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THE EFFECTS OF NUCLEOSIDES ON NUCLEIC ACID SYNTHESIS IN NORMAL AND CHRONIC LYMPHOCYTIC LEUKEMIC LYMPHOCYTES

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

NANCY WU MARCHAND

Oklahoma City, Oklahoma

1972

THE EFFECTS OF NUCLEOSIDES ON NUCLEIC ACID SYNTHESIS IN NORMAL AND CHRONIC LYMPHOCYTIC LEUKEMIC LYMPHOCYTES

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ACKNOWLEDGMENTS

I would like to thank Professor Creed W. Abell for extending to me the opportunity to study under his guidance and to pursue the subject of this research. His excellent advice, continued encouragement and great patience throughout the course of this work are greatly appreciated.

I also wish to thank the faculty members of the Department of Biochemistry and Molecular Biology, University of Oklahoma, for extending their facilities for my use. To the many colleagues with whom I have associated during my studies here, I extend my thanks for providing stimulating discussions and offering many helpful suggestions concerning various aspects of this work. In particular, I wish to extend my appreciation to Mrs. C. W. Kamp, Mr. W. J. Stith, Mrs. P. J. Johnson, and Miss D. K. Shirey for their friendship and willingness to help which made the completion of this work an enjoyable task.

My deepest thanks go to my husband, Alan, for his enduring support and true understanding have helped to make the long road a short and happy one and finally, to my parents who helped lay the foundation and the motivation which made the completion of this work possible.

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CHAPTER I

INTRODUCTION

Chronic Lymphocytic Leukemia

Leukemia was first recognized over a century ago, when Bennett (1) and Virchow (2) reported two individual cases independently. The complexity of this disease became increasingly evident over the years that followed, as more cases of leukemia were identified. Research revealed that several types of white cells played a role in the disease process, each having a different site of formation. Leukemia may be defined briefly as "a generalized, abnormal, neoplastic, self-perpetuating proliferation (slow or rapid) of one of the leukocytic tissues, often associated with abnormal white blood cell counts and eventually leading to anemia, thrombocytopenia, and death" (3).

Clinically, leukemia is classified according to (a) the white blood cell type, (b) the pattern of leukocytic proliferation, and (c) the leukocyte counts and differential counts. Leukocytes include lymphocytes, granulocytes, monocytes, and plasma cells. Thus at least four basic types of leukemia exist: lymphocytic, granulocytic, mono-

cytic and plasmocytic. Leukemia may progress slowly, as in the chronic form, or rapidly, as in the acute form.

While leukemia has a world-wide incidence, its epidemiology seems to vary considerably in different geographic areas (4) as well as among different races (4-8). For example, chronic lymphocytic leukemia (CLL) is virtually absent among non-Caucasians, i.e., the Japanese (6), Chinese (7), and New Zealand Maoris (8). This apparent racial resistance may be congenitally conferred, or it may relate to the lack of environmental factors capable of inducing the leukemic process, or both influences may act in concert. Genetic involvement as a possible etiologic factor has been explored and reviewed extensively (3,9,10). The low incidence of CLL in some Oriental groups and the tendency of CLL to occur repeatedly in the same family (11-13) suggest that a genetic factor may, indeed, be important.

Viruses have been implicated as the specific etiologic agents in certain animal leukemias (14-17). The recent discovery of an RNA-dependent DNA polymerase strongly supports the hypothesis that leukemia is virally transmitted (18,19). This RNA-dependent DNA polymerase can use RNA as a template to transcribe a complementary copy of DNA, which is then converted to double-stranded DNA, cleaved, and integrated into the host DNA during replication of the host genome. Spiegelman et al. (20) were able to demonstrate a homology between the RNA from leukocytes of leukemic patients and the RNA from the murine Rauscher leukemia virus, but not the RNA of an unrelated mouse mammary tumor virus. Using the molecular hybridization technique, with radiolabeled DNA complementary to the RNA of the murine leukemia virus, they

demonstrated this homology in 89% of the leukocytes from patients with CLL, acute lymphocytic leukemia, acute myelocytic leukemia, and monocytic leukemia. Other recent work suggests, however, that the RNA-dependent DNA polymerase is associated, not only with oncogenic viruses (21), but with non-oncogenic viruses (22) and a wide range of host cells, including lymphocytes from normal donors (23).

There is no definite indication that CLL can be induced by a single etiologic agent, however. Endogenous factors, such as genetic makeup, hormonal influences, and aging, are known to be important (24,25). Exogenous factors, such as viruses (26), chemical exposure (27), and irradiation (28), may also play major roles in producing CLL.

Non-stimulated Normal and CLL Lymphocytes

Numerous studies, devoted to comparing lymphocytes from healthy subjects with those from leukemic patients, have established that the small circulating peripheral lymphocytes, without exogenously supplied mitogens, do not normally undergo cell division in culture. These investigations have centered on the metabolism of carbohydrates for energy production, the glycolytic pathway of glucose degradation, and the biosynthesis of polysaccharides. The biochemical aspects of the resting normal and leukemic lymphocytes have been reviewed extensively by Elves (29) and Beck (30).

Cooper and Fitzgerald (31), studying a pure lymphocyte population, found that glycolytic oxidation predominated more under anaerobic conditions than under aerobic conditions. Frei and his coworkers (32) further concluded that the metabolic status of

lymphocytes resembles that of most other normal tissues in that they can use the oxidative pathway, and are also capable of small amounts of glycolysis under aerobic or anerobic conditions.

The independent investigations of glycolysis by Stjernholm (33) and Beck (34) revealed that leukemic lymphocytes produce lactic acid at a substantial lower rate than normal cells. This observation supports the previous findings that CLL lymphocytes possess little or no glycolysis under aerobic conditions and low to high glycolysis under anaerobic conditions (35-37).

Excess glycogen has been reported in CLL lymphocytes (38). Studies of enzymes, such as UDPG glucosyltransferase (39), α-D-glucosidase (40), and phosphorylase (41), which are involved either in the synthesis or catabolism of glycogen, failed to demonstrate that leukemic lymphocyte glycogen storage is related to abnormalities of these enzymes.

Brody et al. (42) took a different approach by studying the hexose monophosphate shunt pathway, which may lead to an accumulation of glycolytic intermediates; these then enter the glycogen synthesis cycle. The study of this pathway for glycogen synthesis in human lymphocytes has produced controversial results. Brody et al. (42) demonstrated that CLL lymphocytes produce significantly less radioactive carbon dioxide from glucose-1-^{14}C than normal lymphocytes, suggesting that the hexose monophosphate shunt is reduced in CLL lymphocytes. However, this finding was inconsistent with Beck's report (43) that a higher percentage of utilized radioactive glucose traverses the hexose monophosphate shunt in leukemic cells than in normal cells.

PHA-stimulated Normal and CLL Lymphocytes

One of the most promising advances in lymphocyte research was the discovery of Hungerford et al. (44) in 1959, that phytohemagglutinin (PHA) could induce the transformation of small lymphocytes in cell culture. When PHA, a glycoprotein extract from the red kidney bean Phaseolus vulgaris, was added to the leukocyte culture, some of the cultured cells underwent marked morphologic changes. Hungerford's observation was followed up by Nowell (45), who first described the mitogenic factor clearly in 1960.

In dividing cell populations, the sequence of metabolic events from one mitosis to the midpoint of a succeeding mitosis may be expressed in the "cell cycle" concept. A cell cycle comprises four main stages: G1, S, G2, and M. The resting or non-stimulated cell resides in the G1 phase, the interval between mitosis and the onset of DNA synthesis. When a mitogen such as PHA is added, sequential changes occur as cells go through the S phase (the DNA synthesis period), the G2 phase (the interval between complete DNA replication and mitosis), and finally the mitotic phase M, in which the cells divide.

The following account of the lymphocyte "cell cycle" has been reviewed by Abell (46). The cell membranes of lymphocytes change within minutes after PHA is added. Smith et al. (47) observed a significant elevation of adenyl cyclase, a membrane-bound enzyme, as well as a 25-300% increase in cyclic AMP levels in homogenates of human peripheral blood lymphocytes within 1-2 minutes after PHA stimulation. After 6 hours, however, the cyclic AMP level in these

cells usually fell to the level of the non-stimulated control cells. After 24 hours, the level dropped below that of the controls. In contrast, Novogrodsky and Kutchalski (48) reported that PHA had no detectable effect on either adenyl cyclase activity or cyclic AMP level in rat lymph node lymphocytes. Further study on the effects of extracellular cyclic-nucleotides failed to support the hypothesis that cyclic AMP alone can initiate the complex series of metabolic alterations which culminate in lymphocyte transformation (49). On the other hand, Cross and Ord (50) found that, in the lymphocytes of pig peripheral blood, dibutyryl cyclic AMP elicited an immediate burst of RNA synthesis, followed by DNA synthesis and eventual transformation. This was indistinguishable in timing and magnitude from that produced by PHA. Therefore, the hypothesis that cyclic AMP may be an important intracellular messenger in PHA-stimulated lymphocytes is plausible. The regulatory role of cyclic AMP on lymphocyte metabolism will be discussed in greater detail later in this chapter.

Also, within minutes after PHA addition, Fischer and Mueller (51) observed (a) accelerated incorporation of $^{32}PO_4^{-3}$ into phosphatidyl inositol, followed by (b) rapid interconversion of a compartmental phosphatidyl inositol and phosphatidic acid, and (c) increased secretion of gamma-immunoglobulin from the cells. The authors postulated that PHA-induced activation of phosphatidyl inositol metabolism leads to the removal of gamma-immunoglobulin from the cell via a protein transport mechanism. This would then allow the expression of cellular genetic information. Recently, the distribution of the stimulated phospholipid synthesis was also

studied (52). When lymphocytes were exposed to PHA for as little as 3 minutes, $^{32}PO_4^{-3}$ incorporation into phosphatidic acid was stimulated more significantly than incorporation into phosphatidyl inositol. Furthermore, the fact that PHA accelerated myoinositol-2-^{3}H and $^{32}PO_4^{-3}$ incorporation into phosphatidyl inositol to a much greater extent than glycerol-2-^{3}H or oleic-1-^{14}C incorporation is consistent with the mechanism of interconversion between phosphatidyl inositol and phosphatidic acid, in which the diglyceride portion is conserved. Evidence was also presented to support PHA stimulation of phosphatidic acid synthesis by diglyceride kinase.

Pogo et al. (53) observed a marked uptake of radioactive acetate into nuclear histone 15 minutes after PHA addition. The acetylation was more pronounced in the arginine-rich fraction than in the lysine-rich histone fraction. Pulse labeling experiments with ^{14}C-uridine and ^{14}C-acetate showed that histone acetylation preceded RNA synthesis. This was interpreted to mean that a change in the chemistry of the basic proteins of the chromosomes, brought about by acetylation of the histone, signaled a later increase in the RNA synthetic activity of the chromatin. MacGillivray (54) also found that PHA increased the labeling of histones of lymphocytes incubated with ^{14}C-methyl-methionine or sodium ^{14}C-acetate, a finding in general agreement with the data of Pogo et al. It has also been reported that the phosphorylation of serine and threonine residues associated with nuclear histones was increased twofold within 1 hour after PHA addition (55).

These modifications -- i.e., acetylation, methylation, and phosphorylation of histones, and histone-DNA interactions suggested

that an alteration of condensed chromatin to a diffused state probably activated the chromatin for RNA synthesis. This possibility has been supported by experiments using labeled uridine or cytidine as precursors to study lymphocyte RNA metabolism. Many workers (56-58) have observed marked incorporation of these isotopes within 30 to 60 minutes after the addition of PHA.

Lucas (59,60) observed that addition of PHA to human lymphocyte cultures produced a 20-fold increase in the rate of ^{3}H-uridine incorporation into RNA, as well as the activity of uridine kinase after 24 hours. Uridine kinase is the enzyme catalyzing the phosphorylation of pyrimidine nucleosides into pyrimidine nucleotides. Lucas also observed the inhibitory effect of cytidine on uridine incorporation and explained it as an end-product feedback inhibition, i.e., uridine kinase inhibition by pyrimidine triphosphate. Other workers (61,62) showed a close correlation between the rate of uridine phosphorylation in lymphocyte extracts and the rate of uridine incorporation by the intact cells. All agreed that uridine incorporation into RNA is limited by the rate of uridine phosphorylation rather than the rate of RNA polymerization.

Conflicting reports have appeared in the literature, however, concerning the rate-limiting step in the uridine incorporation process. Peter and Hausen (63), investigating the kinetics of membrane transport, concluded that the transport of uridine through the cell membrane is the rate-limiting step in uridine uptake in PHA-stimulated bovine lymph node lymphocytes. These authors postulated that PHA induced an increase in the number of functional carrier sites of the membrane

transport system. This hypothesis was further supported by the finding that the transport of the non-metabolizable glucose analogue 3-0-methyl-glucose through the lymphocyte membrane was stimulated immediately upon addition of PHA (64).

Cooper (65,66) reported that accelerated uridine incorporation by PHA-stimulated lymphocytes is accompanied by increased uridine incorporation into ribosomal and transfer RNA as well as polydisperse RNA, which constitutes nearly all the newly made RNA in resting lymphocytes. Thus the rate of RNA synthesis can be determined accurately by using sedimentation studies to estimate the percentage of RNA synthesis attributed to ribosomal RNA (67). By this method, total RNA synthesis increased two to fourfold in 24 hours. Recently, it was reported that the estimation of RNA polymerase activity in nuclei also provides an accurate, simple, and rapid means for determining alterations in RNA synthesis in growing and nongrowing cells (68).

Cooper (69) used methionine-methyl-^{3}H to label newly synthesized ribosomal RNA and made the following observations. First, about one half of the newly synthesized 18S rRNA in resting lymphocytes was immediately degraded. Second, the least newly synthesized rRNA was wasted when cells were in a rapid growth phase after 6-7 hours of incubation with PHA. Finally, after 20 hours of stimulation, rRNA wastage was still low, but rose to a level significantly above that found at 6-7 hours. After 40 hours, at the time of maximal cell growth, rRNA wastage was almost as high as that in resting cells. Cooper suggested that rRNA wastage, in both resting and growing cells, may be a manifestation of a growth-regulating mechanism. In a general

way, reduced wastage might occur only during unstable periods of transition from one stable state (resting) to another (maximal growth). The normal wasteful mode of rRNA synthesis would be re-established on reaching a stable state. The degradation mechanisms of the newly synthesized rRNA were also proposed.

More recently, the rRNA processing in CLL lymphocytes during the first 48 hours of the delayed response to PHA was studied by Rubin (70). He found that CLL lymphocytes failed to conserve 18S rRNA persistently. Subsequently, the over-all rate of RNA synthesis lagged far behind the level attained by normal cultures incubated with PHA.

Small lymphocytes have been shown to incorporate labeled phenylalanine, leucine, isoleucine, and methionine into protein (71). Several authors (72-74) have reported that the PHA-stimulated cultured lymphocytes contain immunoglobulins and even specific antibody. Kay's (75) study indicated that protein synthesis was stimulated after the initiation of RNA synthesis. However, early protein synthesis appeared to occur on pre-existing ribosomes.

Hayden et al. (76) have shown that glucosamine is incorporated into membrane glycoprotein of PHA-stimulated lymphocytes slowly but progressively. By proteolytic digestion, using gel filtration on a Sephadex G-150 column, these authors demonstrated the release of three different glycoproteins containing glucosamine from a purified lymphocyte membrane fraction.

Using labeled thymidine, which is rapidly and specifically incorporated into DNA, Bender and Prescott (77) first elucidated DNA synthesis and mitosis in stimulated human lymphocytes, and thus

introduced the concept of the human lymphocyte cell cycle. Their study revealed that (a) every mitosis is preceded by an in vitro DNA duplication, (b) the duration of the orderly G1, S, and G2 is approximately 24, 12, and 6 hours, respectively, and (c) an individual lymphocyte can divide approximately four times, i.e., the culture life span is limited.

In stimulated normal lymphocytes, Loeb et al. (78) found that (a) the induction of DNA polymerase is parallel in time and magnitude to the ability of the cells to synthesize DNA, (b) the increase in DNase activity also parallels DNA synthesis and the rise in polymerase, (c) the activities of thymidine kinase and thymidylate kinase increase about two to tenfold, and (d) guanylate kinase and deoxyguanylate kinase activities fail to rise significantly.

Abell et al. (79) estimated the rate of DNA synthesis in lymphocyte culture by measuring the rate of incorporation of labeled thymidine into DNA over a period of 8 days. Their study demonstrated that normal lymphocytes reach a maximum DNA synthesis at 3 to 4 days after PHA stimulation, whereas CLL lymphocytes show various delayed responses, depending upon the white blood cell count of the patient. Thus, their CLL cases were arbitrarily divided into three groups. Lymphocytes from patients with low, medium, and high white blood cell counts exhibited maximal DNA synthesis on days 5, 6, and 7, respectively. The observed delay in maximal DNA synthesis in CLL lymphocytes is consistent with the data of Havemann and Rubin (80).

Nucleotide Metabolism in Resting and PHA-stimulated Normal and CLL Lymphocytes

It is well established that nucleotides can be derived either from preformed purine or pyrimidine bases or nucleosides (salvage pathway), or from purines and pyrimidines synthesized by the cells from simple precursors such as ammonia, formate, and CO_2 (de novo pathway). Several comprehensive reviews deal with some of the enzymic reactions of nucleotide biosynthesis and enzyme regulations (81-84).

Indirect evidence that carbamyl phosphate is a precursor of orotate in animal tissues is provided by incorporation of labeled CO_2 into uracil and uridine nucleotides in Ehrlich ascites (85) and Novikoff ascites tumors (86). Carbamyl phosphate synthesis has been directly demonstrated in cell-free extracts of Ehrlich ascites cells, fetal rat liver (87), and mouse spleen (88). Ito and Uchino (89) showed that the initial steps in de novo pyrimidine biosynthesis are present in PHA-stimulated lymphocytes, since increased incorporation of $^{14}CO_2$ into uridine nucleotides and elevated carbamyl phosphate synthetase activity were found in these cells.

Conflicting reports have been published concerning the capacity of lymphocytes to utilize the de novo pyrimidine synthetic route with aspartic acid or orotic acid as precursors. Lucas (59,60) observed substantial incorporation of both labeled aspartic acid and orotic acid into lymphocyte RNA, implying that the pyrimidine de novo pathway is a major synthetic route. On the other hand, Kay and Handmaker (62) did not observe this phenomenon. Enzymes of the de novo pyrimidine pathway, such as aspartate transcarbamylase, dihydroorotase,

dihydroorotic dehydrogenase, and orotidylic decarboxylase, have been demonstrated in leukocytes (90,91).

In Escherichia coli (81) and yeast mutants (83), aspartate transcarbamylase (the enzyme catalyzing the formation of carbamyl aspartate from carbamyl phosphate and aspartic acid) appears to be a regulatory enzyme of de novo pyrimidine synthesis. However, in mouse spleen cells, aspartate transcarbamylase seems relatively insensitive to feedback inhibition (88, 92), whereas carbamyl phosphate synthetase is inhibited by uridine triphosphate (88). Thus a possible alternative means of regulating de novo pyrimidine synthesis may be provided by carbamyl phosphate synthetase.

