procedure for extraction of RNA with alkaline has been widely used. From the data given in this Chapter and the evidence available in the literature it appears that radioactive contamination from protein degradation products results in very large contamination of both the RNA and DNA fractions.

The other procedure used in this study was to carry out acid digestion of the lipid-free residue under conditions which result in RNA hydrolysis to acid-soluble mononucleotides. Under these conditions, essentially all the orcinol-reacting material is solubilized and re-digestion of the precipitate obtained after removal of the RNA hydrolyzate, failed to extract further any RNA. The data in Tables II and III shows that no release of protein material and DNA in acid-soluble form is obtained after "digestion" of the acid-insoluble residue with 1.2 N $HClO_4$ (overnight at 0-4°C) elsewhere PCA.

It is also apparent from Table III that complete removal of DNA by extraction with PCA at 90°C is not obtained. These results confirm the observation made by Hutchison et al. (158). It should be emphasized further that a 90 per cent recovery of DNA is obtained only by careful attention to the conditions of extraction.

The results in Table IV indicate that when glycolysis was

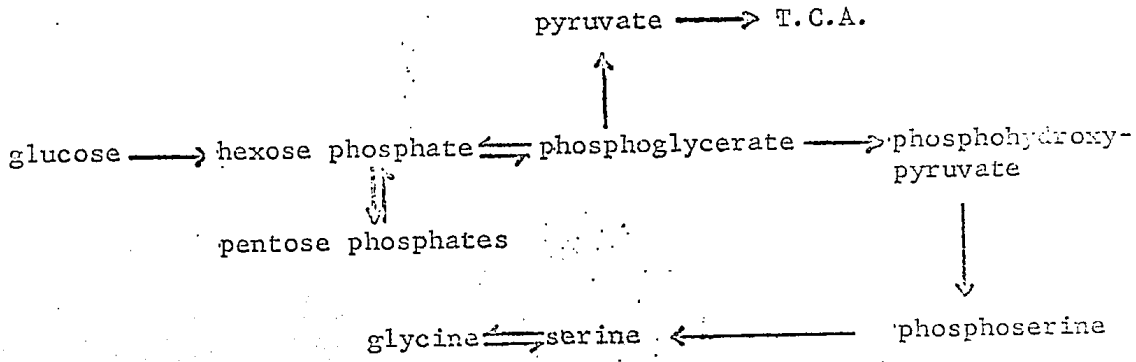
diminished by iodoacetate no increase in the conversion of glucose-U-^{14}C to acid-soluble nucleotides was observed.

Henderson and Khoo (177) have shown that in Ehrlich ascites tumor cells the formation of PRPP is independent of extracellular glucose concentrations above 0.55 mM. It must, therefore, be assumed that in the present study this system is operating at full capacity, since 5 mM glucose is present.

CHAPTER IV

NUCLEOTIDE BIOSYNTHESIS FROM LABELLED GLUCOSE

Studies with labelled adenine, orotic acid, formate and glycine have demonstrated that glucose markedly stimulates the incorporation of all four precursors into acid-soluble nucleotides and nucleic acids (34, 36, 45). Experiments in this laboratory (38) have shown that glucose gives rise to pentose phosphate in Ehrlich ascites tumor cells via the hexose monophosphate shunt and through the transketolase-transaldolase enzyme system. The incorporation of radioactivity from labelled glucose into amino acids, such as L-serine and glycine of tumor cells has also been demonstrated (178, 179). From these studies, the following scheme of reactions for conversion of glucose to glycine has been proposed.



It follows that L-serine and glycine may be formed from glucose and together with ribose-phosphate may be utilized for the synthesis of nucleotides, by the reaction sequences described in Chapter I. By the reactions of glycolysis and the citric acid cycle, glucose may also

give rise to aspartic acid which in turn can be incorporated into the pyrimidine bases, as illustrated in Chapter I.

In the experiments discussed in this Chapter, radioactive glucose was used as a precursor to assess the significance of alternate pahtways in the in vitro labelling of the purine and pyrimidine nucleotides of the acid-soluble, RNA and DNA fractions.

4.1 Incorporation of Glucose-U-^{14}C into Acid-Soluble Nucleotides

The specific radioactivities of the acid-soluble nucleotides after glucose-U-^{14}C incorporation are shown in Table V. The corresponding nucleoside triphosphates and diphosphates were pooled after chromatography since the amounts of both the guanosine and uridine nucleotides were quite small. The monophosphates of uridine and guanosine were not detected and it was not possible to obtain the specific activity of the cytidine nucleotides. This nucleotide is present in small amounts in liver (180) and under the present in vitro conditions only the nucleotides which are present in relatively high quantities will be detected. About 80 per cent of the radioactivity recovered in the acid-soluble nucleotides was contained in the adenosine nucleotides. The average value obtained for ATP was 650 mμmoles per gm of wet weight, the ratio ATP/ADP being about 5/1. The pool sizes of ATP and ADP were 7 to 10 times larger than those of the guanosine or uridine di- and triphosphates. It is therefore not surprising, since nucleoside monophosphates are usually lower than the di- and triphophate derivatives, that uridine and guanosine

TABLE V

Specific Activities of Acid-Soluble Nucleotides after Glucose-U-^{14}C Incorporation into Ehrlich Ascites Cells

Expt. No.	ATP + ADP	GTP + GDP	UTP + UDP
	specific activity, counts/min x 10^{-3}/μmole		
1	34.0	45.0	36.5
2	20.7	38.0	35.2

The specific activity of glucose-U-^{14}C added to the medium was 4 x 10^5 counts/min/μmole. The cells were incubated for 90 minutes as described in Table II. Two incubation mixtures were pooled before separation of the nucleotides by chromatography as described in "Materials and Methods".

monophosphates were not detected. The results presented in Table V show that the specific activities of the guanosine nucleotides are somewhat higher than those of the uridine or adenosine nucleotides. The specific activity of the adenosine nucleotide was always the lower. This is probably explained by the fact that radioactive adenine nucleotides is diluted by a much larger pool of endogenous nucleotides than either radioactive guanine or uridine nucleotides.

4.2 Incorporation of Glucose-U-^{14}C into RNA and DNA Nucleotides

Results concerned with the labelling of the nucleotides of RNA and DNA are shown in Table VI. It can be seen that in the RNA the pattern of labelling of AMP, GMP and UMP is similar to that found in the acid-soluble nucleotides in Table V. The specific activity of GMP is the highest followed by UMP and then AMP. As shown in Table VI, the specific activity of GMP in RNA is only 166 counts/min/μmole while that of UMP is about 2200 counts/min/μmole. This finding was surprising because UTP is the immediate precursor of CTP. In addition, the results obtained in these experiments seem to indicate that the labelling of each nucleotide in RNA is a reflection of the specific activity of the corresponding nucleotide in the acid-soluble fraction. The results in Table V also demonstrated that the smaller guanosine and uridine nucleotide pools reached a higher specific activity than the larger adenine nucleotide pool. Since the pool of CTP is the lowest of all four nucleotide precursors of RNA it might be anticipated that its specific activity and therefore

TABLE VI

Specific Activities and Nucleotide Composition of RNA and DNA After Incubation of Ehrlich Ascites Cells with Glucose-U-^{14}C

Fraction	Nucleotide Isolated	Specific Activity counts/min/μmole		Molar Ratio % Total	$\frac{GC}{AU(T)}$
		Expt.1	Expt.2		
RNA	AMP	1830	1520	18.6	1.75
	GMP	2830	2160	33.5	
	UMP	2220	2118	17.8	
	CMP	166	167	30.1	
DNA	dAMP	133	100	26.6	0.78
	dGMP	66	147	22.6	
	dTMP	150	91	29.3	
	dCMP	147	156	21.3	

The experimental conditions were as described in Table V.

also that of CMP in RNA would be higher or about the same as that of UMP. From the results obtained in the DNA nucleotides, it should be noted that labelled glucose is incorporated to about the same extent into all four deoxyribonucleotides. However, the radioactivity incorporated into DNA was extremely low and did not allow for very accurate measurements. Therefore, no further determinations were made on the deoxynucleotides of DNA.

Alkaline digestion of RNA is known in some instances to give rise to deamination of cytidylic acid (158). The nucleotide composition of the RNA and DNA obtained by the PCA procedure was, therefore, checked very carefully to determine whether such a reaction might be responsible for the low radioactivity obtained in the cytidine nucleotide. Table VI indicates that the RNA of Ehrlich ascites cells contains a high level of CMP and GMP. It is, therefore, unlikely that loss of CMP occurred during the extraction of the RNA by the PCA procedures. It can be seen that the RNA and DNA have a nucleotide composition which is similar to that obtained by various other workers (see Table VII). Further attempts to explain the incorporation of radioactivity from glucose-U-^{14}C into CMP will be discussed in Chapter V.

4.3 Effect of Actinomycin D on Incorporation of Glucose-U-^{14}C into Acid-Soluble Nucleotides and RNA

Cells contain enzymes which catalyze the incorporation of nucleotides into polyribonucleotide chains or their addition to pre-existing chains. It has been demonstrated that Actinomycin D inhibits

TABLE VII

Nucleotide Composition of RNA and DNA from Ehrlich Ascites Cells and Calf Thymus Reported by Other Workers

Fraction	Tissue	Molar proportions (% total)				GC / AU(T)
		A	U(T)	G	C	
RNA	Ehrlich ascites	18.4	19.9	33.3	28.4	1.62
	" *	18.0	19.3	31.0	30.2	1.64
	Calf thymus	19.2	22.8	29.8	28.2	1.38
DNA	Ehrlich ascites	27.7	29.3	20.9	22.1	0.75

The above data was taken from Hadjiolov *et al.* (181).

* Taken from Harel *et al.* (182).

DNA-dependent RNA synthesis (183, 184). Recent studies in cell-free extracts and Ehrlich ascites cells have shown that this compound inhibits RNA polymerase but not the pyrophosphorylase enzymes that are responsible for the terminal addition of nucleotides on existing RNA chains (185, 186). Table VIII shows the effect of Actinomycin D on the incorporation of glucose-U-^{14}C into acid-soluble nucleotides and RNA. The results indicate that glucose-U-^{14}C incorporation into the acid-soluble nucleotides is not affected by Actinomycin D, but further incorporation into RNA is decreased from 319 to 35 counts/min/mg dry weight of cells. Hence, at least 90 per cent of the labelled glucose is incorporated into RNA as a result of net synthesis. These finding are well in accord with the results of Smellie et al. (31) who noted that about 90 per cent of the radioactivity obtained in RNA of Ehrlich ascites tumor cells injected with formate-^{14}C was found in nuclear RNA.