The control of de novo purine biosynthesis by feedback inhibition of phosphoribosylpyrophosphate amidotransferase, the first enzyme of this pathway, has been demonstrated in pigeon liver (93). However, evidence has been presented that non-stimulated human leukocytes cannot utilize the de novo pathway of purine ribonucleotide synthesis, or can do so only poorly (94). Labeled glycine is not incorporated, and labeled formate is incorporated into purines in normal leukocytes only when intermediates such as aminoimidazole carboxamide or its ribonucleotide are present. This indicates that the deficiency exists in the early steps of the purine pathway. The limitations in de novo synthesis of purine ribonucleotides in leukocytes implies that synthesis occurs via the salvage pathway. No clear indication of this, however, has been observed in stimulated lymphocytes.

In the de novo pathway, the deoxynucleotides are formed in vitro by the direct reduction of nucleotides. The responsible enzyme,

ribonucleotide reductase, was found to be present in both non-stimulated normal and CLL lymphocytes, with a slightly higher level in the latter cells (95). Little information is available on regulation of nucleotide reductase synthesis in animal cells, although it is known to occur. In kidney cells cultured directly from the rabbit, cytidine incorporation into deoxycytidine nucleotides begins to increase approximately when DNA formation is initiated (96). This may represent increased ribonucleotide reductase activity, since it is reversibly inhibited by hydroxyurea, an inhibitor of the reductase in Ehrlich ascites cells and L cells (97).

Despite their capacity for de novo nucleotide synthesis, cells of most organisms also synthesize nucleotides from purine and pyrimidine bases and nucleosides. Enzymes of this salvage pathway, including nucleoside kinase, purine phosphoribosyltransferase, and nucleoside phosphorylase, are essential.

Many investigators believe that thymidine kinase is important in the control of DNA synthesis in various cells, because increased activity of this enzyme is associated with rapid growth and accelerated DNA synthesis. That the maximum kinase level is reached at the end of mitosis, coinciding with the onset of DNA replication, was demonstrated in mouse fibroblasts (98). Although the control of thymidine kinase is little understood, studies of liver culture cells have suggested that a phosphorylated derivative of deoxythymidine may be involved (99). In both non-stimulated normal and CLL lymphocytes, thymidine kinase activity was found to be low (100). Other nucleoside kinases, such as uridine kinase and cytidine kinase, rose to 20 times their control levels after PHA treatment (59,60).

In mammalian tissues, formation of purine mononucleotides from the appropriate purine and phosphoribosyl pyrophosphate (PRPP) appears to be a function of at least two enzymes, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase (91). These enzymes are specific for guanine, hypoxanthine, inosine, and adenine. When they were studied in both normal and leukemic leukocytes (91,101,102), there appeared to be no significant reduction in hypoxanthine-guanine phosphoribosyltransferase. However, adenine phosphoribosyltransferase increased two to threefold in CLL cells.

In the Lesch-Nyhan syndrome, hypoxanthine-guanine phosphoribosyltransferase is absent and adenine phosphoribosyltransferase is elevated in the patients' erythrocytes and fibroblasts (103). This syndrome is characterized by neurologic dysfunction, destructive behavior, and overproduction of urate.

The deoxycytidine pathway has also been studied extensively. Rabinowitz et al. (104) found that normal and CLL cells incubated with labeled deoxycytidine or deoxycytidine monophosphate yielded the greatest breakdown to uracil. Myeloblasts and monoblasts, however, showed the greatest recovery of deoxycytidine triphosphate (dCTP), deoxyuridine monophosphate (dUMP) and thymidine triphosphate (TTP). Uracil may be derived from either deoxyuridine or deoxyuridine monophosphate, which, in turn, are formed from deoxycytidine or deoxycytidine monophosphate, respectively. DCTP, dUMP and TTP are products of deoxycytidylate kinase, deoxycytidine deaminase, and thymidylate synthetase, plus thymidylate kinase. Rabinowitz and co-workers also observed that CLL lymphocytes produced appreciable amounts of dCTP,

dUMP, and Th compounds, but did not regularly produce blast cells in culture in response to PHA. On the other hand, cellular extracts of normal lymphocytes, or CLL lymphocytes with a PHA response resembling that of normal cells, did accumulate significant amounts of these compounds compared with initial low values during culture.

The thymidylate synthetase level has been observed to be elevated in chick embryo (105), hepatectomized rat liver (106), and heptomas (107,108). Nothing is known of the role played by metabolites in the regulation of this enzyme synthesis. During rapid growth, thymidylate kinase activity correlates with the rate of DNA synthesis, suggesting that the enzyme level is regulated by repression or induction.

Inhibition of deoxycytidylate deaminase by dTTP has been observed in extracts of rat embryos (109) and of Ehrlich ascites cells (110); in the latter case, dCTP reverses the inhibition. The partially purified enzyme from leukocytes exhibits linear double-reciprocal plots both in the absence of modifiers and in the presence of the activator, dTTP (111).

In conclusion, a precise balance between the de novo and salvage synthesis pathway for purine and pyrimidine is critical to cell function. The intricate relationship between the two pathways may have implications for potential chemotherapeutic application in the treatment of leukemia.

Role of Cyclic AMP in Neoplastic Cell Growth and Lymphocyte Proliferation in Vitro

Cyclic AMP was discovered as the mediator of the hepatic glycogenolytic effect of epinephrine and glucagon by Sutherland and Rall (112). The actions of a wide variety of hormones are now known to be mediated by alterations in the intracellular levels of cyclic AMP in the target tissue responding to the hormones (113). The second messenger function of cyclic AMP has been discussed in detail by Sutherland et al. (114-116).

Evidence exists that cyclic AMP may regulate cell division in at least some mammalian tissues. DNA synthesis increases and cellular proliferation occurs in rat salivary gland in response to isoproterenol (IPT) (117), a derivative of norepinephrine. Thymocytes showed activated DNA synthesis and mitotic activity in response to exogenous cyclic AMP (118). These observations suggest that excessively high levels of cyclic AMP could be a factor leading to malignant cell growth.

In contrast, Ryan and Heidrick (119) found that exogenous cyclic AMP inhibited the growth of HeLa cells and L cells where equimolar concentrations of 5'-AMP and adenosine (metabolites of cyclic AMP) were inactive. Heidrick and Ryan later extended their tissue culture studies to other tumorigenic cell lines and other cyclic nucleotides. Cyclic AMP was found to inhibit the malignant cells markedly, but had only a slight effect on the growth of a nonmalignant diploid cell line (120). Cyclic GMP's (3',5'-GMP and 2',3'-GMP) were less specific, producing similar inhibition in all cell lines. The pyrimidines, cyclic CMP and cyclic UMP, failed to affect the growth of

any of the cells. Gericke and Chandra (121) found that the injection of cyclic AMP inhibited the growth of a subcutaneously transplanted lymphosarcoma in mice. Cyclic UMP and cyclic IMP were also tested; they were less effective than cyclic AMP.

These findings have prompted extensive study of the regulatory role of cyclic AMP in lymphocyte transformation, discussed below. The contradictory results on the cyclic AMP level in PHA-stimulated lymphocytes were described earlier in this chapter.

Using cultured peripheral blood lymphocytes, Hirschhorn et al. (122) observed stimulation of RNA and DNA synthesis in response to cyclic AMP. Other nucleotides and adenosine had no effect or were inhibitory. In suspensions of lymphocytes taken from rat lymph nodes, low concentrations of the dibutyryl cyclic AMP stimulated RNA but not DNA synthesis (48). MacManus and Whitfield (118) demonstrated that additions of cyclic AMP influenced DNA synthesis in thymic lymphocytes; high concentrations inhibited whereas low concentrations stimulated replication.

Abell et al. (79) demonstrated that IPT inhibited DNA synthesis in CLL lymphocytes but not in normal lymphocytes. Furthermore, Johnson and Abell (123) investigated the possibility that IPT might act through the intracellular mediator cyclic AMP. Although cyclic AMP exhibited a moderate inhibitory effect, dibutyryl cyclic AMP and IPT inhibited DNA synthesis to an essentially identical extent over a 100-fold concentration range. These observations strongly suggested that cyclic AMP is involved. Additional support for this concept was provided by Smith et al. (47), who demonstrated marked elevation of cyclic AMP in normal lymphocytes after PHA addition.

General Consideration of Cyclic Phosphodiesterase

The level of cyclic AMP is determined by the activities of two opposing enzymes. One enzyme, adenyl cyclase, catalyzes the formation of cyclic AMP from ATP, whereas the second, cyclic phosphodiesterase, catalyzes its hydrolysis to 5'-AMP. Indirect evidence that adenyl cyclase is present in human leukocytes was provided by Lichtenstein and Margolis (124). They observed that antigen-mediated release of histamine from sensitized human leukocytes was inhibited by aminophylline, IPT, and dibutyryl cyclic AMP. Smith et al. (47) obtained direct evidence for the presence of adenyl cyclase in lymphocytes by measuring early increase in adenyl cyclase activity in lymphocytes exposed to PHA. The enzyme responded conventionally to fluoride, prostaglandin E, and IPT. Although cyclic phosphodiesterase has been identified in many tissues, to date only a few studies have described the physical and chemical properties of the enzyme in any detail (114-116).

Various compounds alter the activity of phosphodiesterase in vitro. Methylxanthines, such as theophylline (125) and caffeine (126, 127), were found to inhibit phosphodiesterase, decrease cyclic AMP degradation, and ultimately raise the level of cyclic AMP in tissues. At the same time, these compounds potentiate the response to the hormone under investigation. Thus, tissue response to methylxanthines in these studies has been generally taken as positive evidence that cyclic AMP is a mediator of hormone action. Other compounds reported to inhibit phosphodiesterase activity included ATP (128), puromycin

(129), and papaverine (130). Other substrates for cyclic phosphodiesterase are cyclic GMP and cyclic IMP. These nucleotides appear to compete with cyclic AMP for the substrate site on the enzyme. The substrate nature of the enzyme will be discussed later.

Kinetic studies have revealed two apparent Michaelis constants for cyclic AMP hydrolysis, ranging from 10^{-6}M to 10^{-4}M. This phenomenon was initially reported by Brooker and Appleman (131) and is consistent with literature on phosphodiesterase activity in muscle (129) and fat cells (132). The implication of the two K_m values is either that these tissues contain two or more forms of cyclic phosphodiesterase or that one form exists with multiple binding sites of different affinities for cyclic AMP. The first of these interpretations was supported by the separation of at least two enzyme forms from brain tissue (133). More recently, separation of multiple forms has been achieved in skeletal muscle, heart muscle, kidney and fat tissues by the use of agarose gel filtration (115,134). Purified phosphodiesterase appeared to be specific for purine 3',5'-mononucleotides. A high-molecular-weight fraction (approximately 400,000) purified from brain, kidney, and fat pads demonstrated a higher affinity for cyclic GMP than cyclic AMP. The K_m for cyclic AMP was 10^{-4}M and that for cyclic GMP was 10^{-5}M. A low-molecular-weight fraction (approximately 200,000) catalyzed no cyclic GMP hydrolysis under normal assay conditions and had a K_m for cyclic AMP of 5×10^{-6}M or lower.

In conclusion, kinetic and physical data concerning cyclic phosphodiesterase have revealed a complicated picture of isoenzymes. Kinetic analysis clearly indicates that the rate of hydrolysis of

cyclic AMP can be directly altered by cyclic GMP and vice versa. However, recent reports suggest that this effect of one cyclic nucleotide on the hydrolysis of the other may not always be inhibitory (135). At the present time, no data were available on phosphodiesterase in human lymphocytes.

Specific Aims

This research was designed to ascertain selected biochemical differences between normal and CLL lymphocytes. We examined (a) the mechanism of the nonspecific inhibitory effect exerted by cytidine on uridine-^{3}H incorporation in both normal and CLL lymphocytes, (b) the effects of purines, purine ribonucleosides, and cyclic purine ribonucleotides on thymidine-^{3}H and uridine-^{3}H incorporation in normal and CLL lymphocytes and the possible mechanisms for their actions and (c) the nature of cyclic phosphodiesterase in both normal and CLL lymphocytes.

Our aim was to elucidate the basic alterations underlying the leukemic process and to broaden our knowledge of the metabolic effects of purine and purine ribonucleosides in human lymphocytes.

CHAPTER II

MATERIALS AND METHODS

Materials

Human Lymphocytes

For the studies on normal lymphocytes, 450 ml samples of peripheral blood were collected from normal female volunteers who had fasted overnight. Each sample was treated with sodium heparin at the time of collection.

For the studies on CLL lymphocytes, 50 ml blood samples were collected from patients with chronic lymphocytic leukemia and were heparinized. These patients had white blood cell counts varying from 15,000 to 400,000 per cubic millimeter. The patients were either untreated for their disease or had received no therapy within 3 months prior to the study.

Cell Culture

Sodium heparin was purchased from the Upjohn Co. (Kalamazoo, Michigan). A column packed with glass wool (Owens Corning, Corning, New York) was used to resolve lymphocytes from other white cells. During the lymphocyte isolation step, cells were suspended in Eagle's minimal essential medium (MEM). For cell culture, McCoy's modified 5AB medium without streptomycin, penicillin, glutamine, and serum was used.

At the time of incubation, the medium was supplemented with glutamine and either autologous serum or fetal calf serum. Eagle's minimal essential medium, modified McCoy's 5AB medium, media supplements, and trypsin solution (0.25%) were all purchased from Grand Island Biological Co. (Grand Island, New York). During the cell-culture manipulations, a laminar-flow hood (Pure Aire Corp., Van Nuys, California) was used to reduce contamination by air-borne organisms. A water-jacketed CO_2 incubator (National Appliances Co., Cherry Hill, New Jersey) was used to incubate the cell cultures. Bone-dry grade CO_2 (Matheson Co., East Rutherford, New Jersey) was used to regulate the incubator at 5% CO_2 in balanced air. Phytohemagglutinin-P was purchased from Difco Laboratories (Detroit, Michigan).

Purine and Pyrimidine Bases, Ribonucleosides and Ribonucleotides

Cytidine, guanine, guanosine, adenine, adenosine, and inosine were purchased from Calbiochem (Los Angeles, California), cyclic AMP from Schwarz Bioresearch (Orangeburg, New Jersey), and cyclic GMP and dibutyryl cyclic AMP from Boehringer Mannheim Corp. (Mannheim, Germany). Thymidine-methyl-^3H, uridine-6-^3H and cyclic AMP-^3H(G) were purchased from the New England Nuclear Corp. (Boston, Massachusetts). Tritium-5-cytidine was manufactured by Amersham Searle (Arlington Heights, Illinois). Tritiated cyclic GMP was purchased from Mann Research Laboratory (New York, New York).

Other Experimental Materials

All common chemical reagents were purchased from Baker Chemical Co. (Phillipsburg, New Jersey). Quantitative studies on DNA,

RNA, and protein were performed with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Paper chromatography was performed on Whatman paper No. 1 (Curtin Scientific Co., Houston, Texas). Ultraviolet-absorbing material on the paper chromatograms was located with a Mineralight UV lamp (Ultra-Violet Products, Inc., San Gabriel, California). Radioactivities were determined either on a Vanguard strip counter (Vanguard Instruments, North Haven, Connecticut) or with a liquid scintillation counter (Nuclear Chicago, Chicago, Illinois).

Methods

Cell Culture

Lymphocyte isolation and culture procedure with or without chemical treatment. Peripheral blood lymphocytes from normal female donors and patients with untreated CLL were prepared for cultures according to the procedure of Abell et al. (123), which is a modified method of Cooper and Rubin (56). One unit (450 ml) of heparinized blood (10,000 units heparin/unit blood) drawn from a fasting female donor was transferred into sterile plastic 50 ml centrifuge tubes with twist caps. The blood was allowed to settle for 1 hour in an incubator at 37°C. The leukocyte-rich plasma layer was separated and mixed with an equal volume of Eagle's minimal essential medium prewarmed to 37°C. A purified lymphocyte population was obtained by passing the cell suspension through a glass wool column. Lymphocytes were separated from other leukocytes due to the selective binding of polymorphonuclear cells to glass wool. Wright stain smears indicated that the effluent contained 97-99% lymphocytes. Since the differential counts of the

leukocytes from patients with CLL showed lymphocyte counts of 73% to 99%, additional resolution was considered unnecessary.

Lymphocyte pellets were obtained by centrifugation at 280 x g for 30 minutes. Isolated lymphocytes were resuspended at a concentration of 2 x 10^6 cells per milliliter of modified McCoy's 5AB medium, which was supplemented with 10% serum and 1% L-glutamine. For normal lymphocyte cultures, autologous serum was used. It was obtained by centrifuging the erythrocyte plasma component remaining in the tubes after removal of the leukocyte-rich plasma supernatant. The serum was aseptically withdrawn after centrifugation at 450 x g for 30 minutes. For CLL lymphocyte cultures, fetal calf serum was added because not enough autologous serum could be obtained. A previous study showed that either fetal calf serum or autologous serum could be used without altering the DNA synthetic pattern or extent (136).

Immediately before use, PHA (40 µg) was added to a 2-ounce prescription bottle containing 5 ml of the lymphocyte suspension, prepared as described above. PHA was added to each culture bottle at zero time to stimulate cell division. The chemical under study was added to the cell culture immediately thereafter. The caps were left on the bottles loosely to allow gas exchange. The lymphocyte cultures were incubated at 37°C for the desired length of time in a humid atmosphere of 5% CO_2 in balanced air.

Assessment of RNA and DNA synthesis. RNA and DNA synthesis were estimated by the uptakes of tritiated uridine (Ur-6-^{3}H, 10.4 Ci/mmole, 2 µCi/ml) and thymidine Th-Me-^{3}H, 2.0 Ci/mmole, 2 µCi/ml), which were added to the cultures 2 to 6 hours, respectively, before

termination of the reaction. The incorporation rate of the radioactive precursors into nucleic acid was determined by liquid scintillation spectrometry, as described in the next section.

Isolation and measurement of DNA. At the end of the 2- and 6-hour incubation period, the cells and media were poured into chilled centrifuge tubes. The CLL lymphocytes, in contrast to normal lymphocytes, adhered somewhat to the glass bottles after PHA addition and, therefore, had to be removed with a 0.25% trypsin solution (1 ml). This was added after the cells had been poured out and incubated at 37°C for 5 minutes. At this time, the 1-ml trypsin solution was transferred to the proper centrifuge tubes and combined with the original cell suspension solution. The chilled cells and media were centrifuged at 0°C at 1,000 x g for 10 minutes. The supernatant was then decanted. To the cell pellet, 1 ml of hypotonic phosphate buffer solution (0.4 mM potassium phosphate, pH 6.7, and 2 mM magnesium chloride) was added. The pellet was resuspended on a Vortex mixer and fast-frozen in acetone and dry ice. Subsequent procedures to remove unwanted cellular constituents and unincorporated radioactive material were performed at 4°C.

Briefly, the procedure was as follows. The cells were broken by freezing and thawing seven times in an acetone-dry ice solution. The nucleic acids were precipitated with 1.6 N cold perchloric acid. The tubes were then chilled for 15 minutes. The standard centrifugation between each addition of solution was 10 minutes at 1,000 x g, after each centrifugation, the supernatant was decanted, the next solution added, and the resuspended pellets were dispersed with glass stirring

rods. The solutions, added sequentially in 3-ml aliquots, were 0.5 N PCA (3 times), 95% ethanol, chloroform:ethanol:ether (2:2:1,V/V), and acetone. The pellet obtained after acetone treatment was dried at 60°C for 30 minutes. Afterwards, the pellet was hydrolyzed in 0.5 ml of 0.5 N PCA at 90°C for 30 minutes.