4.4 Distribution of Radioactivity in the RNA Nucleotides and Corresponding Bases

Since glucose can give rise to pentose phosphate and amino acids that are precursors of purines and pyrimidines it was of interest to determine the extent of conversion of glucose-U-^{14}C into the ribose moiety of the nucleotides of RNA and their constituent bases. In the experiments cited in Table IX, the RNA hydrolyzate obtained by the PCA procedure was separated in two. One aliquot was used to separate the nucleotides by electrophoresis, the other hydrolyzed to free bases, as described in Materials and Methods. In Expt.1, the purine

TABLE VIII

Effect of Actinomycin D (10 ug) on Incorporation of Radioactivity from Glucose-U-^{14}C into Acid-Soluble Nucleotides and RNA

Additions	Counts/min/mg dry weight cells	
	Acid-soluble nucleotides	RNA
Nil	1062	319
Actinomycin D (10 µg)	1091	35

The experimental conditions were as described in Table II. Cells were incubated for 15 minutes with actinomycin D then 5 mM glucose-U-^{14}C (1 µC) was added. The mixture was then incubated for 60 minutes.

and pyrimidine bases or nucleotides were pooled after their separation in order to obtain sufficient radioactivity in the bases. It may be seen that only 4.6 and 6.7 per cent of the total radioactivity is incorporated into the purine and pyrimidine bases, respectively. It is interesting to note that although labelling in the bases is low, Ehrlich ascites cells can synthesize purine and pyrimidine bases from glucose. These result are in agreement with those of Hager et al. (80) and indicate that these tumor cells do have the ability to synthesize the pyrimidine nucleotide by the de novo pathway. In Expt.2, radioactivity incorporated into each nucleotide of RNA and into the constituent base was determined. The percentage of radioactivity from glucose-U-^{14}C incorporated into the adenine moiety is 4.9 per cent while that in guanine is only 1 per cent. The specific activity for CMP is again seen to be much lower than any of the other three nucleotides of RNA. The per cent radioactivity incorporated into the bases of the pyrimidine nucleotides is 2.6 and 6.8 per cent for uracil and cytosine, respectively.

4.5 Effect of L-Glutamine on Glucose-U-^{14}C Incorporation Into Acid-Soluble Nucleotides and RNA

The fact that little radioactivity was found in the bases of the nucleotides could be due to a number of factors. The most obvious that Ehrlich ascites contain very little free glutamine (40, 41). The dependence on glutamine to provide the nitrogen atoms for the de novo synthesis in these tumor cells for both purine and pyrimidine bases was discussed in Chapter I. On addition of glucose a three-fold increase

TABLE IX

Distribution of Radioactivity in the RNA Nucleotides in the Purine and Pyrimidine Bases of the Nucleotides

Expt. No.	Counts/min/μmole				Percent Incorporated in Base
	Specific Activity of the Nucleotides		Specific Activity of the Bases		
1	Adenylic and Guanylic acid	2,700	Adenine Guanine	125	4.6
	Uridylic and Cytidylic acid	1,200	Uracil Cytosine	80	6.7
2	Adenylic acid	13,034	Adenine	634	4.9
	Guanylic acid	15,353	Guanine	155	1.0
	Uridylic acid	22,021	Uracil	570	2.6
	Cytidylic acid	1,590	Cytosine	108	6.8

The experimental conditions were as described in Table V. The specific activity of glucose-U-^{14}C added to the medium in Expt.1 and 2 were 4×10^5 and 1×10^6 counts/min/μmole, respectively.

$$\frac{\text{Specific activity of base}}{\text{Specific activity of nucleotide}} \times 10^2$$

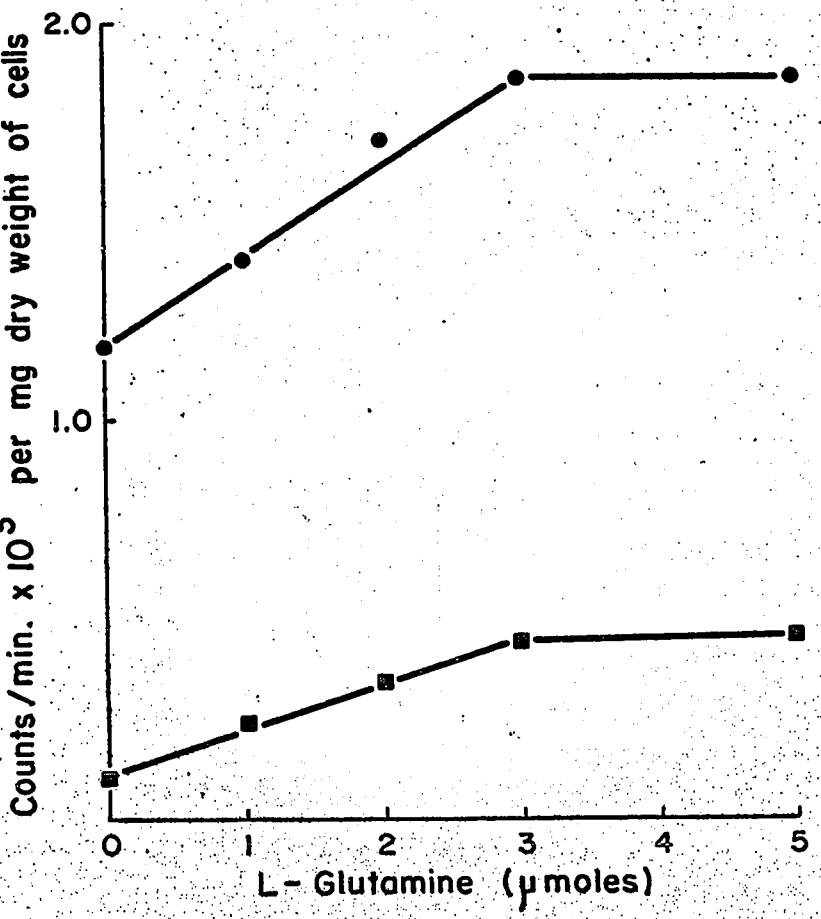
in the concentration of glutamate is obtained in Ehrlich ascites cells (37) and this compound in the presence of ATP, ammonia and the enzyme, glutamine synthetase, can give rise to L-glutamine. Little is known about this reaction in Ehrlich ascites tumor cells but low levels of glutamine synthetase activity in tumor have been reported (187) which suggests a decreased ability of tumor to convert glutamate to glutamine.

In view of these observations on the metabolism of glutamine and the known requirement for glutamine in the synthesis of nucleotides, cells were incubated with various concentrations of L-glutamine to determine the effect of this compound on the incorporation of labelled glucose into nucleotides. As illustrated in Fig.7 glutamine stimulates the incorporation of glucose-U-^{14}C into both the acid-soluble nucleotides and RNA fraction. These results are in agreement with those of previous investigators who showed that the combined effect of glucose and glutamine enhances the incorporation of glycine and formate into the purine nucleotides (36, 37). The optimum concentration of glutamine is 1 mM. The data show that at this concentration, glutamine effected about 70 per cent stimulation of the labelling in the acid-soluble nucleotides compared with 28 per cent in the RNA.

4.6. Effect of L-Glutamine on Glucose-U-^{14}C Incorporation into Acid-Soluble and RNA Nucleotides

Since as shown above L-glutamine increased the incorporation of glucose-U-^{14}C into the acid-soluble and RNA fraction, it was decided to investigate whether this amino acid stimulated de novo synthesis

FIG. 7: Effect of L-Glutamine on Glucose-U-^{14}C Incorporation into Acid-Soluble Nucleotides and RNA



The experimental conditions were as described in Table V. The incubation medium contained 5 mM glucose-U-^{14}C (3 μC) and further additions as noted. ● , acid-soluble nucleotides; ■ , RNA.

of both purine and pyrimidine nucleotides. The results in Table X show that L-glutamine stimulates the incorporation of glucose-U-^{14}C into the purine nucleotides but not into the pyrimidine nucleotides of the acid-soluble fraction; the stimulation of incorporation into adenine nucleotides being 100 per cent and in the guanine nucleotide only 10 per cent. The results in Table XI show the incorporation of glucose-U-^{14}C into the various nucleotides of RNA and their constituent bases. In the presence of L-glutamine the per cent radioactivity incorporated in the adenine, 5.1 per cent, and guanine, 1.4 per cent has remained the same as in the control, which contained no glutamine. This indicates that L-glutamine effected net synthesis of new adenine and guanine nucleotides from glucose. These results indicate, also, that the interconversion reactions of AMP to GMP via IMP is probably not of great significance in these in vitro conditions since the specific activity of the adenine nucleotides of the acid-soluble fraction can be increased to a much greater extent than the specific activity of the guanine nucleotides. This will be substantiated further in Chapter V.

4.7 Effect of Various Compounds on Glucose-U-^{14}C Incorporation into Acid-Soluble Nucleotides

It seemed of interest to determine whether any other nucleotide precursor might limit the rate of de novo synthesis of nucleotides in these in vitro conditions. To test this possibility the effect of glycine, formate, L-serine, L-aspartate on glucose-U-^{14}C incorporation into

TABLE X

Effect of L-Glutamine on Glucose-U-^{14}C Incorporation Into Acid-Soluble Nucleotides

Glutamine added	Specific Activity counts/min x 10^{-3} /µmole		
	ATP + ADP	GTP + GDP	UTP + UDP
Nil	56	145	169
1 mM	107	159	171

The experimental conditions were those described Expt. 2 in Table IX.

TABLE XI

Effect of L-Glutamine on Glucose-U-^{14}C Incorporation in RNA Nucleotides and Bases of These Nucleotides

Additions	Counts/min/μmole				Percent Incorporated in base
	Sp.ac.of nucleotides		Sp.ac. of bases		
Nil	AMP	13,034	Adenine	634	4.9
	GMP	15,353	Guanine	155	1.0
	UMP	22,021	Uracil	570	2.6
	CMP	1,590	Cytosine	108	6.8
L-Glutamine 1 mM	AMP	27,300	Adenine	1400	5.1
	GMP	17,400	Guanine	240	1.4
	UMP	22,200	Uracil	640	2.9
	CMP	1,500	Cytosine	130	8.3

The experimental conditions were as described in Table X.

acid-soluble nucleotides was studied. It is evident from the results in Table XII that the addition of these amino acids has no effect on the incorporation of labelled glucose into acid-soluble nucleotides. In the incubation mixture supplemented with L-glutamine, some decrease in the labelling of the nucleotides is observed after addition of the amino acids to the incubation mixture. Since glutamine increases de novo synthesis this small decrease in the labelling is probably due to dilution of the radioactive amino acids formed from glucose-U-^{14}C.

4.8 Effect of Azaserine on de novo and Salvage Pathways

Two pathways are known by which nucleotides are synthesized in tumor cells. One is de novo synthesis and results from the utilization of small molecules. The other pathway results from the use of preformed bases or nucleosides. The data on the distribution of radioactivity in the nucleotides of RNA and corresponding bases, showed that approximately 90 per cent of the labelling was found in the ribose moiety of the purine and pyrimidine nucleotides. This was true even when L-glutamine was added to the incubation flask to supplement the endogenous amino group donor. The differences in labelling between the ribose and the bases are due partly to differences in the rate of utilization of the newly formed radioactive amino acids and the rate of utilization of PRPP and partly to differences between the sizes of the endogenous pools which dilute the newly formed radioactive molecules. The dependence of Ehrlich ascites

TABLE XII

Effect of Glycine, Formate, L-Serine, L-Glutamine, and L-Aspartic Acid on Glucose-U-^{14}C Incorporation Into Acid-Soluble Nucleotides

Additions 1 mM	Counts/min/mg dry weight of cells
Nil	991
Glycine	999
Formate	871
L-Serine	946
L-Glutamine	1847
L-Glutamine + Glycine + Formate	1759
L-Glutamine + Glycine + Formate + L-Aspartate	1681

The experimental conditions were as described in Fig. 7.

cells upon an exogenous ribose source for nucleotide synthesis has been demonstrated and indicates that very small amounts of ribose phosphate are present in these cells while high levels of the amino acids, glycine, serine, aspartate have in general been found in tumor cells (186). It was of interest, therefore, to discover whether the low radioactivity in the bases reflected low de novo synthesis of both purine and pyrimidine nucleotides and to determine the amount of ribose utilized by this pathway in contrast to the salvage pathways.

Azaserine (O-diozoacetyl-L-serine) is an antimetabolite which inhibits de novo synthesis of purines and pyrimidines by acting as an analogue of glutamine (188). It was found to inhibit the incorporation of ^{14}C-labelled glycine and formate into purines (189, 190) and the incorporation of radioactive CO_2 into carbon-2 of the uracil in Ehrlich ascites cells (80). Preliminary experiments were conducted to determine the effect of azaserine on de novo and salvage pathways in these in vitro conditions. The data in Table XIII indicate that adenine-8-^{14}C and uracil-2-^{14}C incorporation into acid soluble nucleotides or nucleic acid is not affected by azaserine while de novo synthesis of purine, as measured by glycine-2-^{14}C incorporation into nucleic acid, is inhibited by 85 per cent.

4.9 Effect of Azaserine on Glucose-U-^{14}C Incorporation into RNA

The data in Table XIV shows the effect of azaserine on glucose-U-^{14}C incorporation into RNA nucleotides. In the presence of

TABLE XIII

The Effects of Azaserine on Glycine-1-^{14}C, Adenine-8-^{14}C and Uracil-2-^{14}C Incorporation Into Acid-Soluble Nucleotides and RNA

Additions	Counts/min/mg dry weight of cells	
	Acid-soluble nucleotide	RNA
Adenine-^{14}C	6,320	520
Adenine-^{14}C + Azaserine	6,269	528
Uracil-^{14}C	2,430	793
Uracil-^{14}C + Azaserine	2,381	755
Glycine-^{14}C	-	339
Glycine-^{14}C + Azaserine	-	61

The experimental conditions were as described in Table II.

The incubation medium contained 0.3 mM adenine-8-^{14}C (0.5 µC), uracil-2-^{14}C (0.5 µC), glycine-1-^{14}C (2.5 µC) and 1.5 µg Azaserine as noted.

TABLE XIV

Effect of Azaserine on Glucose-U-^{14}C Incorporation Into RNA in the Presence and Absence of L-Glutamine

Additions	Specific Activity, counts/min/umole			
	UMP	CMP	GMP	AMP
Nil	3490	290	3480	2120
Azaserine	3660	330	3540	2300
Glutamine	4430	450	4170	4800
Glutamine + Azaserine	3780	380	3100	2010

The experimental conditions were as described in Table V. The incubation medium contained 1 mM glutamine and 1.5 ug Azaserine as noted.

azaserine the specific activities of all four nucleotides of RNA are the same as that of the control. The results also demonstrate that the increase in the specific activities of the nucleotides obtained by the addition of L-glutamine is inhibited by the presence of azaserine. It is interesting to note that in these experiments L-glutamine was found to stimulate glucose-U-^{14}C incorporation into pyrimidine nucleotides, which decreased after the addition of azaserine. Assuming that azaserine inhibits in these in vitro conditions the de novo pathways of both purine and pyrimidine nucleotides, it may be concluded that almost all of radioactive ribose-phosphate derived from glucose-U-^{14}C is incorporated into the various nucleotides of RNA by utilization of the salvage pathways.

4.10 Effect of Azaserine and L-Glutamine on Glucose-U-^{14}C Incorporation into RNA Nucleotides of Ehrlich Cells Incubated in Ascitic Fluid

Since it was desired to relate the observed utilization of glucose for nucleotide synthesis by the various pathways to possible physiological significance it was important to determine the effect of L-glutamine and azaserine on the incorporation of glucose in Ehrlich cells incubated in their ascitic fluid.

In earlier studies Smellie et al. (31, 32) found that the inability of Ehrlich ascites cells to incorporate formate-^{14}C into purine nucleotides in vitro was largely due to the absence of glucose in the incubation medium. They concluded that in vivo these tumor cells probably receive a continuous supply of glucose to provide the ribose-

phosphate for de novo synthesis. The results in Table XV indicate that the amount of glucose-U-^{14}C incorporated into the nucleotides of RNA by the de novo pathways, in the absence of added L-glutamine, is as previously shown very small.

TABLE XV

Effect of Azaserine and L-Glutamine on Glucose-U-^{14}C Incorporation Into RNA Nucleotides of Ehrlich Cells Incubated in Ascitic Fluid.

Additions	Specific Activity, counts/min/μmole			
	UMP	CMP	GMP	AMP
Nil	4190	320	3330	2740
Azaserine	3670	350	3400	2030
L-Glutamine	5170	810	4640	4850
L-Glutamine + Azaserine	4870	440	2540	3340

The experimental conditions were as described in Table XIV.

SUMMARY OF CHAPTER IV

Glucose-U-^{14}C is readily incorporated into adenine, guanine and uridine nucleotides of the acid-soluble fraction. Radioactivity was also incorporated into the purine and pyrimidine nucleotides of RNA and DNA. The specific radioactivity of CMP of RNA was about 10 times lower than the specific activity of the other 3 nucleotides of RNA. In addition the radioactivity incorporated into DNA nucleotides was low.

The incorporation of glucose-U-^{14}C into RNA is inhibited 90 per cent by the addition of Actinomycin D to the incubation medium.

The radioactivity incorporated into the various nucleotides of RNA is found mainly in the ribose moiety of the purine and pyrimidine nucleotides.

The addition of L-glutamine, at a concentration of 1 mM doubles the incorporation of glucose-U-^{14}C into the adenine nucleotides of the acid-soluble and RNA fractions and increases the labelling in the guanine nucleotides by about 10 per cent. In some cases, glutamine also stimulates the incorporation of glucose-U-^{14}C into pyrimidine nucleotides of RNA.

Addition of azaserine, an inhibitor of <u>de novo</u> synthesis of purine and pyrimidine nucleotides did not affect the radioactivity incorporated into the purine and pyrimidine nucleotides from ^{14}C-glucose. However, in the incubation mixture incubated with L-glutamine, azaserine inhibited the increased labelling obtained by the addition of L-glutamine.

These studies indicate, that in the absence of exogenous L-glutamine, almost all of the labelled ribose-phosphate obtained from glucose-U-^{14}C is converted to the purine and pyrimidine nucleotides in Ehrlich ascites cell *in vitro* by utilization of preformed bases.

CHAPTER V

INCORPORATION OF VARIOUS LABELLED PRECURSORS INTO ACID-SOLUBLE AND RNA NUCLEOTIDES

The results described have shown that when labelled glucose is incubated with Ehrlich ascites cells in vitro it gives rise to radioactive ribose phosphate, an intermediate of the purine and pyrimidine nucleotides which are incorporated into RNA. The experiments demonstrated that the specific activity of ribose in CMP of RNA was one tenth that of GMP, AMP or UMP. Attempts to determine whether cytidine nucleotides were poorly labelled in the acid-soluble fraction were unsuccessful, since the level of cytidine nucleotides was always too low to be determined. However, the manner in which radioactive ribose phosphate is incorporated into the CMP of RNA could be dependent on one or several of the following factors.

1. The radioactive cytidine nucleotides formed in the cytoplasm may mix slowly with the pool of cytidine nucleotides at the site of RNA synthesis in the nucleus, e.g. there may be separate and specific nucleotide pools in the cytoplasm and the nucleus or there may be enzyme bound nucleotides and free nucleotides (191).

2. Under these in vitro conditions radioactive CTP may not be used as extensively for RNA synthesis as are the other three nucleoside triphosphates.

3. The results presented in the previous chapter indicate that labelling in the AMP, GMP and UMP of RNA is a reflection of the specific activity of the corresponding nucleotides in the acid-soluble fraction. Thus, if the rate of formation of radioactive cytidine nucleotides was much lower or if it was diluted to a greater extent than the above three nucleotides, the specific activity of this nucleotide would be lower in the acid-soluble fraction and therefore in RNA.

To investigate these various possibilities, Ehrlich ascites cells were incubated with various labelled precursors and the labelling pattern of the different nucleotides of RNA was studied.

5.1 Distribution of ^{32}P After Hydrolysis of Labelled RNA

In order to test the possibility that in these experiments on the uptake of ^{14}C from labelled glucose, there might be a limited incorporation of radioactive cytidine nucleotides into RNA, experiments were carried out to determine the distribution of radioactivity in RNA nucleotides after labelling the cells with ^{32}P-orthophosphate. It has been shown that after ^{32}P incorporation the specific activities of uridine, cytidine and guanosine nucleotides in the acid-soluble fraction of E.coli are similar (181,192). Since free nucleotides in the cell have a phosphate group on the 5'-position of the ribose, the labelling in the 5'-mononucleotides of RNA by ^{32}P will reflect the amount of each nucleotide precursor incorporated into RNA. After incubation of cells with ^{32}P-orthophosphate

the RNA was isolated by the phenol extraction procedure and then hydrolyzed by snake venom phosphodiesterase to nucleoside-5'-monophosphates, as described in Materials and Methods. The data in Table XVI demonstrate that the specific activities of CMP and UMP are quite similar. The results, therefore, rule out the possibility that cytidine nucleotides are not available at the site of RNA synthesis or that a limited amount of cytosine nucleotides caused the low specific activity of cytidylic acid in RNA after labelling with glucose-U-^{14}C. It is interesting to note that in Expt.1, the specific activity of GMP is 4,300 counts/min/umole and only one third that of the other three nucleotides of RNA. Similar results have been obtained in a previous study (181). However, in Expt.2 the specific activity of GMP is 11,810 counts/min/umole and is of the same order of magnitude as the specific activity of AMP, UMP and CMP.