After hydrolysis, 0.2 ml of the hydrolysate was added to 15 ml of Bray's solution (137) for counting the radioactivity incorporated into the nucleic acid in lymphocytes. Another 0.2 ml of the hydrolysate was used to assay total DNA content by Burton's diphenylamine method (138). Precursor incorporation of each sample was expressed in disintegrations per minute (DPMs). DPMs were calculated by dividing the counts per minute (CPMs) by the efficiency of the counting system. By adding tritiated water of known radioactivity to several samples after the initial counting, the counting efficiency was determined. DNA content was based upon a standard calf thymus preparation. The results were expressed in specific activities, i.e., labeled precursor incorporated per microgram of DNA. Therefore, the measurements of the incorporation rates of the precursors reflected the proliferative or nucleic acid synthetic phase of the cell cycle after PHA stimulation. Data obtained from the same experiment were also used to quantitate differences due to various treatments, such as different chemical additions. All data represented the average of duplicate cultures; each experiment was performed at least twice.

Cytidine Deaminase Study in Lymphocytes in Culture

The procedure for culturing lymphocytes has been described extensively in the previous section. Briefly, lymphocytes (10^7 cells) were incubated at 37°C in 5 ml McCoy's 5AB medium which was supplemented with either 10% autologous serum or fetal calf serum and 1% L-glutamine. PHA (40 µg), cytidine (10^{-4}M), uridine (1 nM) and 10 µCi cytidine-5-^{3}H (26.8 Ci/mmole) were added to the lymphocyte culture at zero time. Incubation was carried out for various times. To terminate the incubation, the cells were pelleted by centrifugation at 1,000 x g for 10 minutes. Both the medium and cell pellet were saved for paper chromatography. The cell pellets were suspended in 0.5 ml of homogenizing phosphate buffer solution (0.4 mM potassium phosphate, pH 6.7, and 2 mM magnesium chloride) and heated for 3 minutes at 95°C to denature the enzyme. The denatured protein was sedimented by centrifugation at 1,000 x g for 10 minutes.

Descending paper chromatography was used to separate cytidine from its metabolites. Authentic unlabeled cytidine (0.2 mM) and uridine (0.2 mM) were added to each 0.25 ml of the culture medium and the aliquots of the heated preparations. The mixtures were applied to Whatman No. 1 paper strips, which were developed in either of two solvent systems. The first consisted of isobutyric acid, concentrated NH_4OH, water, and 0.1 M EDTA·Na_2 (100:13.3:46.7:1.6, V/V). The second consisted of equal volumes of isoamyl alcohol, tetrahydrofurfural alcohol, and 0.08 M potassium citrate (pH 3.02). The R_f values for the first solvent were cytidine = 0.81 and uridine = 0.60; for the second solvent, they were cytidine = 0.52 and uridine = 0.65. After

development of the paper chromatograms in either solvent for 16 hours, the paper strips were dried and the ultraviolet-absorbing material was located with an UV lamp equipped with a short wavelength filter. Radioactivity was determined on a Vanguard strip counter. The area under each peak on the radiochromatogram was quantitated by carefully weighing the tracing paper replica of that peak. Data obtained from both solvent systems were essentially comparable.

Cytidine Deaminase Study in Lymphocytes in Vitro

Lymphocytes (10^7 cells) were incubated with or without PHA (40 μg) for 4 hours under the same conditions described above. The cells were then harvested and homogenized in a Dounce homogenizer (0.1 ml of homogenizing phosphate buffer solution per 10^7 cells) with 40 strokes. The cell homogenate was centrifuged at 7,000 x g for 10 minutes, and the supernatant and particulate fractions were separated. The assay mixture consisted of 10^{-4}M cytidine, 1.0 μCi cytidine-5-^{3}H (26.8 Ci/mmole), 1 nM of uridine, and either the supernatant or the particulate fraction of 10^7 cells in a final volume of 0.5 ml. The reaction mixture was kept at 37°C for various incubation periods. To stop the reaction, the mixture was heated at 90°C for 3 minutes. The methods for separating cytidine from its metabolites and determining the radioactivities in each component were the same as described in the previous section. The enzyme activity was expressed as the amount of enzyme required to form 1 nmole of uridine from cytidine in the standard assay solution at 37°C.

Cyclic Phosphodiesterase Study in Lymphocytes in Culture

The effects of guanine, guanosine, and cyclic GMP on cyclic phosphodiesterase activity in both normal and CLL lymphocytes were investigated. The procedures used to isolate the lymphocytes from whole peripheral blood samples were the same as described in the previous section. For the study of lymphocytes in culture, either guanine, guanosine, or cyclic GMP at concentrations of 10^{-4}M was added to the cell culture simultaneously with the PHA. At the end point of incubation, the cells were pelleted by centrifugation at 1,000 x g for 10 minutes at 4°C. In the normal lymphocyte preparation, a substantial amount of erythrocytes often appeared to be present in the cell pellet. Hypotonic lysis (139) was necessary to remove the contaminating erythrocytes. In the CLL lymphocytes preparation, this treatment was generally omitted. The cell pellet was then suspended in 20 mM phosphate buffer solution (50 µl buffer solution/10^7 cells), pH 7.5, and homogenized in a Dounce homogenizer with 40 strokes at 4°C. In some experiments (as indicated in Results), the whole homogenate was used directly for enzyme assay and Lowry protein content determination (140). In other experiments, a subcellular fraction (1,000 x g for 10 minutes) was used instead. Protein concentration varied from 2 to 4 mg/ml.

Cyclic phosphodiesterase has been reported to be a stable enzyme, with no appreciable change in activity after storage at -20°C for months (141). However, our study indicates that it is best to use a fresh preparation, since storage at -4°C for 2 days did result in substantial loss of enzyme activity in our crude preparation.

Cyclic Phosphodiesterase Study in Lymphocytes *in Vitro*

The kinetics of cyclic phosphodiesterase in both nonstimulated normal and CLL lymphocytes were studied, and the effects of guanine, guanosine, and cyclic GMP on the cyclic phosphodiesterase of CLL lymphocytes were investigated. As described before, pure lymphocyte populations could be prepared either from normal blood samples or from blood obtained from CLL patients. The lymphocytes were suspended in a 20 mM phosphate buffer solution, pH 7.5 (50 µl buffer solution per 10^7 cells) and homogenized in a Dounce homogenizer with 40 strokes. Either the whole homogenate or a subcellular fraction was used for the enzyme assay and Lowry test.

Cyclic Phosphodiesterase Assay

Phosphodiesterase was assayed by measuring the disappearance of cyclic AMP which was converted to 5'-AMP. Krishna *et al.* (142) described a simple, sensitive method to separate cyclic AMP from 5'-AMP and other nucleotides; i.e., all nucleotides are precipitated with zinc sulfate and barium hydroxide treatment. The principle of this method is that ATP, adenosine diphosphate (ADP), 5'-AMP, adenine, and inorganic phosphate are quantitively precipitated by the addition of zinc sulfate and barium hydroxide solutions, whereas over 99.9% of the cyclic AMP remains in the supernatant fluid. On the other hand, adenosine, which may be derived from 5'-AMP by the action of nucleotidase, is not precipitated by this treatment.

To prove the validity of this assay of cyclic phosphodiesterase, it was essential that all radioactivity found in the supernatant fluid of mixed zinc sulfate and barium hydroxide solution

be associated with cyclic AMP. Thus, the material was applied to Whatman No. 1 filter paper and subjected to descending paper chromatography in two solvent systems. The first consisted of isobutyric acid, concentrated NH_4OH, water, and 0.1 M EDTA·Na_2 (100:13.3: 46.7:1.6, V/V). The second was 95% EtOH and 1 M ammonium acetate, pH 7.4 (70:30, V/V). Only one spot corresponding to cyclic AMP was found on the paper chromatograms. Radioactivity on the paper chromatograms was determined by a Vanguard strip counter. This experiment revealed that all of the radioactivity on the paper chromatograms was associated with cyclic AMP and that the formation of adenosine from 5'-AMP by nucleotidase was negligible in the lymphocyte homogenates.

Three slightly different assay mixtures were used depending upon the nature of the studies. For in culture studies, the incubation mixture consisted of cyclic AMP, 5 x 10^{-4}M, containing about 500,000 DPM of tritiated cyclic nucleotide, 0.05 M Tris-HCl buffer, pH 8.1, magnesium chloride (5 mM), bovine plasma albumin (0.015%), and 100 µl of the cell homogenate (200-400 µg protein) prepared as described earlier in a final volume of 0.25 ml. The incubation mixture for *in vitro* kinetic studies contained various concentrations of cyclic AMP supplemented with 500,000 DPM of tritiated cyclic nucleotide, bovine plasma albumin (0.015%), and 100 µl of a phosphodiesterase preparation in a solution of 0.05 M Tris-HCl buffer, pH 8.1, and magnesium chloride (5 mM) in a final volume of 0.25 ml. The third mixture, used to study the effects of guanine, guanosine, cyclic GMP, and theophylline on cyclic phosphodiesterase activity *in vitro*, contained 25 µM of cyclic AMP with about 100,000 DPM of tritiated cyclic nucleotide, various

concentrations of the compound tested, bovine plasma albumin (0.015%), 100 µl of a phosphodiesterase preparation in a solution of 0.05 M Tris-HCl buffer, pH 8.1, and magnesium chloride (5 mM) in a final volume of 0.25 ml.

Reactions were carried out at 37°C for 30 minutes. A preliminary experiment established this to be the optimal condition. To stop the reaction, the assay mixtures were heated in a water bath at 95-98°C for 60-90 seconds. Control (blank) values were obtained by replacing the enzyme with water. The heated reaction mixtures were centrifuged at 1,000 x g for 10 minutes at 4°C to precipitate the denatured protein. 5'-AMP in the supernatant fluid was precipitated by adding 0.1 ml of 8% zinc sulfate and 0.1 ml of saturated barium hydroxide. The barium sulfate sediment was pelleted by centrifugation at 1,000 x g for 15 minutes. These two steps were repeated twice; 0.2 ml each of 8% zinc sulfate and saturated barium hydroxide were used at the second time. After the last contrifugation, 0.25 ml of the clear supernatant was added to 15 ml of Bray's solution for counting the radioactivity attributed to the tritiated cyclic AMP. Disappearance of tritiated cyclic AMP from the supernatant fluid was thus a measure of phosphodiesterase activity in the homogenate.

CHAPTER III

RESULTS

Effects of Purine and Pyrimidine Bases, Ribonucleosides, and Ribonucleotides on Nucleic Acid Synthesis in Both Normal and CLL Lymphocyte Cultures

The studies reported in this section were designed (a) to confirm Lucas's observation (59,60) of the inhibitory effect of cytidine on uridine-^{3}H incorporation into RNA in normal lymphocytes, (b) to examine the effects of cytidine on nucleoside-^{3}H incorporation into nucleic acid in CLL lymphocytes, and (c) to investigate further other bases, ribonucleosides, and ribonucleotides which specifically inhibit nucleic acid synthesis in CLL lymphocytes.

Cells were maintained under culture conditions in the presence or absence of cytidine or other chemicals as described in Methods. Tritiated uridine (Ur-^{3}H) or thymidine (Th-^{3}H) was incubated with these cells for 2 or 6 hours, respectively, to assess the proliferative capability of the lymphocytes in culture. Figure 1 shows the rate of Ur-^{3}H incorporation into RNA in PHA-stimulated normal and CLL lymphocytes with or without the addition of cytidine or guanosine. The presence of $10^{-4}M$ cytidine depressed the uptake of Ur-^{3}H in both normal and CLL lymphocytes to less than 17% of the control levels. In contrast to cytidine, the addition of $10^{-4}M$ guanosine failed to affect

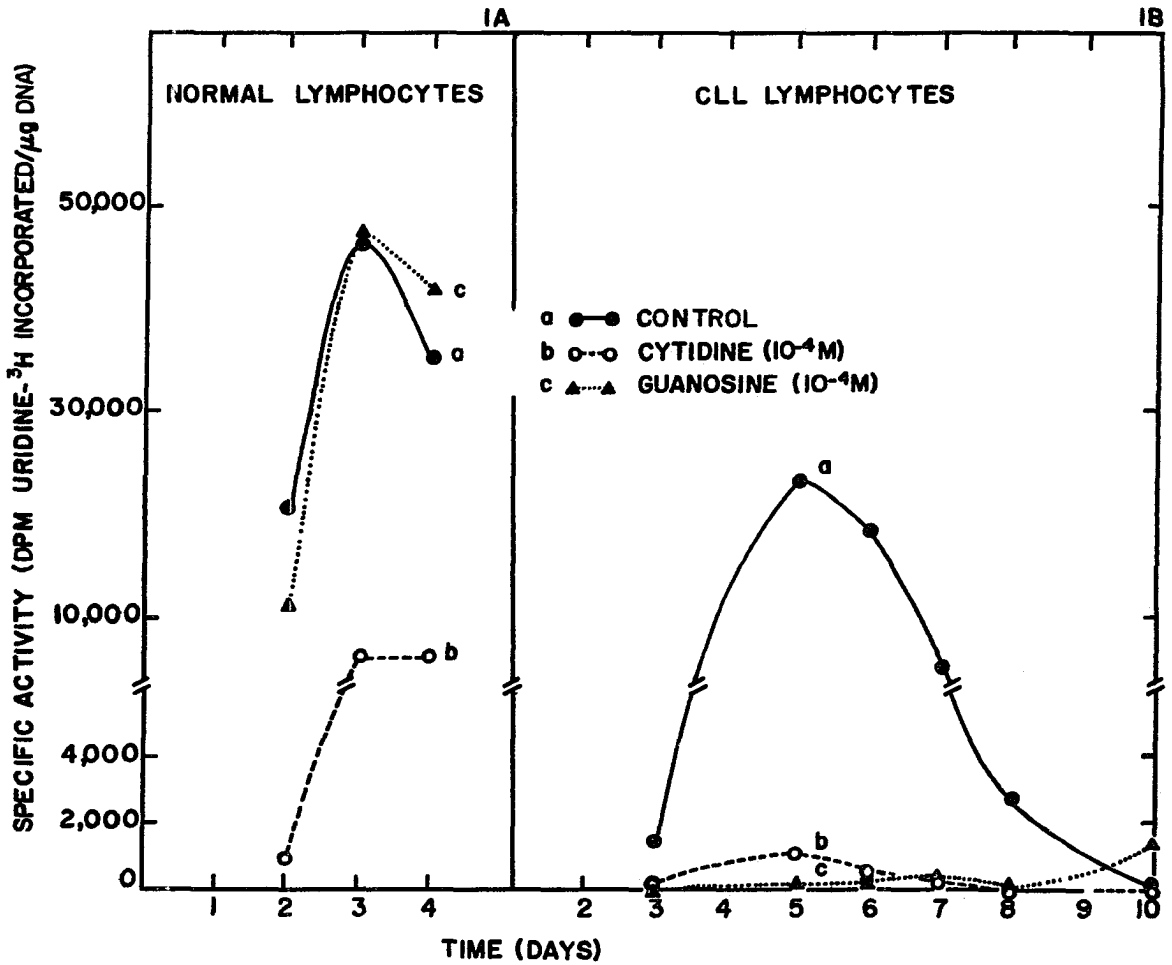

Figure 1 - RNA Synthesis in PHA-stimulated Normal (1A) and CLL (1B) Lymphocyte Cultures with Cytidine and Guanosine Additions at Concentrations of 10^{-4}M

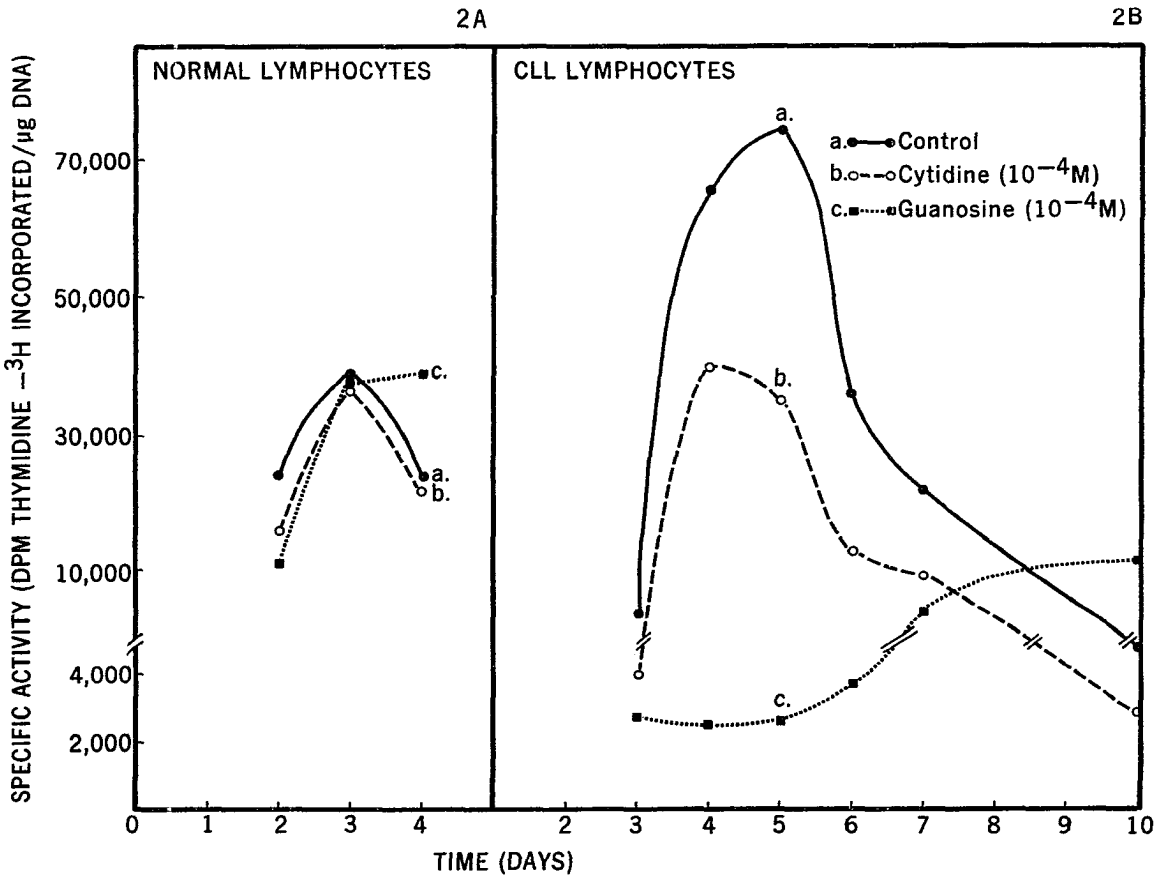

Figure 2 - DNA Synthesis in PHA-stimulated Normal (2A) and CLL (2B) Lymphocyte Cultures with Cytidine and Guanosine Additions at Concentrations of 10^{-4}M