5.2 Effect of Purine and Pyrimidine Bases on Glucose-U-^{14}C Incorporation Into Acid-Soluble and RNA Nucleotides

The results obtained above indicate that the distribution of radioactivity from glucose-U-^{14}C into all four nucleotides of RNA reflects the specific activity of these nucleotides in the acid-soluble fraction. In Chapter IV, it was demonstrated that essentially all the radioactive ribose-phosphate formed from glucose-U-^{14}C was incorporated into purine and pyrimidine nucleotides by utilization of preformed bases. Thus, the specific activity of each nucleotide in

TABLE XVI

Distribution of ^{32}P -Orthophosphate Into RNA Nucleotides

Nucleotide	Specific Activity counts/min/μmole	
	Expt. 1	Expt. 2
5'-AMP	12,090	18,280
5'-GMP	4,330	11,810
5'-UMP	12,080	15,230
5'-CMP	14,250	12,500

Cells were incubated in Krebs-Ringer solution containing 6.7 mM Tris buffer, 3.3 mM phosphate buffer pH 7.4 and 25 μC [^{32}P] orthophosphate. Incubations were carried out at 37°C for 90 minutes as described in "Materials and Methods".

the acid-soluble fraction must be directly proportional to the rate of reaction of its constituent base with radioactive ribose phosphate via the pyrophosphorylase or phosphorylase reactions as shown in schemes 1 and 2 and to the rate of breakdown or utilization of the newly formed radioactive nucleotide.

(1) $\text{Base} + \text{PRPP} \longrightarrow \text{B-R-P}$

(2) $\text{Base} + \text{Ribose-1-PO}_4 \longrightarrow \text{B-R} \xrightarrow{\text{P}} \text{B-R-P}$

In the experiments reported below, the effect of various bases on glucose-U-^{14}C incorporation into the acid-soluble and RNA fractions was studied to determine the relative utilization of these bases for nucleotide and RNA synthesis. It is interesting to note (Table XVII), that the addition of adenine, guanine and uracil in separate incubation mixtures increases only the specific activity of the corresponding nucleotides in the acid-soluble fraction. The specific activity of adenine nucleotides increased from 34 to 152 counts/min/mumole, guanine nucleotides from 45 to 131 counts/min/mumole, uracil nucleotides from 36 to 196 counts/min/mumole, respectively. The addition of the bases also increased the pool sizes of the corresponding nucleotides, about 1.5 to 2 fold. Cytosine exerted no effect on the amount of uridine nucleotides indicating that it is not deaminated but the extent of its incorporation into cytosine nucleotides could not be determined. These results, as indicated previously, demonstrate that under these <u>in vitro</u> conditions, there is no interconversion between AMP and GMP.

TABLE XVII

Effect of Purine and Pyrimidine Bases on Glucose-U-^{14}C Incorporation Into Acid-Soluble Nucleotides

Additions	Specific Activity, counts/min/mumole		
	UTP + UDP	GTP + GDP	ATP + ADP
Nil	36.0	45.0	34.0
Adenine	27.5	41.0	152.2
Guanine	32.0	131.1	35.6
Uracil	196.0	56.0	37.0
Cytosine	34.1	50.5	39.2

The experimental conditions were as described in Table V. The incubation medium contained 0.3 mM of the various bases as noted.

TABLE XVIII

Effect of Purine and Pyrimidine Bases on Glucose-U-^{14}C Incorporated Into RNA Nucleotide

Addition	Specific Activity, counts/min/μmole			
	UMP	CMP	AMP	GMP
Nil	2169	166	1700	2465
Adenine	2085	155	5073	2740
Guanine	2555	242	2249	6531
Uracil	13355	1398	2005	2100
Cytosine	1920	207	2130	2280

The experimental conditions were as described in Table XVII.

The data in Table XVIII demonstrate that the labelling pattern of the RNA nucleotides follows very closely that observed in the corresponding nucleotides of the acid-soluble fraction. In the presence of adenine and guanine the specific activity of AMP increased from 1700 to 5073 counts/min/umole and that of GMP from 2465 to 6531 counts/min/umole, respectively. It should be noted that in the presence of uracil the specific activity of both pyrimidine nucleotides of RNA are increased, UMP from 2169 to 13,355 counts/min/umole, and CMP from 166 to 1398 counts/min/umole. In the presence of cytosine no increased incorporation of glucose-U-^{14}C into CMP or UMP was observed. These results indicate that the incorporation of labelled glucose into the CMP of RNA in contrast to the other three nucleotides, is obtained by the conversion of uridine nucleotides to cytidine nucleotides. The experiments also show that RNA synthesis in vitro is not affected by increases in the size of individual nucleotide pools, but the increased activity of these nucleotides in RNA is due to increased specific activity of the nucleotides in the acid-soluble fraction.

5.3 A Comparison of the Incorporation of Uracil-2-^{14}C and Cytosine-2-^{14}C into Acid-Soluble Nucleotides

The data from the previous experiments showed that cytosine is not utilized for nucleic acid synthesis. Cannellakis (94) demonstrated that uracil-2-^{14}C is not extensively used by rat liver only because it is rapidly oxidized to carbon dioxide. It was of

interest, therefore, to test whether the failure of cytosine to stimulate glucose-U-^{14}C incorporation into CMP was due to the inability of Ehrlich ascites cells to convert cytosine to nucleotides, to rapid breakdown or perhaps to the inability of cytosine to enter the cell. Experiments were performed to compare the incorporation of different concentrations of uracil-2-^{14}C and cytosine-2-^{14}C of equal specific activity. After incubation of the cells with the radioactive precursor, the cells were washed twice with cold Krebs-Ringer solution and the intracellular bases and nucleosides separated from the acid-soluble nucleotides by chromatography. In order to determine the extent of breakdown of uracil-2-^{14}C and cytosine-2-^{14}C, the carbon dioxide was collected at the end of the incubation. The results of these experiments are given in Table XIX. The amount of $^{14}CO_2$ produced from either uracil-^{14}C or cytosine-^{14}C was not more than 0.02 per cent of the initial amount of substrate added and was therefore not tabulated. It is clear from these results that cytosine enters the cells but that it cannot be further utilized by the tumor cell.

5.4 Effect of L-Glutamine on Uracil-2-^{14}C Incorporation into UMP and CMP of RNA

Evidence has been offered that in mammalian tissues the amino group of cytosine nucleotides is derived from L-glutamine (75). Hurlbert et al. (193) have shown that the incorporation of orotic acid-6-^{14}C into CMP of Novikoff ascites hepatoma is increased by the

TABLE XIX

A Comparison of the Incorporation of Uracil-2-^{14}C and Cytosine-2-^{14}C Into Acid-Soluble Nucleotides

^{14}C-substrate mumoles	mumole ^{14}C base & nucleoside present in cells	mumole ^{14}C incorporated into nucleotides
Uracil-2-^{14}C		
12,000	252	10.8
6,000	114	10.8
3,000	57	10.2
1,500	29	7.0
750	13	6.2
75	0.9	0.8
Cytosine-2-^{14}C		
12,000	264	nil
6,000	132	"
3,000	66	"
1,500	33	"
750	17	"
75	1.6	"

The experimental conditions were as described in Table II. The incubation medium contained 0.5 uC of radioactive substrate and 5 mM glucose.

addition of L-glutamine. In the experiments in Chapter IV, addition of L-glutamine to cells incubated with glucose-U-^{14}C did not seem to increase the conversion of uridine nucleotides to cytidine nucleotides. It is possible, however, that higher concentrations of exogenous L-glutamine are required to stimulate the cytidine synthetase enzyme reaction. To test this possibility, cells were incubated with uracil-2-^{14}C and 3 mM L-glutamine. The results, Table XX, indicate that the ratio of the specific activities of CMP/UMP of RNA is similar in the presence or absence of added L-glutamine. If more radioactive uridine nucleotides were converted to cytidine nucleotides, the ratio would increase. It is interesting to compare the results obtained in these experiments with those of Hurlbert et al. (193). In their experiments, the ratio of specific activity of CMP/UMP was increased in the presence of 2 mM L-glutamine from 0.028 to 0.090. The latter value is close to the ratio obtained in the experiments shown in Table XX. Thus, it may be that sufficient L-glutamine is present or formed from exogenous glucose for maximal conversion of UTP to CTP in these experiments.

5.5 Incorporation of Different Concentrations of Uracil-2-^{14}C Into UMP and CMP of RNA

In the experiments of Section 5.2 it was shown that the specific activities of AMP, GMP and UMP of RNA are a reflection of the rates at which the individual bases react with labelled ribose-phosphate to form acid-soluble nucleotides. It was shown further that the labelling of cytidine nucleotides from glucose-U-^{14}C is derived exclusively from the conversion of uridine nucleotides to cytidine nucleotides but the specific activity instead of being comparable to

TABLE XX

Effect of L-Glutamine on Uracil-2-^{14}C Incorporation Into UMP and CMP of RNA

L-Glutamine mM	Specific activity, counts/min/μmole		Ratio of specific activity $\frac{CMP}{UMP}$
	UMP	CMP	
0	5038	318	0.062
3.0	5485	431	0.070

The experimental conditions were as described in Table XIX. The incubation medium contained 0.5 mM uracil-2-^{14}C (0.5 μC), 5 mM glucose and further addition as noted.

uridylic acid, as would be expected since the pool of cytidine nucleotides is small and radioactive UTP is the immediate precursor, is less than one tenth that of UMP.

However, a low specific activity CTP pool would be obtained if the rate of conversion of ^{14}C from radioactive UTP is low (e.g. high Km) and the amount of cytidine nucleotides formed in the soluble fraction during the incubation constitute only a small fraction of the total pool of cytidine nucleotides. The observed specific activity of cytidine nucleotide from glucose-U-^{14}C could also represent inhibition of CTP synthetase by the product. Similar inhibitions are known in many enzyme reactions (155). // According to the latter hypothesis, once the pool of cytidine nucleotides reaches a certain size it would inhibit further conversion of UTP to CTP. To test this possibility, cells were incubated with various concentrations of uracil-2-^{14}C. From the data in Table XXI, it is seen that the amount of ^{14}C-uracil converted rises in parallel fashion in UMP and CMP and reaches a maximum value after the addition of 1.5 µmoles of exogenous uracil-2-^{14}C. It should be noted that after the addition of 0.075 umoles of substrate, the ratio of the specific activity of CMP/UMP is 0.027. At the higher concentrations, this ratio is approximately 0.060. The increase in the CMP/UMP ratio at the higher concentrations of substrate suggest that the conversion of uridine nucleotides to cytidine nucle-

TABLE XXI

Incorporation of Different Concentrations of Uracil-2-^{14}C Into UMP and CMP of RNA

Uracil 2-^{14}C µmoles	UMP % ^{14}C incorp.	UMP Sp.Ac. mumoles incorp./ umole	CMP % ^{14}C incorp.	CMP Sp.Ac. mumoles incorp./ umole	Ratio of specific activity $\frac{CMP}{UMP}$
12	.08	46	.009	3.2	0.069
6	.18	49	.019	3.3	0.067
3	.33	47	.033	3.0	0.063
1.5	.60	47	.053	2.9	0.061
0.75	1.03	39	.085	2.6	0.066
0.075	2.34	8.7	0.111	0.24	0.027

The experimental conditions were as described in Table XIX. The incubation medium contained 0.5 µC Uracil-2-^{14}C.

otides is a reflection of the law of mass action, in the sense that more cytidine nucleotide are formed as the concentration of uridine nucleotides is increased. Table XXI also depicts the per cent of radioactivity incorporated from the different concentrations of uracil-2-^{14}C to UMP and CMP of RNA. It is interesting to note that the amount of radioactivity incorporated into CMP is never more than 10 per cent that in UMP. These results suggest that uridine nucleotides are poorly converted to cytidine nucleotides in the acid-soluble fraction and that after conversion the labelled cytosine compounds mix with a relatively large endogenous pool of cytosine nucleotides before entering RNA.

In order to test further the hypothesis that radioactive cytosine molecules were diluted by a relatively large pool of unlabelled cytidine nucleotides, cells were incubated with uracil-2-^{14}C for different times. It would be expected that with increasing time the specific activity of the cytidine nucleotide would increase and approach that of the uridine nucleotides. The data in Table XXII shows the results obtained after incubation of Ehrlich ascites cells with the labelled substrate for 30, 60, 90, 120 and 180 minutes. The results indicate that regardless of whether the cells were incubated for 30 minutes or 180 minutes, the ratio of the specific activity CMP/UMP remained essentially the same. This indicates that the cytidine nucleotide pool is supplied continuously with cytidine nucleotides which dilute the radioactive cytidine compounds derived from uridine nucleotides.

TABLE XXII

Specific Activities of UMP and CMP After Incorporation of Uracil-2-^{14}C at Different Times

Time Minutes	Specific Activity counts/min/μmole		Ratio of Specific Activity $\frac{CMP}{UMP}$
	UMP	CMP	
30	6320	390	0.061
60	8340	503	0.060
90	11940	760	0.063
120	9000	790	0.086
180	11125	900	0.080

The experimental conditions were as described in Table XIX. The incubation medium contained 2 mM uracil-2-^{14}C (2 μC).

5.6 Incorporation of Various Labelled Precursors Into CMP and UMP of RNA

In view of the results obtained thus far it was of interest to examine the relative incorporation of various labelled precursors into the pyrimidine nucleotides of RNA. The data in Table XXIII provide a comparison for the incorporation of radioactive cytidine, uridine, uracil and cytosine. The results indicate that uridine-2-^{14}C is incorporated to a greater extent than uracil-2-^{14}C into UMP and CMP. The resulting ratios of specific activities of CMP/UMP are 0.064 and 0.062 respectively. Approximately the same ratio is obtained when glucose-U-^{14}C is used as the precursor. Cytosine as was shown previously is not utilized, but cytidine-2-^{14}C labels both pyrimidine nucleotides of RNA, the ratio CMP/UMP obtained is 4.39. This efficient utilization of cytidine by Ehrlich ascites cells is in agreement with the results of Reichard *et al.* (99) who have shown that extracts of these tumor cells convert cytidine to cytidine nucleotides. The incorporation of radioactive cytidine into RNA and DNA has been demonstrated also in rat tissues (195). The results obtained in Table XXIII are consistent with the view that uracil, uridine, cytidine and ribose phosphate are converted to uridine and cytidine nucleotides by the reaction sequences shown below. It is seen from this scheme that cytidine is deaminated to uridine by cytidine deaminase which as shown in previous studies (196, 197) is specific for the nucleoside. These and the previous experiments demonstrate further that Ehrlich ascites cells have the ability to

TABLE XXIII

Comparison of the Specific Activities of CMP and UMP of RNA After Incubation with Various Labelled Precursors

^{14}C Substrate	Specific Activity counts/min/μmole		Ratio of Specific Activity $\frac{CMP}{UMP}$
	UMP	CMP	
Cytidine-2-^{14}C	2,300	10,100	4.39
Uridine-2-^{14}C	15,550	1,000	0.064
Uracil-2-^{14}C	5,040	310	0.062
Cytosine-2-^{14}C	0	0	-
Glucose-U-^{14}C	2,169	166	0.076

The experimental conditions were as described in Table XIX.

The values for glucose-U-^{14}C were taken from Table XVIII.

use various bases and nucleosides, but not cytosine, for the formation of the nucleotides of RNA. In addition, the reactions shown below suggest that the salvage pathway for the formation of pyrimidine nucleotides is not just a mechanism for the random supply of nucleotides but probably a well organized series of events essential for the supply and control of precursors involved in nucleic acid synthesis.

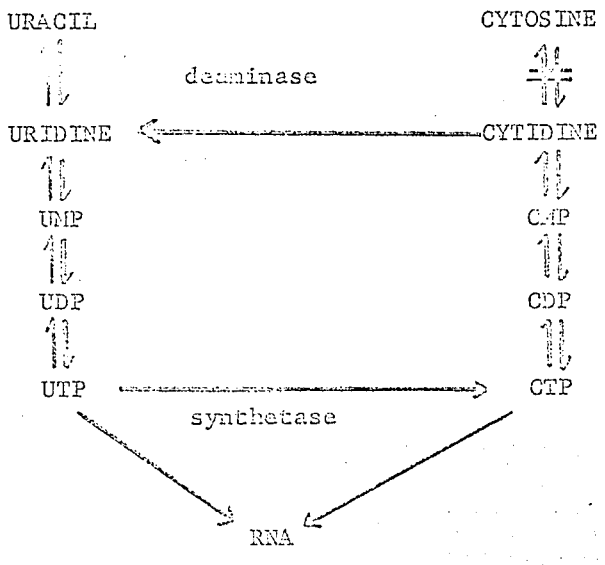


FIG. 8: The interrelationships of the various pyrimidine derivatives in Ehrlich ascites cells.

5.7 The Effect of Pyrimidine Nucleosides on Uridine-2-^{14}C Incorporation Into Acid-Soluble Nucleotides

The regulation of metabolic pathways is often effected through feedback inhibition by the end-product (155). End-product inhibition of pyrimidine biosynthesis de novo is exerted by CTP upon aspartate transcarbamylase, the enzyme that catalyzes the first step in this pathway (146). It has become apparent in recent years that the enzymes of the salvage pathways for the utilization of pyrimidine nucleosides are susceptible to end-product inhibition. The activities of deoxythymidine kinase, deoxyuridine kinase and deoxycytidine kinase are all under the control of deoxythymidine triphosphate (dTTP) and deoxycytidine triphosphate (dCTP), respectively (198) (Fig. 9.). Pyrimidine deoxyribonucleotides synthesis by the other pathways can also be inhibited by the end-products of these reactions (199-202).

The present study has been concerned with the metabolism of pyrimidine ribonucleotides derived mainly by the salvage pathways. These pathways appear to operate at a minimal level in mammalian cells that are not proliferating rapidly, but increase to significant activity in rapidly growing tissues such as regenerating liver and tumor cells (99). The finding of Anderson and Brockman (203) that UTP and CTP inhibit the conversion of uridine to uridine nucleotides in extracts from tissue culture cells suggest that uridine kinase is susceptible to feedback inhibition.

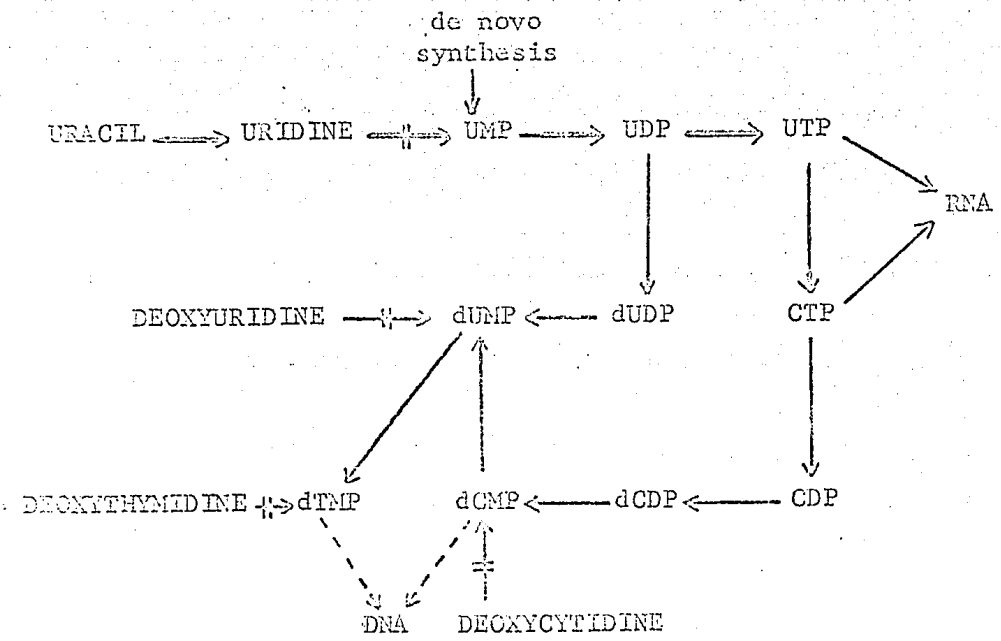


FIG. 9: Feedback inhibition for controlling pyrimidine nucleotide synthesis derived by the salvage pathway. Hatched arrows indicate site of inhibition.