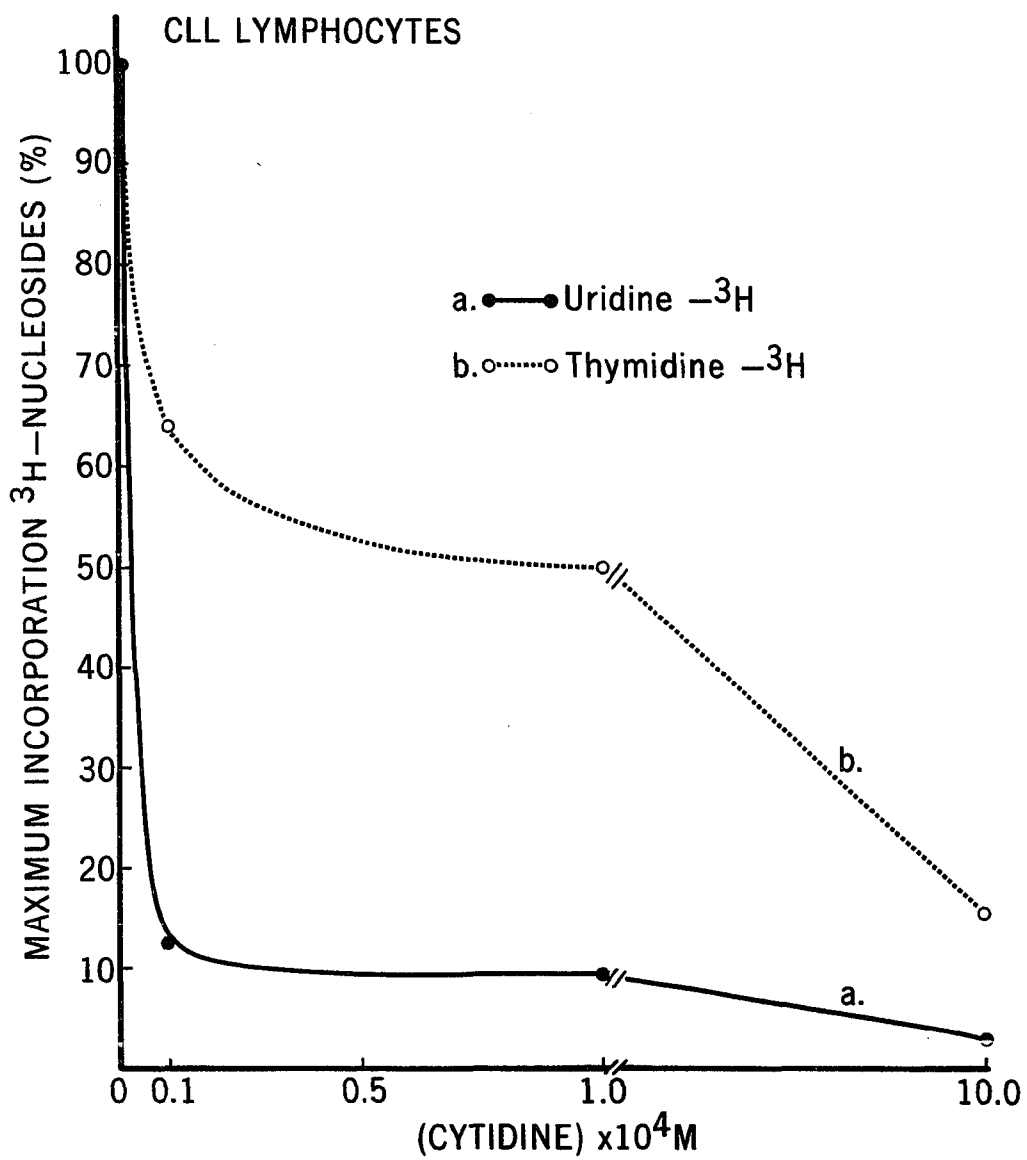

Figure 3 - The Percent Inhibition of Nucleic Acid Synthesis in CLL Lymphocyte Cultures by Various Concentrations of Cytidine

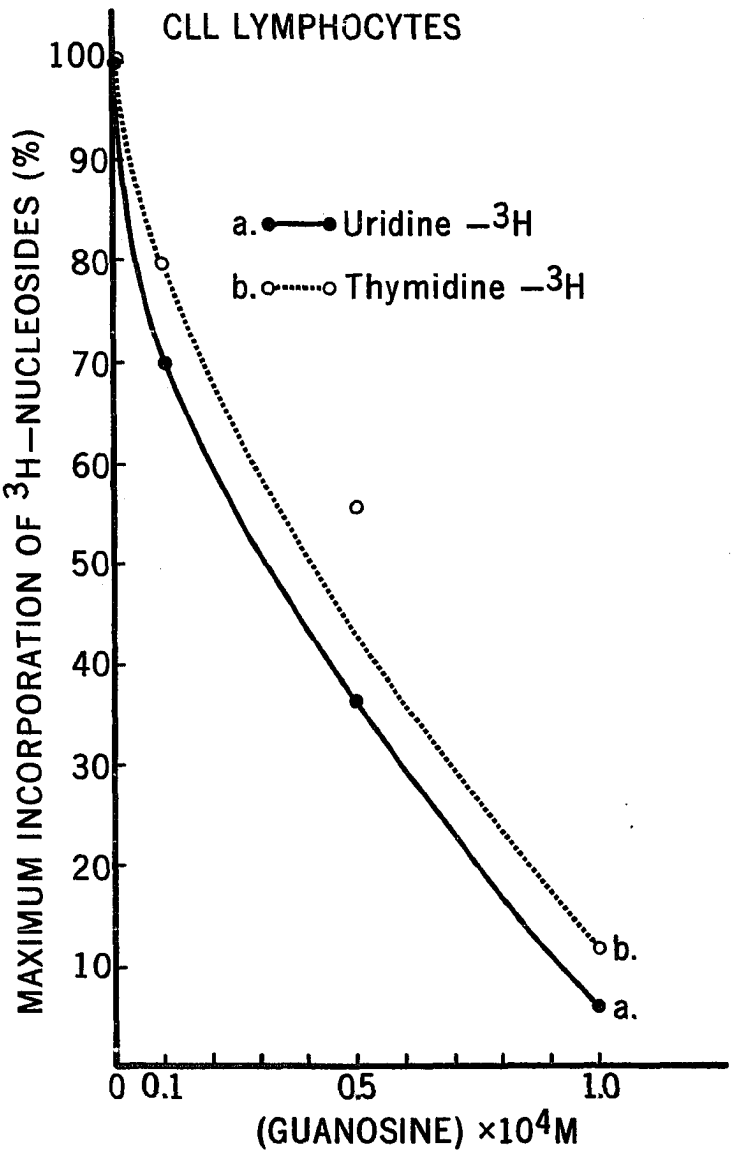

Figure 4 - The Percent Inhibition of Nucleic Acid Synthesis in CLL Lymphocyte Cultures by Various Concentrations of Guanosine

the uptake of Ur-^{3}H into RNA in normal lymphocytes, but it did reduce the Ur-^{3}H incorporation to less than 5% of the control level in CLL lymphocytes.

The inhibition by cytidine and guanosine was further examined by studying their effects on the rate of Th-^{3}H incorporation into DNA in normal and CLL lymphocytes (Figure 2). Cytidine (10^{-4}M) had no effect on Th-^{3}H incorporation into DNA in normal lymphocytes. In CLL lymphocytes, cytidine (10^{-4}M) did inhibit Th-^{3}H incorporation into DNA to a small or moderate extent. Figure 2 also illustrates that guanosine (10^{-4}M) had no effect on Th-^{3}H incorporation into DNA in normal lymphocytes, but in CLL lymphocytes it reduced DNA synthesis almost completely. Figures 1 and 2 illustrate the similar effects of cytidine on nucleic acid synthesis in both normal and CLL lymphocytes. However, while guanosine exerted a distinct inhibitory effect on both RNA and DNA synthesis in CLL lymphocytes, it had essentially no effect in normal lymphocytes.

The effects of various physiologic concentrations of cytidine and guanosine on tritiated nucleoside incorporation were studied with CLL lymphocytes. Cytidine, at concentrations ranging from 10^{-5}M to 10^{-3}M, reduced Th-^{3}H incorporation much less than Ur-^{3}H incorporation (Figure 3). Moreover, cytidine at various concentrations exhibited a hyperbolic pattern of its inhibitory effect on both Ur-^{3}H and Th-^{3}H incorporation into RNA and DNA in CLL lymphocytes, indicating that a simple first-order mechanism was involved.

The extent to which guanosine inhibited both Ur-^{3}H and Th-^{3}H incorporation was comparable (Figure 4). However, the non-hyperbolic

pattern of inhibition suggests higher order effects rather than simple competitive inhibition.

Since guanosine was found to inhibit Ur-^{3}H and Th-^{3}H uptake specifically in CLL lymphocytes, further experiments were undertaken to examine some of the closely related compounds (e.g., guanine, adenine, adenosine, inosine, cyclic GMP, and dibutyryl cyclic AMP). The culture conditions and the experimental scheme were the same as described in Methods. The results are summarized in Table 1. At the same concentration (10^{-4}M), adenosine failed to inhibit nucleoside uptake in either normal or CLL lymphocytes. Both adenine and inosine had no effect on normal cells and only a moderate inhibitory effect on CLL cells. However, guanine, cyclic GMP and dibutyryl cyclic AMP exhibited essentially the same effect as guanosine, i.e., they reduced the Ur-^{3}H and Th-^{3}H incorporation almost completely in CLL lymphocytes without affecting nucleoside incorporation in normal lymphocytes.

The observation that dibutyryl cyclic AMP inhibited CLL lymphocyte proliferation was consistent with Ryan and Heidrick's reports (119,120) on inhibition of cell growth by cyclic AMP and its dibutyryl derivative. A previous study (123) indicated that dibutyryl cyclic AMP inhibited DNA synthesis in CLL cells two to four times more than cyclic AMP. This result agreed well with other observations on blood glucose level (143), stimulation of lipolysis (144), and amylase secretion in rat parotid cells (145). This greater activity of the dibutyryl derivative is generally ascribed to its resistance to hydrolysis by phosphodiesterase and to increased penetration of the cell. Therefore, the sensitivity of the CLL lymphocytes to dibutyryl cyclic AMP was interpreted as a result of increased intracellular cyclic AMP.

TABLE 1

EFFECTS OF BASES, RIBONUCLEOSIDES, AND CYCLIC RIBONUCLEOSIDE MONOPHOSPHATES ON NUCLEIC ACID SYNTHESIS IN NORMAL AND CLL LYMPHOCYTES

Compound	N-L Ur-^{3}H Incorp. DPM/µg DNA	%	N-L Th-^{3}H Incorp. DPM/µg DNA	%
None	46,500	100	39,000	100
Cytidine (10^{-4}M)	7,700	17	36,000	92
Guanosine (10^{-4}M)	47,600	102	38,000	97
None	17,123	100	20,819	100
Adenosine (10^{-4}M)	16,993	99	46,602	224
Guanine (10^{-4}M)	24,860	145	32,009	154
Inosine (10^{-4}M)	16,059	94	31,611	152
None	20,318	100	50,250	100
Adenine (10^{-4}M)	21,700	107	52,400	104
Cyclic GMP (10^{-4}M)	24,000	118	58,108	116
None	46,500	100	39,000	100
Dibutyryl Cyclic AMP (10^{-4}M)	30,000	64	28,500	73
IPT (10^{-4}M)	40,000	86	36,000	92

TABLE 1

EFFECTS OF BASES, RIBONUCLEOSIDES, AND CYCLIC RIBONUCLEOSIDE MONOPHOSPHATES ON NUCLEIC ACID SYNTHESIS IN NORMAL AND CLL LYMPHOCYTES - CONT'D

	CLL-L			
Compound	Ur-^{3}H Incorp. DPM/µg DNA	%	Th-^{3}H Incorp. DPM/µg DNA	%
None	23,400	100	74,400	100
Cytidine (10^{-4}M)	1,100	5	39,500	53
Guanosine (10^{-4}M)	150	0.5	2,680	3
None	13,300	100	69,600	100
Adenosine (10^{-4}M)	15,428	116	90,480	130
Guanine (10^{-4}M)	240	2	2,500	4
Inosine (10^{-4}M)	9,300	70	60,000	86
None	12,000	100	30,000	100
Adenine (10^{-4}M)	6,800	57	12,000	40
Cyclic GMP (10^{-4}M)	1,250	10	5,200	17
None	40,000	100	65,000	100
Dibutyryl Cyclic AMP (10^{-4}M)	300	0.8	1,400	2
IPT (10^{-4}M)	3,600	9	40,000	61

Cytidine Deaminase Activity in Both Normal and CLL Lymphocytes

Previous studies (59,60,62) have shown that uridine incorporation into RNA is limited by the rate of phosphorylation of uridine by uridine kinase. Therefore, the reduced uridine incorporation into RNA by cytidine was explained as end-product inhibition or repression of uridine kinase by cytidine or its phosphorylated derivatives (59). Studies by Kay et al. (146) demonstrated that RNA synthesis in PHA-stimulated normal lymphocytes is obligatory for the onset of DNA synthesis. Since cytidine markedly depressed Ur-^{3}H incorporation, RNA must be synthesized by an alternate method, possibly by the de novo pathway. Unfortunately, studies using aspartic acid demonstrated limited uptake of this precursor (Table 2). Consequently, it did not verify the increased utilization of the de novo synthetic pathway for the pyrimidine nucleotides. However, a potentially competitive reaction, namely the deamination of cytidine to uridine, was not measured. The conversion of cytidine to uridine is catalyzed by cytidine deaminase, an enzyme which is ubiquitous in bacterial cells (147,148), avian cells (149), mammalian cells (148,150), and virus-induced tissue culture lines (151). In this study, we demonstrated the presence of cytidine deaminase in human lymphocytes. We presented evidence suggesting that at least part of the inhibitory effect of cytidine on uridine incorporation results from dilution of the radioactive uridine pool by cold uridine derived from enzymatic deamination of cytidine.

The presence of cytidine deaminase in normal lymphocytes in culture is illustrated in Figure 5. The appearance of uridine was

TABLE 2

INCORPORATION OF PYRIMIDINE NUCLEOTIDE PRECURSORS
INTO RNA OF CLL LYMPHOCYTES

Condition	^{14}C-Aspartic Acid (DPM/µg DNA)	^{3}H-Uridine (DPM/µg DNA)
PHA (Day 3)	13	1,102
PHA (Day 6)	35	10,800
PHA + Cytidine (10^{-4}M) (Day 3)	15	164
PHA + Cytidine (10^{-4}M) (Day 6)	37	325

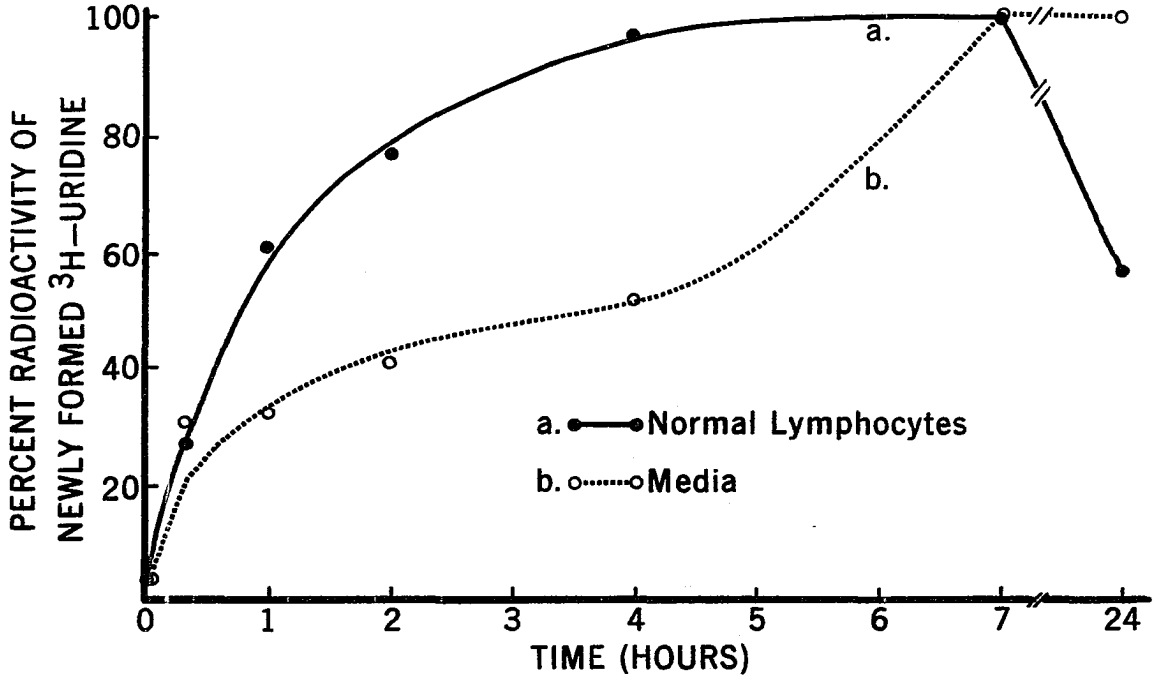

Figure 5 - Conversion of Cytidine into Uridine in Normal Lymphocyte Cultures

assessed in both cell fractions and medium fractions of the lymphocyte cultures. Approximately 90% of the cytidine was converted into uridine within 4 hours. Complete deamination was observed within 24 hours. At the end of 24 hours of incubation, 42% of the radioactivity was recovered in nucleotides and polynucleotides. The rate of uridine production in the media, however, was slightly slower than that in the cells. This phenomenon could possibly be due to a "membrane barrier", preventing free exchange of cytidine and uridine through the cell membrane.

Figure 6 shows the formation of uridine from cytidine in both the cell fractions and medium fractions of CLL lymphocyte cultures. During a 2-hour incubation period, 90% of the cytidine was deaminated into uridine; complete conversion was reached within 4 hours. The deamination of cytidine occurred much faster in CLL lymphocytes than in normal lymphocytes (Table 3). This observation suggests that CLL lymphocytes possess either more of the same cytidine deaminase or a different cytidine deaminase with higher activity. The rate of uridine production observed in the media of the CLL cell cultures, however, was much slower than that in the media of the normal cell cultures. This phenomenon could be explained by differences in membrane permeability between normal and CLL lymphocytes.