The finding in the previous experiments that only small amounts of uracil-2-^{14}C are incorporated into cytidine nucleotides, coupled with the inability of Ehrlich ascites cells to utilize cytosine, suggested that the pathway of pyrimidine synthesis might be very sensitive to higher concentrations of intracellular cytidine nucleotides. This possibility was studied by preincubating the Ehrlich ascites cells for 15 minutes with cytidine, uridine or cytosine arabinoside. The cells were removed from the medium and washed with Krebs-Ringer solution, to remove intracellular nucleoside, and incubated in fresh medium containing

uridine-2-^{14}C for 30 minutes. The results obtained are shown in Table XXIV. The values on the left in the right hand column show the per cent decrease of uridine-2-^{14}C incorporation into acid-soluble nucleotides compared to the control, pre-incubated without nucleoside. Uridine effected about 60 per cent decrease, and cytidine 83 and 90 per cent at concentrations of 0.5 and 1 mM. Since the cells were pre-incubated with unlabelled nucleoside, these results must represent to some extent dilution of the labelled substrate by intracellular accumulation of uridine from both uridine and cytidine. However, since uridine is a closer precursor of uridine nucleotides than cytidine the inhibitory effect of the latter on the conversion of uridine-2-^{14}C to acid-soluble nucleotides may be obtained by comparison with the values obtained from the cells pre-incubated with uridine. These values shown (in brackets) in the right hand column of Table XXIV demonstrate that 60 and 75 per cent inhibition of uridine-2-^{14}C conversion to nucleotides is obtained by the addition of 0.5 and 1 mM cytidine. That this inhibition is obtained from intracellular accumulation of CTP is suggested from the results illustrated in Table XXVI. It was found that the cytidine that was taken into the cells was converted mainly to its triphosphate derivative. It was not possible to detect intracellular CDP, CMP or cytidine.

Cyt[illegible]side, an analog of cytidine, is used as an anticancer agent (204). Kessel et al. (204) have demonstrated that the effectiveness of this drug in inhibiting cell growth is

TABLE XXIV

Effect of Pyrimidine Nucleosides on Uridine-2-^{14}C Incorporation Into Acid-Soluble Nucleotides

Uridine-2-^{14}C + substrate added for preincubation	counts/min/mg dry weight of cells	% decrease of uridine incorporation	
Nil	1,777	0	-
Uridine (0.5 mM)	717	59.7	(0)
" (1.0 mM)	732	59.0	(0)
Cytidine (0.5 mM)	304	83.0	(60)
" (1.0 mM)	185	89.6	(75)
Cytosine Arabinoside (1.0 mM)	1,625	-	-

The experimental conditions were as described in Table XIX. Cells were incubated with 0.5 mM uridine-2-^{14}C (0.5 μC) at 37°C for 30 minutes.

dependent on the extent of conversion of cytosine arabinoside to its phosphorylated derivatives. It seemed interesting to determine whether this compound would inhibit the conversion of uridine-2-^{14}C to the nucleotides. The data in Table XXIV shows that the radioactivity incorporated into acid-soluble nucleotides from uridine-2-^{14}C is unaffected by the presence of cytosine arabinoside, although it must have been converted to the nucleotides since in experiments not reported in this thesis the incorporation of uridine-2-^{14}C into DNA was inhibited by the addition of this compound.

The effect of uridine and cytidine nucleotides on uridine kinase was studied by incubating uridine-2-^{14}C with a cell-free extract of Ehrlich ascites cells in the presence of the above compounds. After the incubation, the amount of radioactivity which appeared in UMP, UDP and UTP was determined as described in Table XXV. In comparing the radioactivity incorporated into the various nucleotides of uridine it was found that 30 per cent of the uridine added was phosphorylated during 15 minutes of incubation. 70 per cent of the radioactivity incorporated into the nucleotides was in the form of UTP. This is in accord with previous findings that uridine kinase is the rate limiting reaction in the conversion of uracil to uridine nucleoside triphosphates (98, 100). Preliminary experiments were carried out to determine the concentration of uridine needed to saturate the uridine kinase reaction and the amount of ATP required for maximum phosphorylation of uridine-2-^{14}C. These optimal conditions were used in the experiments described below. The results (Table XXV) demonstrate in accord with the previous experiments, that CTP is a very potent inhibitor of uridine

TABLE XXV

Effect of Pyrimidine Nucleotides on Uridine-2-^{14}C Phosphorylation in Cell-Free Extracts

Addition mM	Uridine Phosphorylated % of control
Nil	100
UTP 0.3	83
UTP 3.0	11
UDP 0.3	77
CTP 0.3	44
CTP 3.0	7
CDP 0.3	55

The experimental conditions were as described in "Materials and Methods". The reaction mixture contained 0.25 umole Uridine-2-^{14}C (0.25 uC). Samples were incubated at 37°C for 15 minutes and deproteinized with heat. ^{14}C-labelled UMP, UDP and UTP were isolated by descending chromatography in the isobutyric solvent system and assayed for radioactivity as described in "Materials and Methods".

kinase. At a concentration of 0.3 mM 66 per cent inhibition of uridine phosphorylation was obtained by CTP and 17 per cent by UTP. At high concentrations of CTP and UTP (3 mM) 89 and 93 per cent inhibition was obtained. UDP and CDP also decreased the phosphorylation of uridine. However, it is probable that UDP and CDP were converted to the triphosphate derivatives and as such inhibited uridine kinase. It was therefore judged not useful to determine the effect of the pyrimidine nucleoside monophosphates since they would also be converted rapidly to their respective triphosphates.

5.8 Effect of Cytidine on the Incorporation of Adenine-8-^{14}C Into ATP, RNA and DNA

The results in the above experiments indicate that cytidine nucleotides inhibit the net synthesis of pyrimidine nucleotides from uridine. Cannellakis (94, 95) suggested that the presence of active uridine phosphorylase and uridine kinase in rapidly growing tissues might be indicative of an homeostatic mechanism for the regulation of nucleic acid synthesis in animal cells. To examine the effect of cytidine nucleotides on RNA and DNA synthesis, adenine-8-^{14}C incorporation was used. The results in Table XXVI demonstrate that the addition of 0.1 mM cytidine increased the cytidine nucleotide pool to 116 μ μmole/mg dry weight of cells. At this concentration cytidine has no effect on adenine-8-^{14}C incorporation into ATP or RNA. In contrast, the incorporation of adenine-8-^{14}C into DNA was increased by 42 per cent. At cytidine concentrations greater than 0.1 mM there was a progressive

increase in the concentrations of the cytidine nucleotides in the acid-soluble fraction, but no further increase of adenine-8-^{14}C incorporation into ATP, RNA or DNA.

TABLE XXVI

Effect of Cytidine on the Incorporation of Adenine-8-^{14}C Into ATP, RNA and DNA

Addition mM	Cytidine Nucleotides µmoles/mg dry wt.of cells	Counts/min/mg, dry weight of cells		
		ATP	RNA	DNA
Nil	N.D.	2,465	526	64
Cytidine (0.1)	116	2,555	595	91
" (0.5)	190	2,471	546	92
" (1.0)	304	2,082	505	81

The experimental conditions were as described in Table XIX. The incubation medium contained 0.3 mM adenine-8-^{14}C (0.5 µC), 5 mM glucose and further additions as noted in a total volume of 6 ml.

N.D. not detected on the chromatography paper.

SUMMARY OF CHAPTER V

The distribution of radioactivity from ^{32}P in RNA nucleotides was determined and it was demonstrated that all four nucleotides were labelled to approximately the same extent.

Radioactivity incorporated into the various nucleotides of the acid-soluble fraction and in RNA was increased by the addition of adenine, guanine, or uracil, but not cytosine. This together with the failure of cytosine-2-^{14}C to be incorporated (even at high concentrations) in the corresponding nucleotides indicated that Ehrlich ascites cells cannot utilize this base although it penetrates, as well as uracil, into the cell.

The experiments with uracil-2-^{14}C demonstrate that the specific activity of CMP in RNA is never [illegible] per cent of its precursor UMP even when the cells are incubated for longer period of time to dilute the unlabelled cytosine nucleotides with radioactive nucleotides obtained from uridine nucleotides. The radioactivity incorporated from uracil-2-^{14}C into CMP is about 10 per cent that of UMP. These results suggest that the radioactive uridine nucleotides are poorly converted to cytidine nucleotides which are then diluted by a continuous supply of unlabelled cytidine nucleotides.

The incorporation studies also demonstrate that cytidine, probably by its conversion to CTP, caused significant inhibition of

the conversion of uridine-2-^{14}C to its nucleotides. The experiments with cell-free extracts of Ehrlich ascites cells demonstrate that CTP inhibits the uridine kinase enzyme. This reaction was also inhibited to some extent by the same concentration of UTP.

The addition of cytidine to Ehrlich ascites cells increased the incorporation of adenine-8-^{14}C into DNA, by about 40 per cent.

CHAPTER VI

DISCUSSION

Glucose is metabolized through the Embden-Meyerhof glycolytic sequence to pyruvate and lactate. These compounds can be metabolized further through the citric acid cycle. From a series of reactions which branch from glycolysis and the citric acid cycle, glycine, L-serine, L-aspartic acid, L-glutamine and ribose phosphate may be formed and utilized for the synthesis of purine and pyrimidine nucleotides. It has been suggested that neoplastic tissues are characterized by a permanent imbalance between those enzymes which utilize essential metabolites in the growth process and the enzymes which convert the metabolites to pathways not involved in growth (205). Schmitz et.al.(205, 206) working with rats bearing the Flexner-Jobling carcinoma found higher labelling in the nucleic acids and nucleotides from glucose-1-^{14}C in the tumor than in normal rat tissues. Kit et al.(178, 179) demonstrated that labelled glucose was converted to L-serine, glycine, L-aspartic acid and L-glutamic acid by Ehrlich ascites cells. In the present work the incorporation of ^{14}C from uniformly labelled glucose into purine and pyrimidine nucleotides of nucleic acids was examined to obtain information concerning the pathways involved in nucleotide synthesis and to make an estimate of the overall rate of nucleic acid synthesis.

6.1 Utilization of Glucose for Nucleotide Synthesis in Ehrlich Ascites Cells

The results presented in Chapter IV show that when Ehrlich ascites cells are incubated *in vitro* with glucose-U-^{14}C the purine and pyrimidine nucleotides are labelled mainly in the ribose moiety. Previous studies in tumors indicate that the low rate of *de novo* synthesis of nucleotides *in vitro* is largely due to the absence of L-glutamine and glucose to supply the amide and ribose phosphate, respectively (34, 36, 80). It was shown that glucose increases the amount of glutamic acid available for the formation of L-glutamine in Ehrlich ascites cells (37). Herscovics and Johnstone (37) demonstrated that in some experiments the incorporation of ^{14}C-formate into purine nucleotides in the presence of glucose was not increased by the addition of L-glutamine. They concluded that in the presence of glucose the increased formation of glutamate may lead to sufficient synthesis of L-glutamine to stimulate *de novo* synthesis of purine nucleotides in Ehrlich ascites cells.

The present data are in accord with the concept that glucose and glutamine control *de novo* synthesis of nucleotides in Ehrlich ascites cells since of the numerous precursors required for nucleotide synthesis only L-glutamine has a stimulatory effect on the incorporation of glucose-U-^{14}C into the nucleotides. The present data indicate, however, that the presence of glucose alone is not sufficient to satisfy all the requirements for *de novo* synthesis of

nucleotides in the Ehrlich ascites cells. It was shown that the addition of 1 mM L-glutamine doubles the incorporation of ^{14}C from glucose-U-^{14}C into adenine nucleotides, increases by 10 per cent the label in guanine nucleotides and in some cases, stimulates the incorporation of glucose-U-^{14}C into the pyrimidine nucleotides of RNA. The variable effect of L-glutamine on the incorporation of ^{14}C from labelled glucose into pyrimidine nucleotides may be dependent on the following factors. Different cell preparations probably differ with respect to their intracellular concentrations of L-glutamine and also to the synthesizing capacity of L-glutamine from exogenous glucose which may vary depending on the

tumor. Although the above would be expected to effect the purine nucleotides in the same manner, it may be that the pyrimidines are more sensitive to these variations. In addition, concentrations of other factors necessary for de novo synthesis of pyrimidines may be low in certain cell preparations. The results, however, are in agreement with those of Hager and Jones (80) and Mayfield et al. (82) and indicate that L-glutamine can provide the nitrogen for de novo synthesis of pyrimidine nucleotides.

The data in Table XIV show that, in the absence of L-glutamine, radioactive ribose phosphate derived from glucose-U-^{14}C is incorporated into the various nucleotides of RNA mainly by utilization of preformed bases. Azaserine, a known inhibitor of de novo synthesis of nucleotides, did not affect the radioactivity incorporated from labelled glucose into the ribose moiety of the purine

and pyrimidine nucleotides of RNA. The amount of radioactivity incorporated into the purine and pyrimidine nucleotides by de novo pathways, about 10 per cent, is too close to the standard error and will not be detected in these experiments.

The possibility that in vivo the Ehrlich tumor cells might be supplied by the ascitic fluid with L-glutamine or some other precursors necessary for de novo synthesis was also investigated. The results, Table XV, demonstrated that addition of the ascitic fluid has no significant effect on the amount of glucose-U-^{14}C incorporated into the nucleotides by the de novo pathways. Moreover, de novo synthesis of purine and pyrimidine nucleotides was again seen to be dependent on L-glutamine. These results favor the view that Ehrlich ascites tumor cells, due to their limited capacity to synthesize L-glutamine, may be dependent to a large extent on the availability of preformed bases to supply them with sufficient nucleotides for nucleic acid synthesis. Of course, no evidence was provided in this study to indicate that de novo synthesis of nucleotides in vitro parallels that in vivo. In addition, it is possible that in vivo glutamine synthetase enzyme shows greater activity or is provided in greater amounts when necessary.

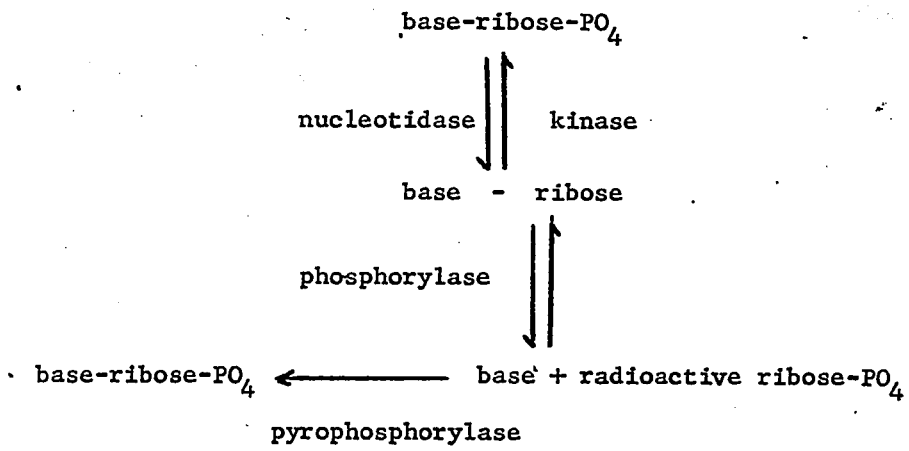
6.2 Labelling of the RNA Nucleotides

The results obtained indicate that in the presence of radioactive glucose, the pattern of labelling of the RNA nucleotides

is a direct reflection of the specific activity of the various nucleotides in the acid-soluble fraction. It is interesting to note that the specific radioactivities of adenine, guanine and uracil nucleotides are of the same order of magnitude despite the fact that radioactive ribose phosphate incorporated into adenosine nucleotides must be diluted by an intracellular pool of endogenous nucleotides which is 7-10 times larger than uridine or guanosine nucleotides. These observations suggest that during incubation, free radioactive ribose phosphate comes into isotopic equilibrium with the various nucleotide precursors of RNA. There are two possible mechanisms by which radioactive ribose phosphate can be incorporated into the nucleotides under these in vitro conditions. One involves the reaction of free bases with labelled ribose phosphate by the reactions of the salvage pathways described in Chapter I. The other involves the interconversion of the ribose moiety of endogenous nucleotides with labelled ribose phosphate derived from glucose-U-^{14}C. Although there are probably small amounts of bases in cells attempts to measure the intracellular concentrations of bases in Ehrlich escites cells have not been successful since the level of bases is extremely low. In addition, no exogenous bases can be obtained from the host in vitro and it seems therefore unlikely that the radioactive ribose phosphate is incorporated into the nucleotides, to any significant extent, by utilization of free bases.

Support for the concept that the endogenous nucleotides

exchange their ribose moiety with free radioactive ribose phosphate is derived from the following; Creaser et al. (207) have shown that glucose has no effect on the incorporation of ^{32}P-orthophosphate into acid-soluble nucleotides of Ehrlich ascites cells, indicating that the radioactivity was not incorporated by de novo synthesis of nucleotides. They showed also that after 45 minutes of incubation the specific activities of the endogenous nucleotides are in isotopic equilibrium with the ^{32}P present in the cell. The above workers concluded from this, that the phosphates are probably removed from the endogenous nucleotides and then rephosphorylated with the ^{32}P in the medium. Recent studies (208) have shown the presence of 5'-nucleotidase enzymes for the dephosphorylation of the four nucleoside monophosphates, AMP, GMP, UMP and CMP. A consequence of this is that nucleosides are intermediates in these reactions. Paterson (209) has shown that the ribosyl group of a purine or pyrimidine nucleoside can be transferred to another purine and pyrimidine base. The transfer is apparently carried out by two separate nucleoside phosphorylases with free ribose-1-phosphate as a probable intermediate (209). The ability of Ehrlich ascites cells to metabolise purine and pyrimidine nucleosides to free bases and ribose-1-phosphate has been demonstrated (210). Thus radioactive ribose phosphate derived from glucose-U-^{14}C may be utilized by the various bases that can be formed from the coupled action of nucleotidase and phosphorylase enzymes present in Ehrlich ascites cells, as shown below.



If the equilibrium of the above reactions favors nucleotide formation, there will be little free bases or nucleosides at any one time, but a constant non-expandable pool will be present. In addition, the rate of these reactions during the incubation will determine the specific activity of the various nucleotide pools derived from labelled ribose phosphate and not the size of the endogenous pools of nucleotides.

The results concerned with the specific activity of CMP in RNA after labelling the cells with glucose-U-^{14}C (Table VI) were difficult to understand in terms of the existing concepts of the relationship between uridine and cytidine nucleotides. Based on the results from the ^{32}P labelling experiments (Table XVI) which showed that radioactive cytidine is not limiting or poorly incorporated into RNA, the low specific activity of CMP in RNA appeared to be a direct reflection of the metabolism of cytidine nucleotides in the acid-soluble fraction. The results obtained for the other nucleotide

precursors of RNA indicated that the radioactive ribose phosphate was incorporated into nucleotides by exchanging with the ribose moiety of endogenous nucleotides. It seemed reasonable to assume that under these in vitro conditions the conversion of UTP to CTP was insignificant and that only a small exchange of radioactive ribose-phosphate with the ribose moiety of this nucleotide was obtained. The experiments concerned with the utilization of the free bases showed that cytidine phosphorylase is absent in Ehrlich ascites cells and explained the apparent non exchangeability of the ribose of this nucleotide with the free radioactive ribose phosphate derived from glucose-U-^{14}C. These experiments showed that the radioactivity in the ribose moiety of CMP in RNA was obtained by the conversion of uridine to cytidine nucleotides in the acid-soluble fraction.

The experiments in which uracil-2-^{14}C was used to label the pyrimidine nucleotides of RNA suggested that the low specific activity in CMP is probably due to the following factors; poor conversion of radioactivity from uridine nucleotides in the acid-soluble fraction and dilution of the radioactive cytidine nucleotides by a continuous supply of unlabelled cytidine nucleotides. Bucher and Swaffield (211) and Hadjiolov et al.(212) have found that after labelling in vivo with orotic acid-6-^{14}C the ratio of the radioactivity of CTP to UTP in the acid-soluble fraction of rat liver was low and remained unchanged throughout a time course experiment. These results are in close agreement with the data of the present work.