Cytidine deaminase was further studied <u>in vitro</u> in both normal and CLL lymphocytes. Cytidine deaminase was found in both the supernatant and particulate fractions (7,000 x g for 10 minutes) of the cell homogenates from both normal and CLL lymphocytes preincubated with or without PHA. Figure 7A shows that in the supernatant fractions of

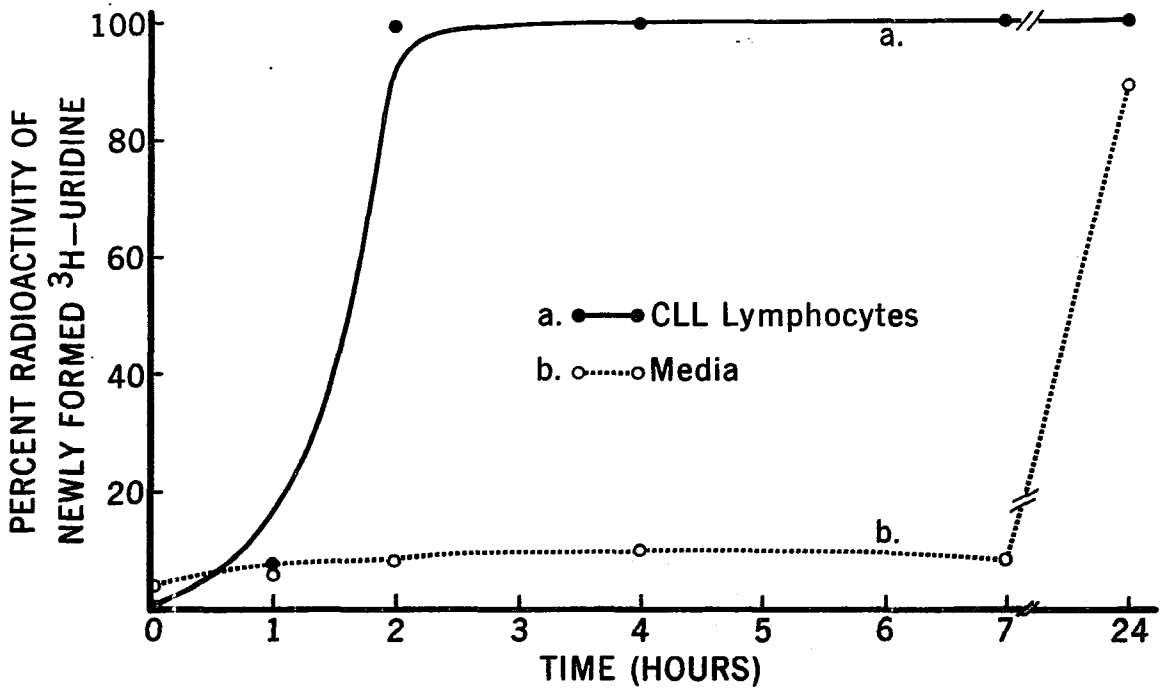

Figure 6 - Conversion of Cytidine into Uridine in CLL Lymphocyte Cultures

TABLE 3

CONVERSION OF CYTIDINE INTO URIDINE IN NORMAL AND CLL LYMPHOCYTE CULTURES

Substance Assayed	Incubation Time	N-L % Cytidine Remaining	% Uridine Formed
Cells	0 time	96	4
	10 min.	73	27
	1 hr.	39	61
	2 hr.	23	77
	4 hr.	3	97
	7 hr.	0	>99
	1 day	0	*58
Media	0 time	96	4
	10 min.	69	31
	1 hr.	67	33
	2 hr.	59	41
	4 hr.	48	52
	7 hr.	0	>99
	1 day	0	>99

*Approximately 42% of the radioactivity was found in nucleotides and polynucleotides

TABLE 3

CONVERSION OF CYTIDINE INTO URIDINE IN NORMAL AND CLL LYMPHOCYTE CULTURES - CONT'D

		CLL-L	
Substance Assayed	Incubation Time	% Cytidine Remaining	% Uridine Formed
Cells	0 time	100	0
	10 min.	-	-
	1 hr.	92	8
	2 hr.	0	>99
	4 hr.	0	>99
	7 hr.	0	>99
	1 day	0	>99
Media	0 time	96	4
	10 min.	-	-
	1 hr.	94	6
	2 hr.	92	8
	4 hr.	90	10
	7 hr.	92	8
	1 day	12	88

either PHA-stimulated or nonstimulated normal lymphocytes (Curve a and b, respectively), 100% of the original cytidine was converted into uridine within a 1-hour incubation period. In the particulate fractions of the same cultures (Curve c and d referring to PHA-stimulated and nonstimulated normal lymphocytes), approximately 20% and 40% of cytidine was deaminated within 1 and 2 hours of incubation, respectively. Figure 7B illustrates that in CLL lymphocyte cultures, the supernatant fractions of either the PHA-stimulated or nonstimulated lymphocyte homogenates (expressed in Curve a and b, respectively) demonstrated that at least 90% of the original cytidine was converted into uridine within 3 hours of incubation, whereas in the particulate fractions of the stimulated (Curve c) and nonstimulated (Curve d) cell homogenates, 10% and 25% of the original cytidine were deaminated, respectively, within the same incubation period.

In summary then: (a) deaminase activity predominated in the supernatant fraction; the small amount of activity observed in the particulate fraction was probably due to contamination from the supernatant, (b) PHA had little or no effect on either the rate or the extent of cytidine deaminase activity, and (c) the activity of cytidine deaminase in CLL lymphocytes was essentially comparable to that in the normal lymphocytes; in contrast to the previous observation of cytidine deaminase in culture, however, the deamination in vitro occurred slightly faster in normal lymphocytes than in CLL lymphocytes.

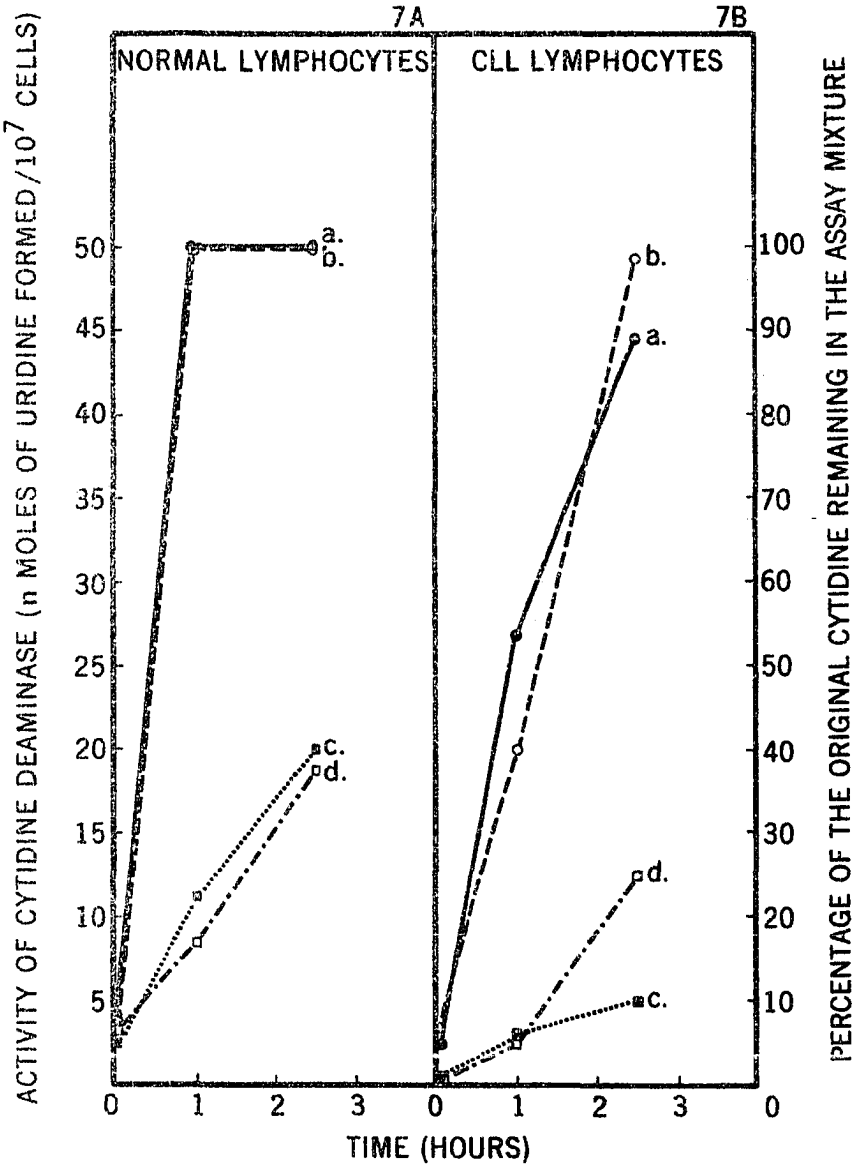

Figure 7 - The in vitro Study of Cytidine Deaminase in Normal (7A) and CLL (7B) Lymphocytes

a. Supernatant Fraction from PHA-stimulated Lymphocytes
b. Supernatant Fraction from Nonstimulated Lymphocytes
c. Particulate Fraction from PHA-stimulated Lymphocytes
d. Particulate Fraction from Nonstimulated Lymphocytes

Cyclic AMP Phosphodiesterase Activity in Both Normal and CLL Lymphocytes Preincubated with Guanine, Guanosine and Cyclic GMP

Many reports in the literature have indicated that high concentrations of cyclic GMP consistently inhibit cyclic AMP phosphodiesterase (152-156) and thus increase the cellular content of cyclic AMP (154). Johnson and Abell's (123) investigation on the inhibitory effect of IPT and dibutyryl cyclic AMP on lymphocyte DNA synthesis strongly suggests that cyclic AMP is involved in lymphocyte proliferation. Furthermore, Ramsden's (157) report that guanosine and guanine partially inhibit the enzymic hydrolysis of cyclic AMP led us to explore the possibility that in CLL lymphocytes, guanine, guanosine, and cyclic GMP may act through the cyclic phosphodiesterase and alter intracellular cyclic AMP level, ultimately resulting in the inhibition of nucleic acid synthesis.

Cyclic AMP phosphodiesterase was assayed in different preparations obtained from either normal or CLL lymphocyte cultures. Figures 8 and 9 show the rate of cyclic AMP hydrolysis in lymphocyte homogenates as a function of incubation time and protein concentration. These results indicate that the rate of degradation of cyclic AMP is linear with respect to both time and enzyme concentration.

An experiment was carried out in which the cyclic AMP phosphodiesterase was prepared from normal lymphocyte cultures treated with guanine (10^{-4}M), guanosine (10^{-4}M), or cyclic GMP (10^{-4}M) over a period of 2 to 3 days. Data illustrated in Table 4 demonstrate that preincubation of the normal lymphocytes with guanine moderately reduced

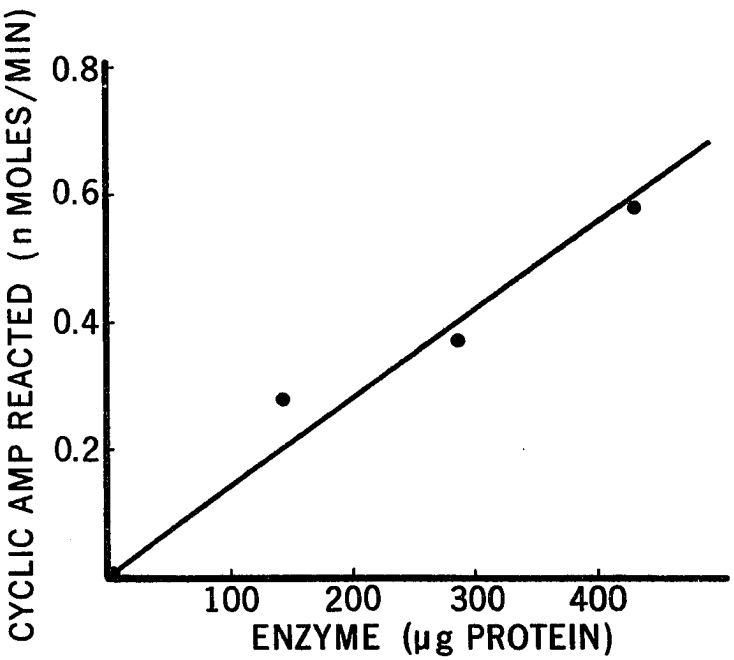

Figure 8 - Cyclic AMP Phosphodiesterase Activity of Human Lymphocytes As A Function of Enzyme Concentration

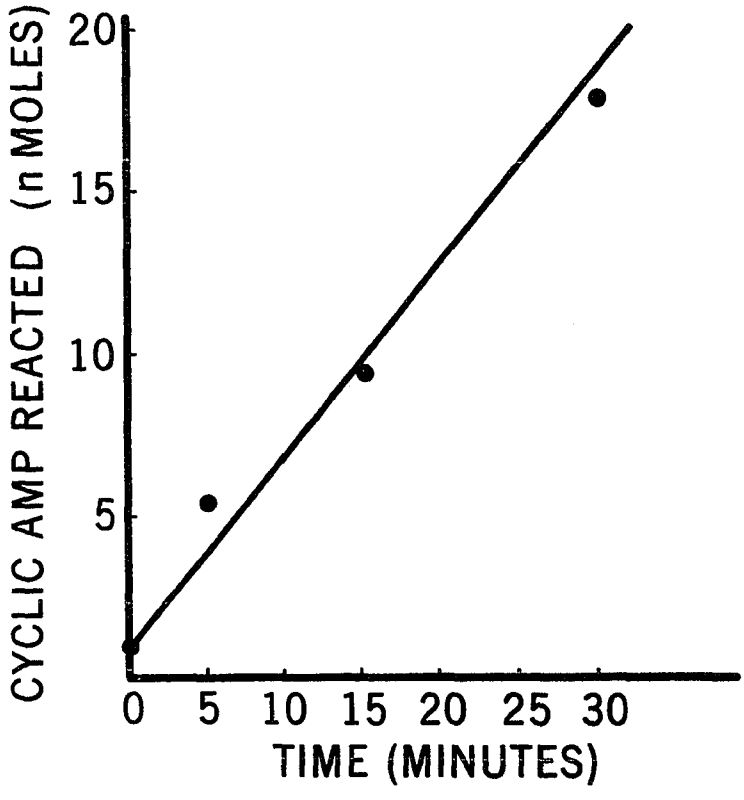

Figure 9 - Cyclic AMP Phosphodiesterase Activity in Crude Homogenate of Human Lymphocytes As A Function of Time

the activity of cyclic phosphodiesterase initially. However, the enzyme exhibited the same level as the control after a longer incubation with guanine. Normal lymphocytes preincubated with guanosine contained twice as much activity as the control cells on Day 2, although the activity decreased and was about 1.5 times above that of the control cells on Day 3. Cyclic GMP had a very slight stimulatory effect on the phosphodiesterase activity in normal cells. Increases of 23% and 20% in enzyme activity were obtained by normal cell culture with cyclic GMP treatment for 2 and 3 days, respectively.

The effects of guanine, guanosine, and cyclic GMP were also studied in CLL lymphocytes. In the first case, lymphocytes were obtained from a patient with a white blood cell (WBC) count of 48 x 10^3/mm^3. Results shown in Table 5 illustrate that CLL lymphocytes in culture treated with 10^{-4}M guanine for 3 and 5 days contained 87% and 77% of the cyclic phosphodiesterase activity, respectively. CLL lymphocytes treated with 10^{-4}M guanosine for 3 and 5 days contained 91% and 88% of the enzyme activity, respectively. Treatment of CLL lymphocytes with 10^{-4}M cyclic GMP, however, produced a slight stimulatory effect on cyclic phosphodiesterase. Increases of 50% and 25% in enzyme activity were observed in these cultures on Days 3 and 5, respectively.

A second case of CLL with a WBC count of 280 x $10^3/mm^3$ was also investigated. As shown in Table 6, marked inhibition was observed in all cultures treated with guanine (10^{-4}M), guanosine (10^{-4}M), or cyclic GMP (10^{-4}M). Lymphocytes cultured for 3 days showed 23%, 5%, and 13% of cyclic AMP phosphodiesterase activity corresponding to

TABLE 4

CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN NORMAL LYMPHOCYTES CULTURED WITH GUANINE (10^{-4}M), GUANOSINE AND CYCLIC GMP (10^{-4}M)

Pre-treatment of Cell Culture	Cyclic AMP-^{3}H Hydrolyzed nmoles/mg protein/30 minutes			
	Day 2	% of Activity	Day 3	% of Activity
PHA (control)	14.8	100	16.8	100
PHA + Guanine (10^{-4}M)	3.0	20.3	16.6	98.8
PHA + Guanosine (10^{-4}M)	33.2	224.3	27.4	163.1
PHA + Cyclic GMP (10^{-4}M)	18.2	122.9	20.2	120.2

TABLE 5

CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN LYMPHOCYTES FROM A CLL PATIENT WITH A MEDIUM PERIPHERAL LEUKOCYTE COUNT ($48 \times 10^3/mm^3$) CULTURED WITH GUANINE ($10^{-4}M$), GUANOSINE ($10^{-4}M$) OR CYCLIC GMP ($10^{-4}M$)

Pre-treatment of Cell Culture	Cyclic AMP-^{3}H Hydrolyzed nmoles/mg protein/30 minutes			
	Day 3	% of Activity	Day 5	% of Activity
PHA (control)	20.8	100	23.9	100
PHA + Guanine ($10^{-4}M$)	18.1	87.0	18.5	77.4
PHA + Guanosine ($10^{-4}M$)	19.0	91.3	21.1	88.3
PHA + Cyclic GMP ($10^{-4}M$)	32.0	153.8	30.0	125.5

TABLE 6

CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN LYMPHOCYTES FROM A CLL PATIENT WITH A HIGH PERIPHERAL LEUKOCYTE COUNT ($280 \times 10^3/mm^3$) CULTURED WITH GUANINE ($10^{-4}M$), GUANOSINE ($10^{-4}M$) OR CYCLIC GMP ($10^{-4}M$)

Pre-treatment of Cell Culture	Cyclic AMP-^{3}H Hydrolyzed nmoles/mg protein/30 minutes			
	Day 3	% of Activity	Day 5	% of Activity
PHA (control)	22.3	100	28.5	100
PHA + Guanine ($10^{-4}M$)	5.2	23.3	1.7	5.9
PHA + Guanosine ($10^{-4}M$)	1.1	4.9	5.2	18.2
PHA + Cyclic GMP ($10^{-4}M$)	3.0	13.4	2.3	8.1

guanine, guanosine and cyclic GMP treatment. Lymphocytes obtained from similar cultures with guanine, guanosine or cyclic GMP added for 5 days contained 6%, 18%, and 8% of the control level, respectively.

To summarize these three tables, the cyclic AMP phosphodiesterase level in cell homogenates of normal lymphocytes or CLL lymphocytes of a medium WBC count was generally affected to only a small extent by guanine, guanosine or cyclic GMP. However, in CLL lymphocytes obtained from a donor with a high WBC count, these compounds reduced the enzyme activity to at least 20% of the control level. In addition, the cyclic AMP phosphodiesterase levels of the untreated control cells could be arranged in a decreasing order of CLL lymphocytes (high WBC count) > CLL lymphocytes (medium WBC count) > normal lymphocytes (low WBC count).

Kinetic Studies of Cyclic AMP Hydrolysis by Lymphocyte Preparations

To evaluate the possibility that the phosphodiesterase activities in normal and CLL lymphocytes differed functionally, some of the kinetic parameters of cyclic AMP hydrolysis were measured in enzyme preparations of both lymphocytes. The rate of cyclic AMP hydrolysis by phosphodiesterase from a normal lymphocyte preparation is shown in Figure 10 as a function of substrate concentration ranging from 0.5 to 400 μM. The Eadie-Hofstee plot of the data was found to be nonlinear (Figure 11). This observation suggested that the cyclic AMP phosphodiesterase exists in more than one form. Furthermore, this plot gave an approximate estimation of the values of two apparent maximal velocity (V_{max}) values of 0.75 and 1.32 nmoles of cyclic AMP hydrolyzed

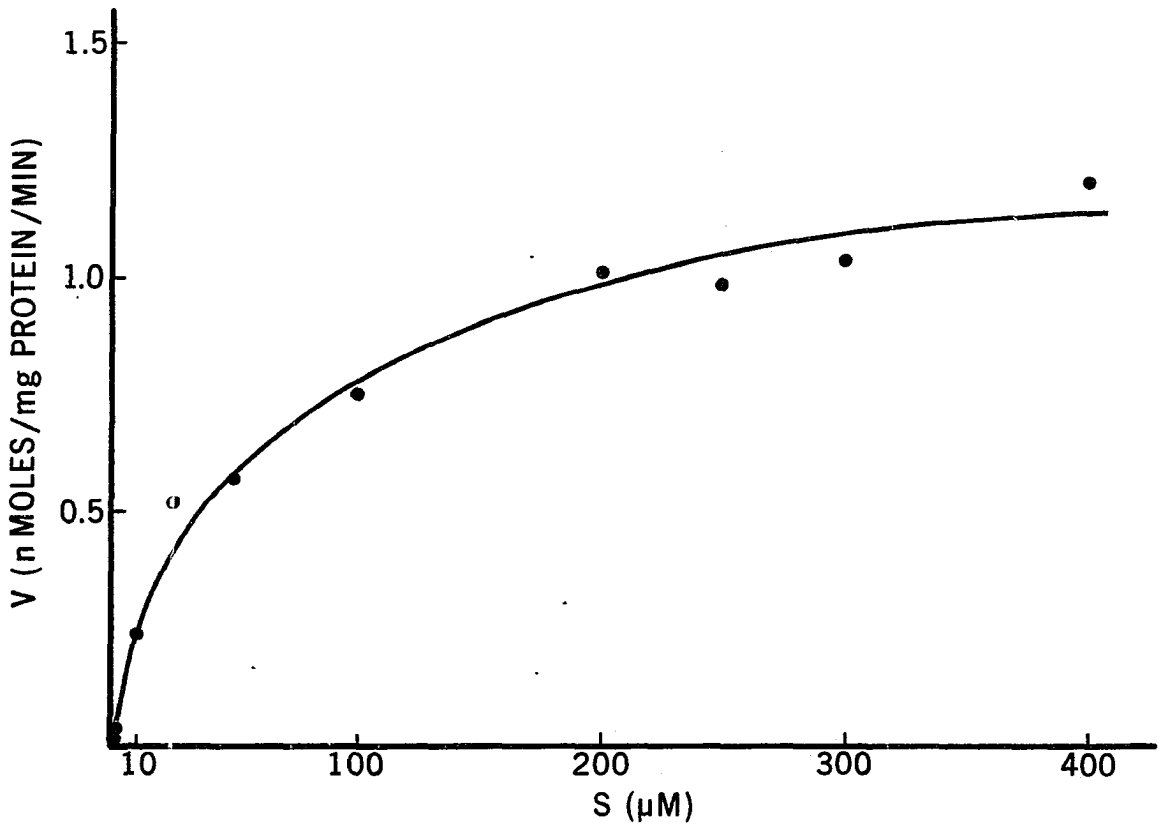

Figure 10 - Cyclic AMP Phosphodiesterase Activity of Normal Lymphocytes As A Function of the Cyclic AMP Concentration

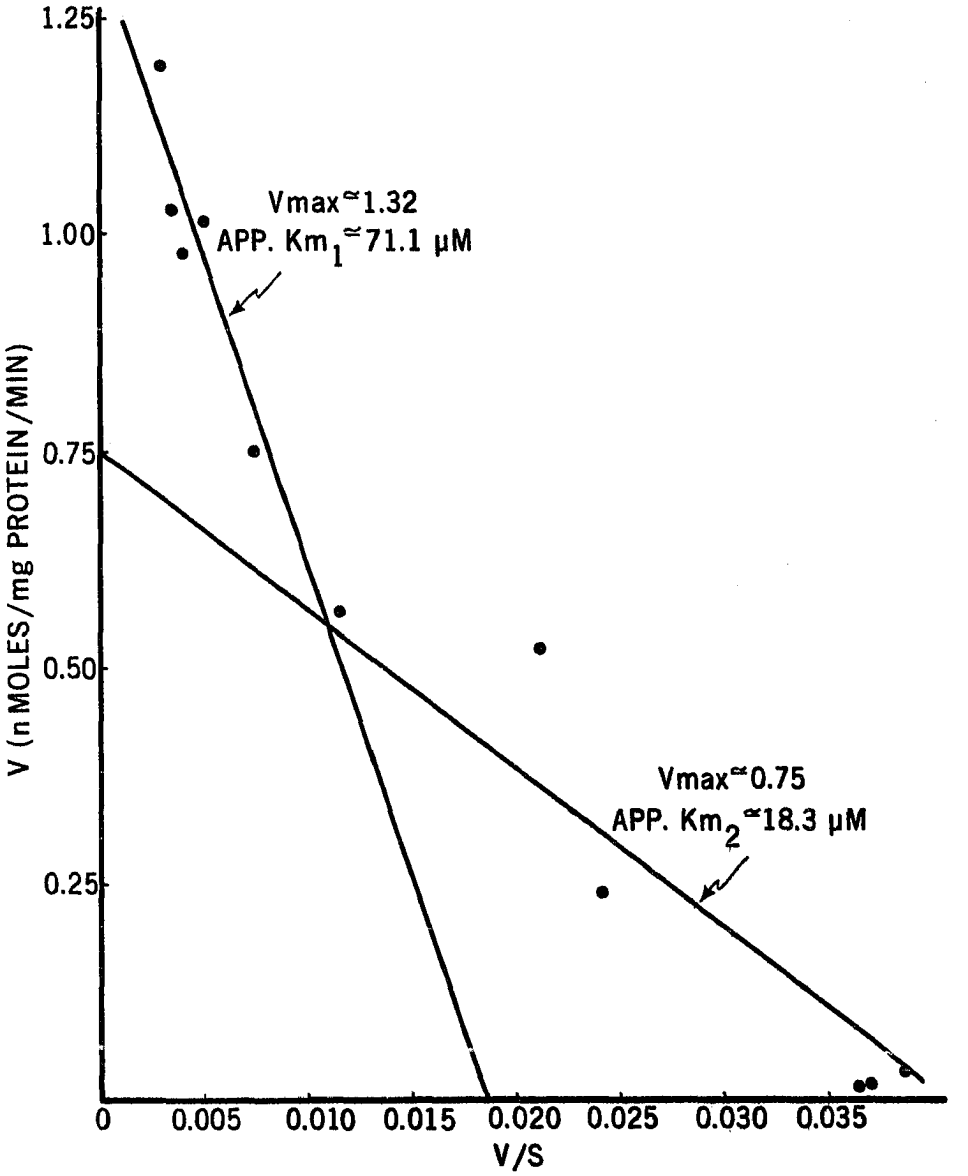

Figure 11 - Eadie-Hofstee Plot of the Rate of Cyclic AMP Hydrolysis by A Supernatant Fraction (1,000xg, 10min.) of Cyclic Phosphodiesterase Preparation Obtained from Normal Lymphocytes

per milligram of protein per minutes, as well as two Michaelis-Menten constants (K_m), one at 1.8 x 10^{-5}M and the other at 7.1 x 10^{-5}M. A different treatment of the data expressed as S/V vs. S (Figure 12) revealed two apparent V_{max} values of 0.8 and 1.38 and two apparent K_m values of 1.8 x 10^{-5}M and 7.7 x 10^{-5}M. In an Eadie-Hofstee plot, V_{max} is obtained as the intercept of the straight line on the vertical axis and K_m is determined by the slope of the straight line, whereas in the Dixon plot V_{max} is the reciprocal of the slope and K_m is the product of V_{max} and the intercept of the vertical axis.

Cyclic AMP phosphodiesterase obtained from CLL lymphocytes was assayed over a wide range of substrate concentrations between 1 μM and 500 μM. Both the Eadie-Hofstee (Figure 13) and the Dixon plot (Figure 14) indicated that CLL lymphocytes possessed two cyclic AMP phosphodiesterase activities, one with a low K_m of 1.3 x 10^{-5}M and one with a high K_m of 2.9 x 10^{-4}M. The corresponding V_{max} values are 0.31 and 2.20 nmoles of cyclic AMP hydrolysis per milligram of protein per minute.

The above kinetic data indicate the existence in either normal or CLL lymphocytes of more than one cyclic AMP phosphodiesterase activity. It seemed likely that at least one of the enzyme activities was affected by the addition of guanine, guanosine, or cyclic GMP. In order to test this possibility, the hydrolysis of cyclic AMP-^{3}H in the presence of varying amounts of one of the nonlabeled inhibitors was measured. The substrate concentration was 20 μM based on the low K_m value of the enzyme. As shown in Table 7, over a wide range of concentrations (5 μM to 100 μM), guanine or guanosine had little or no

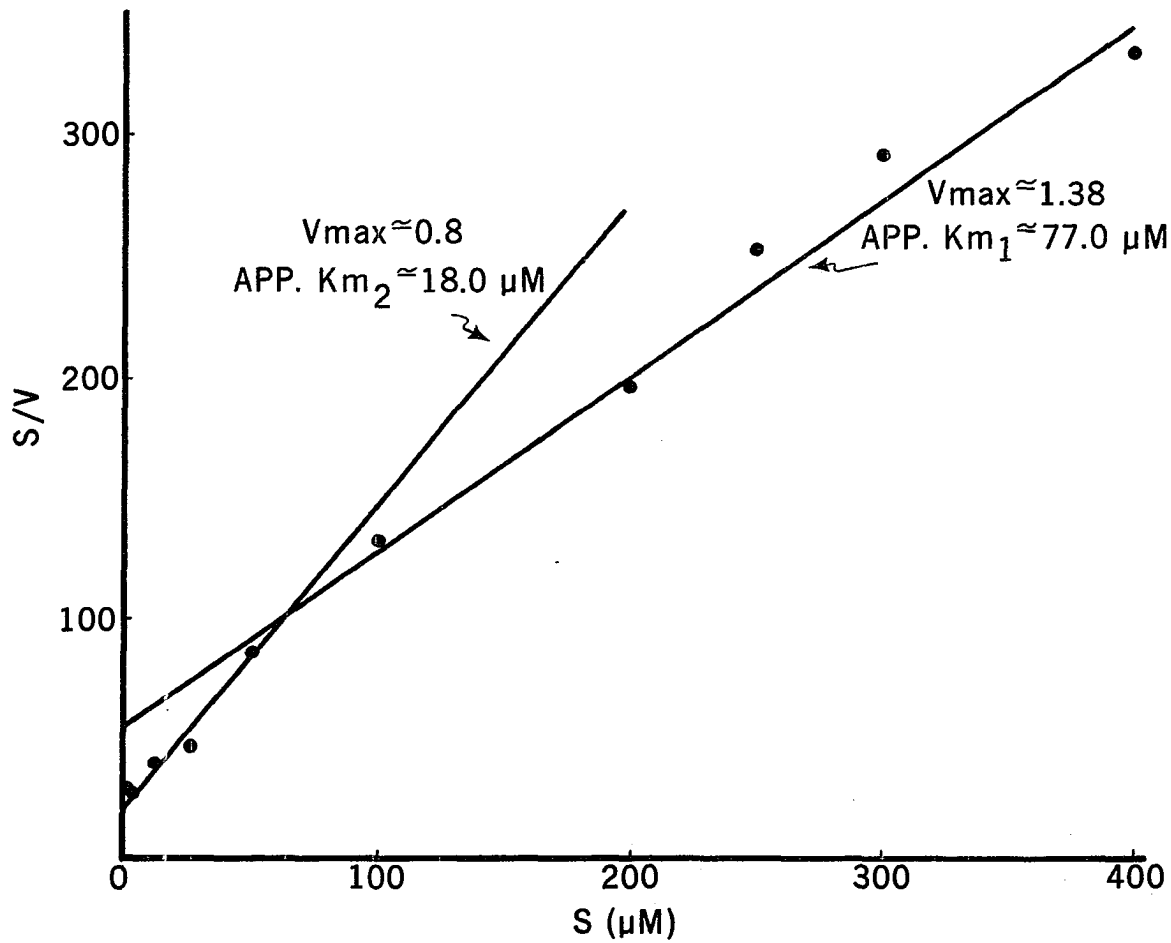

Figure 12 - Dixon Plot of the Rate of Cyclic AMP Hydrolysis by A Supernatant Fraction (1,000xg, 10min.) of Cyclic Phosphodiesterase Preparation Obtained from Normal Lymphocytes

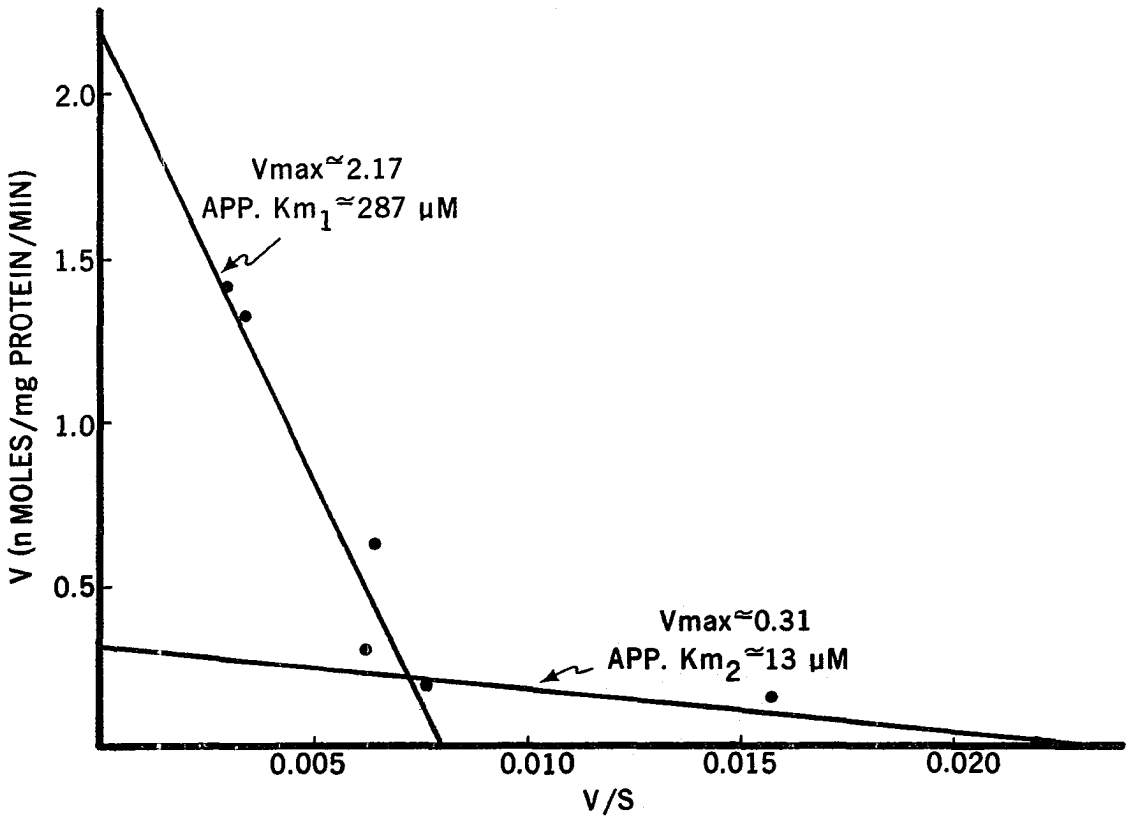

Figure 13 - Eadie-Hofstee Plot of the Rate of Cyclic AMP Hydrolysis by A Supernatant Fraction (1,000xg, 10min.) of Cyclic Phosphodiesterase Preparation Obtained from CLL Lymphocytes

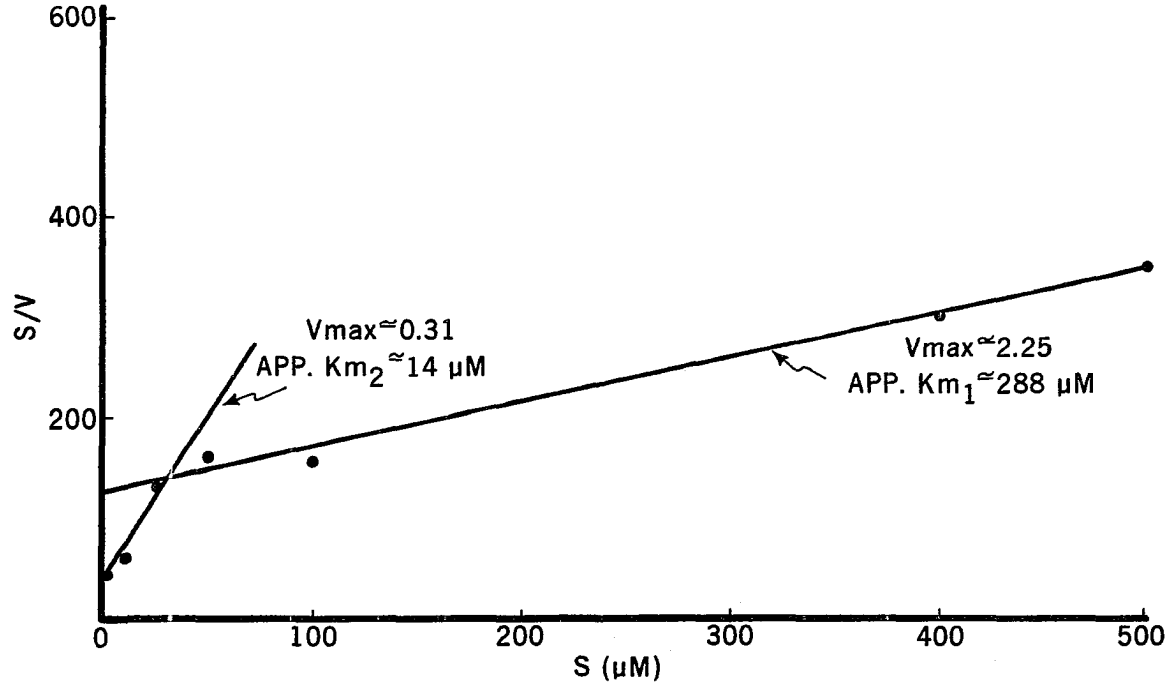

Figure 14 - Dixon Plot of the Rate of Cyclic AMP Hydrolysis by A Supernatant Fraction (1,000xg, 10min.) of Cyclic Phosphodiesterase Preparation Obtained from CLL Lymphocytes

TABLE 7

EFFECT OF GUANINE, GUANOSINE, CYCLIC GMP AND THEOPHYLLINE ON THE ACTIVITY OF CYCLIC PHOSPHODIESTERASE ($K_{m2} \simeq 1.3 \times 10^{-5}$M) FROM CLL LYMPHOCYTES

Inhibitor	Concentrations (μM)	Activity (nmole Cyclic AMP Hydrolyzed/ mg protein/minute)
None	0	0.38
Guanine	5	0.37
	25	0.34
	50	0.33
	100	0.38
Guanosine	5	0.37
	25	0.49
	50	0.35
	100	0.34
Cyclic GMP	5	0.25
	25	0.32
	50	0.34
	100	0.24
Theophylline	5 mM	0.31
	10 mM	0.25

effect on the enzyme activity. However, an appreciable amount of reduction on the enzyme activity was observed in the presence of 5 or 100 μM cyclic GMP, and slight inhibition was attained with 25 or 50 μM cyclic GMP. Therefore, cyclic GMP did inhibit cyclic AMP hydrolysis moderately, but the degree of inhibition was inconsistent with the concentration of the inhibitor. In addition, theophylline also inhibited cyclic AMP phosphodiesterase appreciably, supporting the validity of the assay.