Two possibilities may be cited to explain the continuous dilution of radioactive cytosine nucleotide. It has been shown that conditions that stop cell growth result in rapid turnover of RNA, and lead to a supply of nucleotides which are reutilized for RNA renewal (213). Since Ehrlich ascites cells cannot divide in vitro (39), studies of isotope incorporation into nucleic acids in vitro probably reflect primarily the process of turnover of the nucleic acids. The results discussed above indicate that the pools of adenine, guanine and uracil nucleotides also turnover by exchanging both their phosphate and ribose moieties with pentose and phosphate in the medium. It follows that the ribose of the nucleotides formed by the degradation of RNA during the incubation will, except for cytidine, exchange with free radioactive ribose phosphate and the specific activity of the adenosine, guanosine, and uridine nucleotides will probably remained unchanged. Since cytidine cannot exchange its ribose moiety with radioactive ribose phosphate unlabelled cytidine nucleotides, derived from RNA, will provide a continuous supply to dilute the radioactive cytidine nucleotides derived from the uridine nucleotides. If the radioactive precursor is uracil, the non-radioactive uridine molecules, obtained from RNA breakdown, may be simply diluted out because the amount of nucleotides obtained from the radioactive base is probably much greater than that obtained from the breakdown of RNA. In addition, the same exchange as with the ribose probably also takes place between the radioactive base and the unlabelled uracil.

Finally, another possibility for diluting the radioactivity

of the cytidine nucleotides comes from the studies of Harel et al. (182) who have shown that the terminal nucleotide sequence of soluble RNA, pCpCpA, undergoes rapid breakdown. Although this RNA amounts to only a few per cent of the total RNA, it could also contribute to the dilution of the labelled cytidine.

It must be assumed also from these experiments that the nucleotides released from RNA breakdown are mostly unlabelled, since the ratio of cytidylic acid/uridylic acid was not significantly changed over a period of 3 hours. If only the labelled RNA was degraded, the specific activity of the cytidine nucleotides would increase with time and the ratio of cytidylic acid/uridylic acid should be altered.

6.3 A Regulatory Role for Cytidine Nucleotides

The inability of the cells to utilize cytosine for nucleotide synthesis and the apparent low conversion of uridine nucleotides to cytidine nucleotides suggested that the phenomena concerned may represent a feedback control relationship of cytidine nucleotides on the rate limiting uridine kinase enzyme. This concept prompted a study designed to explore the effect of cytidine nucleotides on the conversion of uridine-2-^{14}C to its corresponding nucleotides in intact cells and cell-free extracts. The incorporation studies with uridine-2-^{14}C demonstrated that the addition of cytidine to the intact cells increases the concentration of CTP in the cell and inhibits the formation of isotopic uridine nucleotides 60 and 75 per cent. The ex-

periment with the cell-free extracts showed that the formation of uridine phosphates from uridine could indeed be inhibited by CTP. This reaction was also decreased by UTP, but to a much lesser extent. Although the inhibition of uridine kinase by CTP has been demonstrated (203), it has not been previously shown that an increase in the end product, CTP, in the intact cell results in a decrease of the conversion of uridine to the nucleotides. It is also possible to speculate that the apparent inability of Ehrlich ascites cells to synthesize pyrimidine nucleotides by the de novo pathway, observed in these and in previous experiments (214), may be due to strong inhibition by intracellular CTP on aspartate transcarbamylase. It is possible that the de novo synthesis of nucleotides is increased significantly only during conditions of rapid removal of CTP for DNA and RNA synthesis.

The present experiments also demonstrate that the addition of cytidine to Ehrlich ascites cells increased the incorporation of adenine-8-^{14}C into DNA, by about 40 per cent. Pertinent to this finding is the demonstration that addition of high concentrations of thymidine in the culture medium of mammalian cells inhibit cell growth (215). This inhibition can be relieved if deoxycytidine is added to the culture medium containing thymidine (215). It was shown in subsequent studies (216) that thymidine triphosphate (dTTP) inhibits the conversion of cytidine nucleotide to the deoxycytidine derivative, as shown in Fig. 10. Thus, the effect of thymidine on cell growth is obtained because of deprivation

of deoxycytidine triphosphate in the cell and DNA synthesis stops (216). Reichard et al. (217, 218) showed that the conversion of cytidine diphosphate to deoxycytidine diphosphate was also inhibited by deoxyguanosine triphosphate (dGTP), deoxyadenosine triphosphate (dATP) but not by deoxycytidine triphosphate (dCTP). It has been suggested that this control over the reduction of CDP may be part of a regulatory mechanism for the synthesis of DNA (219).

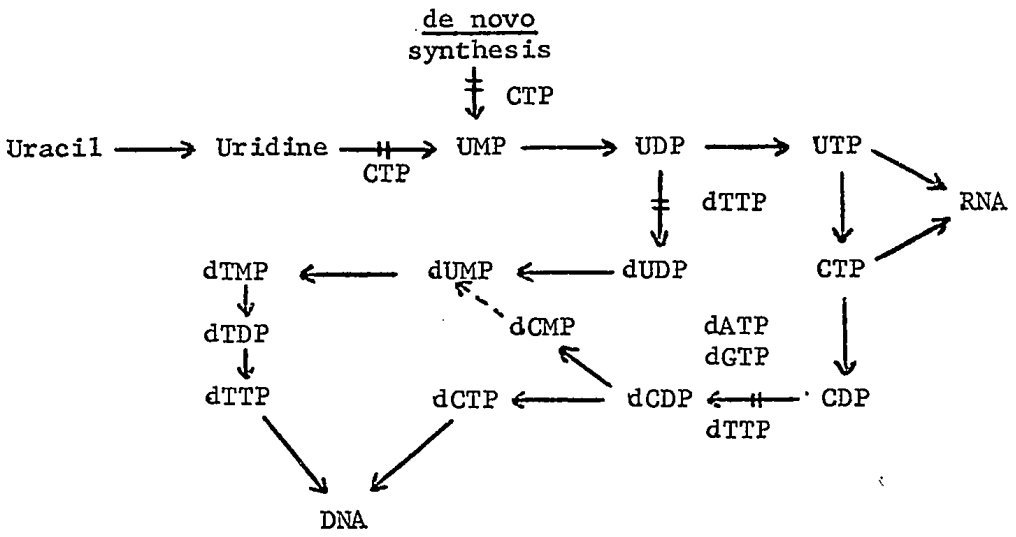


FIG. 10: Pyrimidine nucleotide synthesis. Hatched arrows indicate reactions inhibited in cell-free systems by compounds shown. Pointed arrow denotes that the significance of this reaction is unknown in Ehrlich ascites cells.

It could be reasonned further that when intracellular cytidine nucleotides are sharply increased, the cell is unable to exert sufficient control over the amount of deoxycytidine nucleotides formed from the reduction of CDP. Since dCTP has not effect on this reaction

and the amount of thymidine phosphates formed from cytidine nucleotides might not be sufficient (220) to prevent the accumulation of deoxycytidine nucleotides. The data in Table XXVI showed that in the presence of higher intracellular concentrations of cytidine nucleotides the conversion of adenine-8-^{14}C into DNA is significantly increased. Two possibilities may be offered for this observation. One is that cytidine nucleotides or its deoxyderivatives increase the conversion of adenine-8-^{14}C to deoxyadenosine nucleotides. This would increase the specific activity of the deoxyadenosine nucleotide pool and more radioactive molecules would be incorporated into DNA. The other possibility is that the exogenously derived deoxycytidine nucleotides increased the synthesis of DNA from adenine-8-^{14}C. This would be in accord with the concept that DNA synthesis may be related to the control of the intracellular pools of deoxynucleotides.

CLAIMS TO ORIGINAL RESEARCH

1. A method was described which prevented radioactive contamination of RNA and DNA fractions, isolated from Ehrlich ascites cells after their incubation with labelled glucose.

2. At various time intervals following the addition of glucose-U-^{14}C to Ehrlich ascites cells <u>in vitro</u>, the radioactivity incorporated into acid-soluble nucleotides, RNA and DNA was determined.

3. After 90 minutes of incubation with glucose-U-^{14}C <u>in vitro</u>, the acid-soluble compounds were subjected to two dimensional chromatography and were shown to contain the adenosine, guanosine and uridine di- and triphosphates. The specific activities of these compounds were determined.

4. The RNA and DNA were separated from each other and hydrolyzed to give the mononucleotides, and the specific activity of each nucleotide was determined.

5. In the acid-soluble fraction, the specific activity of guanosine nucleotides was somewhat higher than uridine and adenosine nucleotides.

6. In the RNA, the specific activity of GMP was the highest followed by UMP and AMP. In contrast, the specific activity of CMP was about ten times lower than the above three nucleotides.

7. In the DNA the specific activity of all four deoxyribonucleotides was approximately the same.

8. The radioactivity of the acid-soluble and RNA nucleotides was shown to be located primarily in the ribose moiety.

9. In the presence of L-glutamine, the radioactivity incorporated from glucose-U-^{14}C into purine nucleotides of RNA was increased in the adenine, guanine and ribose moieties. In some cases, L-glutamine also stimulated the incorporation of labelled glucose into the pyrimidine nucleotides of RNA.

10. Addition of azaserine, an inhibitor of de novo synthesis of purine and pyrimidine nucleotides affected only the increased incorporation of glucose-U-^{14}C obtained after the addition of L-glutamine. This indicated that in the absence of exogenous L-glutamine almost all the radioactive ribose phosphate obtained from glucose-U-^{14}C was converted to the purine and pyrimidine nucleotides in Ehrlich ascites cells in vitro by utilization of preformed bases.

11. In order to determine whether cytidine nucleotides were limiting in these in vitro experiments, the distribution of ^{32}P in RNA nucleotides was determined. The results demonstrated that all four nucleotides were labelled to approximately the same extent.

12. The addition of adenine, guanine, uracil, but not cytosine, increased the incorporation of glucose-U-^{14}C into acid-soluble and RNA nucleotides. This together with the inability of cytosine-2-^{14}C to be incorporated into acid-soluble nucleotide indicated that Ehrlich ascites have no salvage pathway for the conversion of this base to its nucleotide.

13. Studies with uracil-2-^{14}C demonstrated that the specific activity of CMP in RNA is never more than 8 per cent that of UMP, its pre-

cursor. Even incubating uracil-2-^{14}C for long periods did not increase the specific activity of CMP of RNA. It is postulated that the radioactive cytosine nucleotides derived from uracil nucleotides is diluted by a continuous supply of unlabelled cytosine nucleotides.

14. The experiments also demonstrated that incubation of Ehrlich ascites cells with cytidine 15 minutes before addition of uridine-2-^{14}C resulted in 60 and 75 per cent inhibition of the conversion of labelled uridine to its nucleotides. In cell-free extracts CTP was found to inhibit the uridine kinase enzyme. This reaction was also inhibited to some extent by UTP.

15. Incorporation studies also demonstrated that the addition of cytidine to the incubation medium containing the Ehrlich ascites cells increased the incorporation of adenine-8-^{14}C into DNA by 42 per cent. At present it is not possible to determine whether this is due to an enhancement of the rate of DNA synthesis.

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