CHAPTER IV

DISCUSSION

The effects of several purines, pyrimidines and their nucleosides on nucleic acid synthesis in both normal and CLL lymphocytes were measured in order to elucidate differences in the synthesis and regulation of nucleic acid between these two cells. The actions of purine and pyrimidine containing derivatives were characterized by (a) either the reduction of Ur-^{3}H incorporation into RNA or Th-^{3}H incorporation into DNA, or both, (b) the specific sensitivity of CLL lymphocytes toward the chemicals or the nonspecific sensitivity displayed by both normal and CLL lymphocytes, and (c) the pattern of inhibition on Ur-^{3}H and Th-^{3}H incorporations by various concentrations of the agents employed. On the basis of the above characteristics, results of this study indicated that purine and pyrimidine exert their inhibitory effect on nucleic acid synthesis in human lymphocytes via different mechanisms.

Cytidine (10^{-4}M;10^{7} cells) was found to inhibit markedly the rate of Ur-^{3}H incorporation into RNA without apparent effect on Th-^{3}H incorporation into DNA in both normal and CLL lymphocytes. Cytidine was also found to display a hyperbolic inhibition pattern at various concentrations. These observations were interpreted by Lucas (59) as results of end-product inhibition or repression of uridine kinase.

According to studies by Kay et al. (146), RNA synthesis in the G1 phase is obligatory for subsequent DNA synthesis. Therefore, during cytidine inhibition RNA must be synthesized by an alternate mechanism, most likely the de novo pathway.

It is well established in bacterial systems, that de novo pyrimidine ribonucleotide synthesis is subject to feedback regulation (82,83). Eucaryotic cells in general have not displayed this stringent feedback regulatory mechanism. Yip and Knox (158) have recently observed that the modulation of carbamyl phosphate synthetase activity by nucleotides is less prominent in mammalian cells than in bacteria. In vivo investigation of aspartate transcarbamylase from various eucaryotic sources (159,160) indicated that the activity of this enzyme is not regulated by nucleotides in the same manner as it is in bacteria (161). In the presence of cytidine, Lucas (59) observed that the activity of the pyrimidine salvage enzyme (uridine kinase) was reduced in the fast proliferating lymphocyte culture. At the same time, an increase in de novo synthesis of pyrimidine might be expected to occur. However, our attempt to detect a significant amount of incorporation of aspartic acid failed to verify the increased utilization of the de novo synthetic pathway of the pyrimidine nucleotides.

It is possible that the conversion of cytidine into uridine resulting in the dilution of the radioactive uridine pool may account for the inhibitory effect of cytidine. We decided to pursue this possibility. The formation of uridine from cytidine is catalyzed by cytidine deaminase, which is generally present in bacterial cells (147, 148), avian cells (149), mammalian cells (140,150), and virus induced

tissue culture lines (151). It is also known that cytidine is deaminated during the hydrolysis of nucleic acid under strongly acidic conditions (162). Shapiro and Klein (163) reported that cytidine and cytosine can be hydrolytically deaminated at 95°C by a variety of aqueous buffer solutions of pH≤5. The equilibrium between cytidine and its deamination products (uridine plus ammonia) was further investigated in the presence of bacterial cytidine deaminase by Cohen and Wolfender (164). Their study revealed that deamination is strongly favored by the extensive protonation of ammonia at pH≈7. The best opportunities for observing the reverse reaction occur at pH≈9. Thus, the conditions used to carry out the cytidine deamination reaction in the present study seemed to be optimal; no spontaneous hydrolytic deamination occurred as solutions of low pH were avoided.

It is known that in man, both liver and serum contain cytidine deaminase (165). This study provided evidence that cytidine deaminase is present also in human lymphocytes. The rates of cytidine deamination in both normal and CLL lymphocyte cultures were measured in the cell fractions as well as in the medium fractions. Our results revealed that deamination takes place in both lymphocytes, and the reaction is faster in CLL cells than in normal cells. However, in normal cell culture media, uridine appears earlier than in CLL cell culture media. This phenomenon suggests that there are differences in membrane structure and permeability between normal and CLL lymphocytes. In fact, Kornfeld (166) did demonstrate that CLL lymphocytes have less surface sialic acid than normal lymphocytes, although the effect of sialic acid concentration on membrane permeability of nucleosides is not clear.

Studies in vitro demonstrated the presence of cytidine deaminase in homogenates obtained from either normal cells or CLL cells. The enzyme activity is mainly distributed in the supernatant fraction, and only a small amount of activity is detected in the particulate fraction. In contrast to the in culture investigation, it was found that the deamination is carried out at a slightly higher rate in normal cells than in CLL cells.

The effectiveness of arabinosyl cytosine (ara-C, one of the common drugs for treatment of leukemia) in the treatment of leukemia can be correlated with the rate of uptake of the molecule in vitro and its subsequent phosphorylation (167). In man, this nucleoside is also rapidly deaminated to produce arabinosyl uridine which has no therapeutic activity (168). These characteristics suggest that the susceptibility of the leukemic cell to ara-C is related to the activity of cytidine deaminase. This possible relationship between lymphoblastic leukemic bone marrow cell cytidine deaminase concentrations and therapeutic response were eveluated by Steuart and Burke (169). It was found that leukemic bone marrow cells in "responders" contained six times less cytidine deaminase than the cells of "non-responders". Thus, cytidine deaminase activity in CLL lymphocytes could be used as an indicator for the effectiveness of ara-C treatment.

The inhibitory effects exhibited by guanine, guanosine and cyclic GMP are quite different from that of cytidine. These compounds, at 10^{-4}M, have no discernible effect on either RNA or DNA synthesis in normal lymphocytes. In CLL lymphocytes, however, they demonstrated marked inhibition on both Ur-^{3}H and Th-^{3}H incorporation into

RNA and DNA, respectively. Also, the non-hyperbolic inhibition pattern of various concentrations of guanosine indicated a situation more complicated than simple competitive inhibition.

Several mechanisms, summarized in Figure 15, may be proposed to account for their effects. First, the permeability of the lymphocyte membrane may be reduced in the presence of exogenous nucleosides. Steck et al. (170) demonstrated that the transport of radioactive nucleosides into mammalian cells can be blocked by high levels (10^{-3}M) of heterologous nucleosides. Presumably, the excessive amounts of unlabeled nucleosides compete with the trace amounts of the radioactive nucleoside at a specific permeation site. The plausibility of this mechanism is further supported by Peter and Hausen's (63) report that the transport of uridine through the cell membrane is the rate-limiting step in uridine uptake in PHA-stimulated bovine lymph node lymphocytes. If this mechanism applies to lymphocytes, the preferential inhibitory effects of guanine, guanosine and cyclic GMP on CLL lymphocytes suggest that the surface of leukemic lymphocytes is different from that of the normal cells. As mentioned before, evidence for membrane difference was given by the observation that less sialic was found on CLL lymphocyte surface than on normal lymphocyte surface (166). Again, the significance of this finding with respect to the permeation sites for exogenous compounds is still unknown. Furthermore, our studies show that the rate of uridine incorporation into RNA is not directly proportional to the guanosine concentration in the medium. Thus, the different responses of CLL lymphocytes and normal lymphocytes toward guanine, guanosine and cyclic GMP may not all be entirely attributed to the transport of radioactive nucleoside into cells.

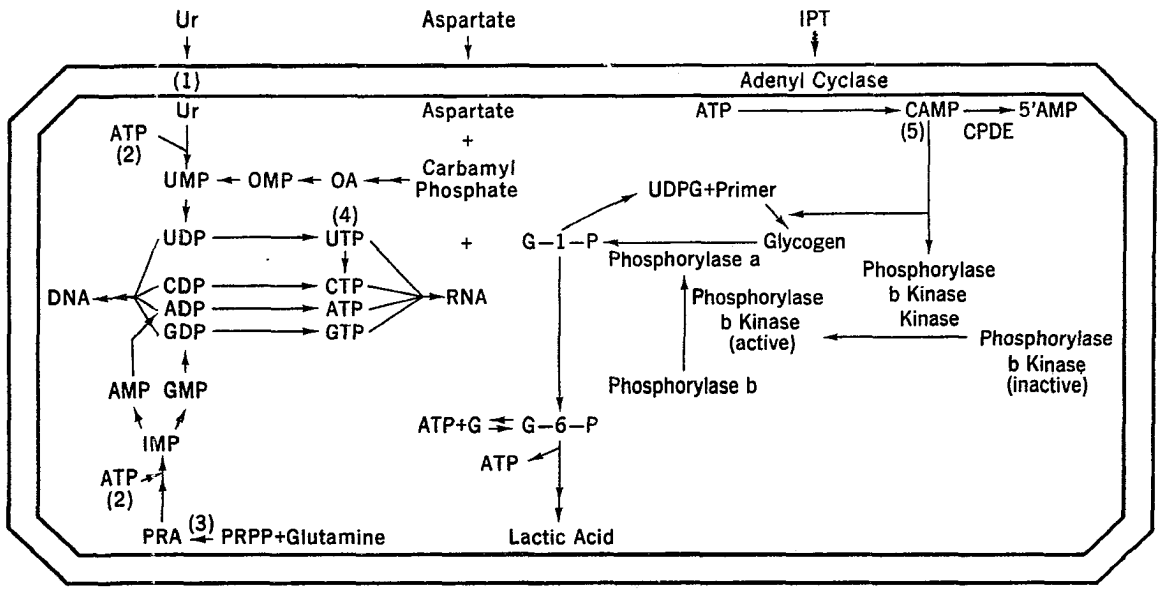

Figure 15 - Possible Mechanisms of Guanine, Guanosine or Cyclic GMP Action

(1) Membrane Permeability to Exogenous Nucleosides
(2) ATP Levels in Normal and Chronic Leukemic Lymphocytes
(3) Salvage vs. de novo Pathway in RNA Metabolism
(4) UTP Utilization for Glycogenesis and RNA Synthesis
(5) Cyclic AMP Levels in Normal and Chronic Leukemic Lymphocytes

An alternative hypothesis suggests that guanine, guanosine or cyclic GMP exert their inhibitory effect by competing with other nucleosides for ATP (mechanism 2). As shown in the purine nucleotide salvage synthetic pathway (Figure 16), the combined action of purine nucleoside phosphorylase and hypoxanthine/guanine phosphoribosyl-transferase converts guanine and guanosine into GMP. Cleavage of cyclic GMP by phosphodiesterase also results in the formation of GMP. Thus, ATP is required in the phosphorylation of GMP by guanylate kinase to form GDP and GTP. Rabinowitz and Wilhite (100) studied the levels of thymidine kinase activity in both normal and CLL lymphocytes. Their results clearly demonstrated that the amount of thymidine triphosphate formed from thymidine depended upon the ATP concentration. This finding suggests that the changes in the ATP levels in cells may function in regulating thymidine metabolism. Since ATP is also required in the phosphorylation of uridine by uridine kinase, ATP availability could regulate uridine metabolism as well. Thus, if the ATP concentration is limited in CLL lymphocytes, guanine, guanosine and cyclic GMP may exert their inhibitory effect by this mechanism.

A third possible mechanism involves the control of alternate pathways (mechanism 3), namely, the salvage pathway vs. the de novo pathway for purine nucleotide synthesis (Figure 16). It is most likely that direct control of de novo purine synthesis is exerted at the first obligatory step. This first step is catalyzed by phosphoribosyl pyrophosphate (PRPP) amidotransferase. It seems reasonably well established that PRPP amidotransferase is inhibited by monophosphates, particularly AMP and GMP. Thus, the addition of guanine, guanosine or

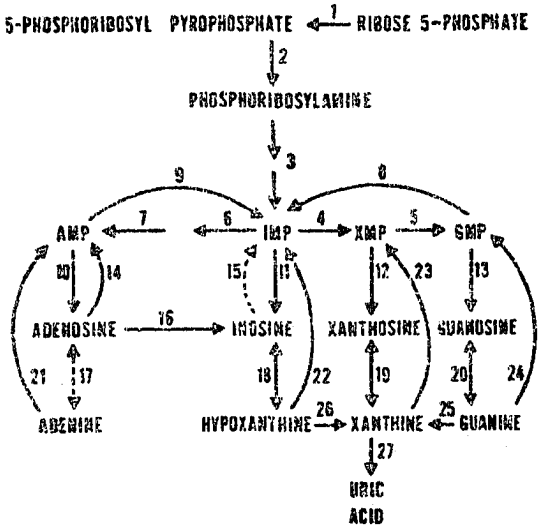

FIGURE 16–Purine biosynthesis, interconversion, and degradation in animal tissues. The numbers refer to the following enzymes or sequences of enzymes. 1: PRPP synthetase; 2: PRPP amidotransferase; 3: the enzymes of de novo purine biosynthesis; 4: IMP dehydrogenase; 5: XMP aminase; 6: adenylosuccinate synthetase; 7: adenylosuccinate lyase; 8: GMP reductase; 9: AMP deaminase; 10, 11, 12, 13: 5'-nucleotidase; 14: adenosine kinase; 15: inosine kinase; 16: adenosine deaminase; 17, 18, 19, 20: purine nucleoside phosphorylase; 21: adenine phosphoribosyltransferase; 22, 23, 24: hypoxanthine/guanine phosphoribosyltransferase; 25: guanine deaminase; 26, 27: xanthine oxidase.

Reactions indicated by dashed lines have only been shown to have low activities in animal tissues and are discussed further in the text; this does not necessarily indicate that they lack physiological importance.

(From Murray, A.W. 1971 The biological significance of purine salvage, Ann. Rev. Biochem., 40: 812.)

cyclic GMP could rapidly inhibit de novo purine nucleotide synthesis resulting from the elevation of intracellular GMP concentration as indicated previously. Normal cells when challenged by various inhibitors may reduce their utilization of the de novo pathway, but they nevertheless may continue to synthesize nucleic acid by the salvage pathway. However, CLL lymphocytes may not be able to change to the alternate pathway as readily. Reduced utilization of the de novo pathway in the presence of guanine, guanosine or cyclic GMP could result in a decrease in nucleic acid synthesis. Very recently, it was found that in PHA-stimulated human lymphocytes, no de novo purine synthesis was detected using labeled glycine as precursor (171). However, it is possible that CLL lymphocytes undergo a certain amount of de novo purine synthesis. If this is true, the presence of guanine, guanosine or cyclic GMP could have variable degrees of inhibitory effect, depending upon the extent of de novo purine synthesis. That is, an increase in GMP level due to the addition of guanine, guanosine or cyclic GMP would show no effect on purine synthesis in normal lymphocytes, but a significant effect on purine synthesis in CLL lymphocytes would be expected.

A fourth mechanism which also involves the control of alternate pathway may be suggested. Several investigators have demonstrated that large particles of glycogen are present in the cytoplasm of CLL lymphocytes (172-174). The glycogen particles were found to disappear after IPT treatment (136). None of the glycogen particles are seen in the normal lymphocytes. Previous studies also demonstrated that DNA synthesis in CLL lymphocytes is markedly inhibited by IPT (79). The significance of the presence of glycogen particles in the CLL cells is

indicated by the observation that IPT inhibits DNA synthesis, presumably by depleting the stored glycogen content. As shown in Figure 15, glycogen synthesis and nucleic acid synthesis are related by their mutual requirement for UTP. The coordination of the synthesis of these two macromolecules is obviously subject to precise regulation. In CLL lymphocytes, however, the UTP pool for RNA synthesis may be reduced or depleted because of the relatively large amount required for glycogen synthesis. Furthermore, guanine, guanosine or cyclic GMP could exert additional stress upon the regulation of glycogen synthesis and upon RNA synthesis resulting in a greater depletion of UTP for obligatory RNA synthesis.

Finally, a fifth possible mechanism can be suggested wherein cyclic AMP may be the primary factor involved in the different biochemical behavior exhibited by normal and CLL lymphocytes. As indicated previously, Abell et al. (79,123) have found that DNA synthesis in CLL lymphocytes is markedly inhibited by IPT or dibutyryl cyclic AMP, whereas DNA in normal lymphocytes is not affected significantly by either of the two drugs under the same conditions. It was also found that large particles of glycogen are present in the cytoplasm of CLL lymphocytes (172-174). These glycogen particles disappeared after IPT treatment, and none of the glycogen particles was observed in normal lymphocytes (136). These results indicated that the presence of glycogen implies that an alteration has occurred in the metabolic pathway associated with the leukemic nature of the cell. Furthermore, investigation of glycogen metabolism in lymphocytes showed that the phosphorylase a activity is increased in PHA-stimulated normal lymphocytes but not in PHA-stimulated CLL lymphocytes (175).

The action of IPT and of dibutyryl cyclic AMP can be explained by the "second messenger" concept introduced by Sutherland (112), i.e., cells respond to hormones by increasing the intracellular concentration of cyclic AMP. Increases in cyclic AMP have been related to various cellular processes such as glycogenolysis, lipolysis, steroidogenesis and protein synthesis (114-116). Of particular interest is the effect of cyclic AMP on glycogenolysis. The activation of phosphorylase by cyclic AMP was first recognized in liver by Sutherland and Rall (176). The process of phosphorylase activation in muscle has been most extensively studied by Krebs et al. (177,178). The sequence of this process initiates as cyclic AMP increases the activity of a protein kinase (also referred to as kinase II or phosphorylase kinase kinase) by allosteric interaction. This protein kinase catalyzes the phosphorylation of phosphorylase b kinase and results in the activation of this enzyme. The activated phosphorylase b kinase then catalyzes the inactive phosphorylase b to produce the active phosphorylase a. On the basis of the above observations, it is conceivable that the sensitivity of the CLL lymphocytes to IPT and dibutyryl cyclic AMP is due to an increase in intracellular cyclic AMP which consequently regulates the cell cycle by degrading the stored glycogen.

The level of cyclic AMP in cells is controlled by both adenyl cyclase and cyclic AMP phosphodiesterase. In attempts to obtain further information regarding the possible role of cyclic AMP in growth regulation, the activities of cyclic AMP phosphodiesterase in both cultured normal and CLL lymphocytes were measured. The effects of guanine, guanosine and cyclic GMP on cyclic AMP phosphodiesterase in

both cells were determined. In normal lymphocytes, the level of phosphodiesterase was determined on day 3 (maximal DNA synthetic day). Guanine (10^{-4}M) had no inhibitory effect; guanosine (10^{-4}M) had a moderate stimulatory effect, and cyclic GMP had a small stimulatory effect. In one CLL case with a medium WBC count, the activities of cyclic AMP phosphodiesterase were inhibited very mildly by guanine and guanosine on day 5 (maximal DNA synthetic day). The enzyme activity was increased slightly by cyclic GMP. However, in another CLL case with a high WBC count, drastic reductions of cyclic AMP phosphodiesterase were observed in cultures treated with guanine, guanosine, or cyclic GMP. It appears that the response of cyclic AMP phosphodiesterase in lymphocytes toward guanine, guanosine, or cyclic GMP is variable, possibly depending upon the WBC count of the blood donor. The stimulatory effect of cyclic GMP on the hydrolysis of cyclic AMP seems to be in agreement with other observations made with rat liver (156) as well as with rat thymic lymphocytes (153). Furthermore, a parallel between the non-treated control levels of lymphocyte phosphodiesterase and the WBC counts of the donors were demonstrated. The enzyme contents of the non-treated control cells are in a decreasing order of CLL lymphocytes (high WBC count) > CLL lymphocytes (medium WBC count) > normal lymphocytes (low WBC count).

The foregoing observations are consistent with the fifth possible mechanism. Provided that all other factors are constant, the cyclic AMP phosphodiesterase activity varies inversely with the concentration of cyclic AMP. As a consequence of low cyclic AMP present in cells, less active phosphorylase a will be formed. Eventually, the accumulation of glycogen is unavoidable.

Kinetic studies of the phosphodiesterase of the cell extracts indicated that both lymphocytes possessed two phosphodiesterase activities, one with a low apparent K_m between 1×10^{-5} and 2×10^{-5}M and another with an apparent K_m of 1×10^{-4} to 3×10^{-4}M. Similar findings have been reported by others (131,141,153,156,179-181). The ranges of apparent K_m values obtained from all these studies are summarized in Table 8. The apparent K_m's for the low-K_m phosphodiesterase activities are comparable among different tissues from different animals, whereas the apparent K_m's for the high K_m-enzyme are 100 fold different between the highest and lowest values, corresponding to human blood platelets (179) and rat thymic lymphocytes (153), respectively.

The two activities could also be distinguished by their specificity for cyclic AMP and cyclic GMP (115,153,156). We have tested the substrate specificities of only the one enzyme with the low apparent K_m, but found that guanine or guanosine had essentially no effect, whereas cyclic GMP had a small to moderate inhibitory effect. However, there is no consistent relationship between the extent of inhibition and the concentration of the inhibitor.

Concerning the inhibition of cyclic AMP phosphodiesterase by guanine or guanosine observed in cultured CLL lymphocytes, the molecular basis for the response of the enzyme to these two inhibitors is not known. Similarly, it is not clear whether or not the inhibition by guanine or guanosine reflects some physiological regulatory control of the enzyme. The cyclic AMP level in human lymphocytes was found to be around 2 picomoles/10^7 cells (47). The K_m of the low K_m-phosphodiesterase falls closer to the range of cyclic AMP concentration present in cells and

TABLE 8

K_m VALUES FOR PHOSPHODIESTERASE PREPARED FROM VARIOUS SOURCES (SUBSTRATE WAS CYCLIC AMP)

Source	K_m	Reference
Bovine Brain	1.4×10^{-4}M, 1×10^{-6}M	141
Rat Brain	1.3×10^{-4}M, 1×10^{-6}M	131
Pig Cerebrum	1×10^{-4}M, 4×10^{-6}M	180
Bovine Heart	25×10^{-6}M, 0.8×10^{-6}M	156
NISI-67	$1\text{-}2 \times 10^{-4}$M, $1\text{-}1.5 \times 10^{-6}$M	181
HeLa-S3-67	$0.8\text{-}1.7 \times 10^{-4}$M, $1\text{-}1.5 \times 10^{-6}$M	181
LG-67	$1.5\text{-}4 \times 10^{-4}$M, $1\text{-}2 \times 10^{-6}$M	181
LB-67	$1\text{-}1.5 \times 10^{-4}$M, $1\text{-}1.5 \times 10^{-6}$M	181
Rat Thymic Lymphocytes	8×10^{-6}M, 0.9×10^{-6}M	153
Human Blood Platelets	7×10^{-4}M, 7×10^{-5}M	179
Human Lymphocytes (normal)	1×10^{-4}M, 2×10^{-5}M	This Study
Human Lymphocytes (CLL)	2.9×10^{-4}M, 1.3×10^{-5}M	This Study

thus could play a role in the regulation of cyclic AMP levels in cells. It is therefore of interest that both normal and CLL lymphocytes contain low-K_m phosphodiesterase of similar kinetic characteristics. Since the apparent K_m is about 4 orders of magnitude higher than the cyclic AMP concentration of cells, the physiological significance of the high-K_m phosphodiesterase is uncertain. However, the absolute concentration of cyclic AMP in various regions of the cell may be appreciably higher than is indicated by the average values because of a possible compartmentalization of cyclic AMP in cells. No information is available regarding this point. Our finding that the phosphodiesterase activities are distributed mainly in the supernatant fraction (1,000 x g, 10 minutes) of the cell homogenate is in agreement with reports of the two activities in various animal tissues (156,179,182).

CHAPTER V

SUMMARY

Human peripheral blood lymphocytes obtained from both healthy normal donors and patients with chronic lymphocytic leukemia (CLL) were maintained in a cell culture system. The effects of several purines, pyrimidines and their nucleosides were studied in these two lymphocytes in order to elucidate possible differences in nucleic acid synthesis and its regulation.

Cytidine (10^{-4}M) was found to inhibit markedly the rate of uridine-^{3}H incorporation into RNA without apparently affecting thymidine-^{3}H incorporation into DNA in both normal and CLL lymphocytes. This study presented evidence that the inhibitory effect of cytidine is at least partially attributed to the cytidine deamination reaction occurring in both normal and CLL lymphocytes. The conversion of cytidine into uridine by cytidine deaminase results in the dilution of the radioactive uridine pool and in the reduction of uridine-^{3}H incorporation into RNA.

Guanine, guanosine and cyclic GMP, at a concentration of 10^{-4}M have no discernible effect on either RNA or DNA synthesis in normal lymphocytes. In CLL lymphocytes, however, they demonstrated marked inhibition on both uridine-^{3}H and thymidine-^{3}H incorporation into RNA and DNA, respectively.

In order to obtain information regarding the possible role of cyclic AMP in the regulation of nucleic acid synthesis in lymphocytes, the activities of cyclic AMP phosphodiesterase were measured in both normal and CLL lymphocytes. The effects of guanine, guanosine and cyclic GMP on cyclic AMP phosphodiesterase in both cells were also determined. Our results indicated that the level of cyclic AMP phosphodiesterase as well as the inhibition by guanine, guanosine or cyclic GMP were in a decreasing order of CLL lymphocytes (high WBC count) > CLL lymphocytes (medium WBC count) > normal lymphocytes (low WBC count).

Kinetic studies of the phosphodiesterase of the cell extracts further revealed that both lymphocytes possessed two phosphodiesterase activities. For normal lymphocytes, one K_m is 1×10^{-4}M and the other is 2×10^{-5}M. For CLL lymphocytes, one K_m is 2.9×10^{-4}M and the other is 1.3×10^{-5}M. The K_m's for the low-K_m phosphodiesterase activities are comparable between normal lymphocytes and CLL lymphocytes, whereas the K_m for the high-K_m phosphodiesterase activities in CLL lymphocytes is 3 fold greater than that in normal lymphocytes.

The substrate specificities of the low-K_m phosphodiesterase were also investigated. No significant inhibitory effect by guanine or guanosine was found. Cyclic GMP exerted a moderate inhibitory effect, but the extent of its inhibition is not proportional to its concentration.

BIBLIOGRAPHY

1. Bennett, J. H. 1845 Case of hypertropy of the spleen and liver, in which death took place from suppuration of the blood, Edinburgh M. and S. J., 64: 413.

2. Virchow, R. 1845 Weisses blut, Froriep's Notizen., 36: 151.

3. Dameshek, W. and Gunz, F. 1964 Leukemia. Second Edition. Grune and Stratton, New York.

4. World Health Organization. 1960 Epidemiol. Vital Statist. Rep., 8: 34.

5. Smith, R. L. 1957 Recorded and expected mortality among the Indians of the United States with special reference to cancer, J. Nat. Cancer Inst., 18: 475.

6. Tekeda, K. 1960 Geographical pathology of leukemia in Japan, Acta Un. Int. Cancr., 16: 1629.

7. Wells, R. and Lau, K. S. 1960 Incidence of leukemia in Singapore and rarity of chronic lymphocytic leukemia in Chinese, Brit. Med. J., 1: 759.

8. Gunz, F. W. 1961 Incidence of some aetiological factors in human leukemia, Brit. Med. J., 1: 326.

9. Zarafonetis, C. J. D. 1968 Proceedings of the International Conference on Leukemia-Lymphoma. Lea and Febiger, Philadelphia.

10. Dameshek, W. and Dutcher, R. M. 1968 Perspective in Leukemia. Grune and Stratton, New York.

11. Reilly, E. B. Rapaport, S. I. Karr, N. W. Mills, H. and Carpenter, G. E. 1952 Familial chronic lymphatic leukemia, Arch. Intern. Med., 90: 87.

12. Gunz, F. W. and Dameshek, W. 1957 Chronic lymphocytic leukemia in a family including twin brothers and a son, J. Amer. Med. Ass., 164: 1323.

13. Fraumeni, J. F. Jr., Vogel, C. L. and DeVita, V. T. 1969 Familial chronic lymphocytic leukemia, Ann. Intern. Med., 71: 279.

14. Gross, L. 1961 Oncogenic Viruses. Pergamon Press, New York.

15. University of Texas and M. D. Anderson Hospital and Tumor Institute. 1963 Proceedings of 17th Ann. Symposium on Fundamental Cancer Research. Williams and Wilkins Co., Baltimore.

16. Symposium on the Possible Role of Viruses in Cancer, 1960 Cancer Res., 20: 669.

17. Burdette, W. J. 1966 Viruses Inducing Cancer, Implications and Therapy. University of Utah Press. Provo.

18. Baltimore, D. 1970 Viral RNA-dependent DNA polymerase, Nature, 226: 1209.

19. Temin, H. M. and Mizutani, S. 1970 RNA-dependent DNA polymerase in virions of Rous sarcoma virus, Nature, 226: 1211.

20. Hehlmann, R., Kufe, D. and Spiegelman, S. 1972 RNA in human leukemic cells related to the RNA of a mouse leukemia virus, Proc. Nat. Acad. Sci. USA, 69: 435.

21. Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M. and Watson, K. 1970 Synthetic DNA-RNA hybrids and RNA-RNA duplexes as templates for the polymerase of the oncogenic RNA virus, Nature, 228: 430.

22. Stone, L. B., Scolnick, E., Takemoto, K. K. and Aaronson, S. A. 1971 Visna Virus: a slow virus with a RNA dependent DNA polymerase, Nature, 229: 257.

23. Scolnick, E. M., Aaronson, S. A., Todaro, G. J. and Parks, W. P. 1971 RNA dependent DNA polymerase activity in mammalian cells, Nature, 229: 318.

24. Andervont, H. B. 1957 Genetic, hormonal and age factors in susceptibility and resistance to tumor-inducing viruses, Texas Rep. Biol. Med., 15: 462.

25. Kirschbaum, A. 1951 Rodent leukemia: recent biological study, Cancer Res., 11: 741.

26. Temin, H. M. 1971 Protovirus hypothesis, J. Nat. Cancer Inst., 46: III.

27. Kirschbaum, A., Shapiro, J. R. and Mixer, H. W. 1953 Synergistic action of leukemogenic agents, Cancer Res., 13: 262.

28. Dunlap, C. E. 1942 Effects of radiation on the blood and the hemopoietic tissues, including the spleen, the thymus and the lymph nodes, Arch. Path., 34: 562.

29. Elves, M. W. 1967 The Lymphocytes. Year Book Medical Publishers, Inc., Chicago.

30. Beck, W. S. 1968 Proceedings of the International Conference on Leukemia-Lymphoma. Lea and Febiger, Philadelphia.

31. Cooper, E. H. and Fitzgerald, M. G. 1958 Respiration and glycolysis of isolated rat lymphocytes, Biochem. J., 68: 5.

32. Frei, J., Borel, C., Horvath, G., Cullity, B. and Vannotti, A. 1961 Enzymatic studies in the different types of normal and leukaemic human white cells, Blood, 18: 317.

33. Stjernholm, R. L., Noble, E. P., Nikolay, V. D., Morton, D. J. and Falor, W. H., 1969 Carbohydrate metabolism in leukocytes XII. Metabolism of the human lymphocyte, J. Reticuloendothel. Soc., 6: 590.

34. Beck, W. S. 1958 The control of leukocyte glycolysis, J. Biol. Chem., 232: 251.

35. Barron, E. S. G. and Harrop, G. A. 1929 Studies on blood cell metabolism V. The metabolism of leukocytes, J. Biol. Chem., 84: 89.

36. Kempner, W. 1939 The nature of leukaemic blood cells as determined by their metabolism, J. Clin. Invest., 18: 291.

37. Schlossman, H. 1930 Uber den staff wechsel von lymphocyten, Biochem. Z., 219: 463.

38. Jones, R. U., Goffi, G. P. and Hutt, M. S. R. 1966 Lymphocyte glycogen content in various disease, Amer. J. Med. Sci., 252: 282.

39. Nakai, G. S. and Craddock, C. G. 1966 Uridine-diphosphoglucose-glycosyl-transferase in human leukemic lymphocytes, Biochim. Biophys. Acta, 121: 195.

40. Lisker, S. A. and Brody, J. I. 1968 α-D-glucosidase in normal and leukemic lymphocytes, Experientia, 24: 682.

41. Yunis, A. A. and Arimura, G. K. 1964 Enzymes of glycogen metabolism in white blood cells. I. Glycogen phosphorylase in normal and leukemic lymphocytes, Cancer Res., 24: 489.

42. Brody, J. I., Oski, F. A. and Singer, D. E. 1969 Impaired pentose phosphate shunt and decrease glycolytic activity in lymphocytes of chronic lymphocytic leukemia. Metabolic pathway...? Blood, 34: 421.

43. Beck, W. S. 1958 Occurrence and control of the phosphogluconate oxidation pathway in normal and leukemic leukocytes, J. Biol. Chem., 232: 271.

44. Hungerford, D. A., Donnelly, A. J., Nowell, P. C. and Beck, W. S. 1959 The chromosome constitution of a human phenotype intersex, Amer. J. Hum. Genet., 11: 215.

45. Nowell, P. C. 1960 Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes, Cancer Res., 20: 462.

46. Abell, C. W. 1970 Regulation of cell division in normal and malignant human lymphocytes, Ann. Okla. Acad. Sci., 1: 58.

47. Smith, J. W., Steiner, A. L., Newberry, W. M. and Parker, C. W. 1971 Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after phytohemagglutinin stimulation, J. Clin. Invest., 50: 432.

48. Novogrodsky, A. and Katchalski, E. 1970 Effect of phytohemagglutinin and prostaglandins on cyclic AMP synthesis in rat lymph node lymphocytes, Biochim. Biophys. Acta, 215: 291.

49. Smith, J. W., Steiner, A. L. and Parker, C. W. 1971 Human lymphocyte metabolism. Effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin, J. Clin. Invest., 50: 442.

50. Cross, M. E. and Ord, M. G. 1970 Transformation of lymphocytes by CAMP and consequent changes in histone microstructure, Biochem. J., 120: 21.

51. Fischer, D. B. and Mueller, G. C. 1968 An early alteration in the phospholipid metabolism of lymphocytes by phytohemagglutinin, Proc. Nat. Acad. Sci. USA, 60: 1396.

52. Fischer, D. B. and Mueller, G. C. 1971 Studies on the mechanism by which phytohemagglutinin rapidly stimulates phospholipid metabolism of human lymphocytes, Biochim. Biophys. Acta, 248: 434.

53. Pogo, B. G. T., Allfrey, V. G. and Mirsky, A. E. 1966 RNA synthesis and histone acetylation during the course of gene activation in lymphocytes, Proc. Nat. Acad. Sci. USA, 55: 805.

54. MacGillivray, A. J. 1966 Histones of human leucocytes, Biochem. J., 101: 24p.

55. Kleinsmith, L. J., Allfrey, V. G. and Mirsky, A. E. 1966 Phosphoprotein metabolism in isolated lymphocyte nuclei, Proc. Nat. Acad. Sci. USA, 55: 1182.

56. Cooper, H. L. and Rubin, A. D. 1965 RNA metabolism in lymphocytes stimulated by phytohemagglutinin: initial responses to phytohemagglutinin, Blood, 25: 1014.

57. Epstein, L. B. and Stohlman, F. 1964 RNA synthesis in cultures of normal human peripheral blood, Blood, 24: 69.

58. Kay, J. E. and Cooper, H. L. 1969 Rapidly labeled cytoplasmic RNA in normal and phytohemagglutinin-stimulated human lymphocytes, Biochim. Biophys. Acta, 186: 62.

59. Lucas, Z. J. 1967 Pyrimidine nucleotide synthesis: regulatory control during transformation of lymphocytes in vitro, Science, 56: 1237.

60. Lucas, Z. J. 1969 Proceedings of the Third Leukocyte Culture Conference. Appleton-Century-Crafts, New York.

61. Hausen, P. and Stein, H. 1968 On the synthesis of RNA in lymphocytes stimulated by phytohemagglutinin. I. Introduction of uridine-kinase and the conversion of uridine to UTP, Europ. J. Biochem., 4: 401.

62. Kay, J. E. and Handmaker, S. D. 1970 Uridine incorporation and RNA synthesis during stimulation of lymphocytes by PHA, Exp. Cell Res., 63: 411.

63. Peters, J. H. and Hausen, P. 1971 Effect of phytohemagglutinin on lymphocyte membrane transport. I. Stimulation of uridine uptake, Europ. J. Biochem., 19: 502.

64. Peters, J. H. and Hausen, P. 1971 Effect of phytohemagglutinin on lymphocyte membrane transport. II. Stimulation of "faciliated diffusion" of 3-O-methylglucose, Europ. J. Biochem., 19: 502.

65. Cooper, H. L. 1968 Ribonucleic acid metabolism in lymphocytes stimulated by phytohemagglutinin. II. Rapidly synthesized ribonucleic acid and the production of ribosomal ribonucleic acid, J. Biol. Chem., 243: 34.

66. Cooper, H. L. 1969 Proceedings of the Third Leukocyte Culture Conference. Appleton-Century-Crafts, New York.

67. Cooper, H. L. 1969 The Biochemistry of Cell Division. Charles C. Thomas. Springfield, Illinois.

68. Cooke, A., Kay, J. E. and Cooper, H. L. 1971 Ribonucleic acid polymerase activity as a measure of ribonucleic acid synthesis, Biochem J., 125: 74p.

69. Cooper, H. L. 1969 Ribosomal ribonucleic acid wastage in resting and growing lymphocytes, J. Biol. Chem., 244: 5590.

70. Rubin, A. D. 1971 Defective control of ribosomal RNA processing in stimulated leukemic lymphocytes, J. Clin. Invest., 50: 2485.

71. Trowell, O. A. 1965 Cells and Tissue in Culture: methods, biology and physiology. Vol. 2, Academic Press, New York.

72. Bach, F. and Hirschhorn, K. 1963 γ-Globulin production by human lymphocytes in vitro, Exp. Cell Res., 32: 592.

73. Elves, M. W., Roath, S., Taylor, G. and Israels, M. C. G. 1963 The in vitro production of antibody lymphocytes, Lancet 1: 1292.

74. Ripps, C. and Hirschhorn, K. 1967 The production of immunoglobulin by human peripheral blood lymphocytes in vitro, Clin. Exp. Immun., 2: 377.

75. Kay, J. E. 1966 RNA and protein synthesis in lymphocytes incubated with phytohemagglutinin. In: The Biological Effects of Phytohemagglutinin, The Robert Jones and Agnes Hunt orthopaedic hospital management committee. Oswestry, England.

76. Hayden, G. A., Crowley, C. M. and Jamison, G. A. 1970 Incorporation of glucosamine into membrane glycoproteins of phytohemagglutinin-stimulated lymphocytes, J. Biol. Chem., 245: 5827.

77. Bender, M. A. and Prescott, D. M. 1962 Synthesis and mitosis in cultures of human peripheral leukocytes, Exp. Cell Res., 27: 221.

78. Loeb, L. A., Ewald, J. L. and Agarwal, S. S. 1970 DNA polymerase and DNA replication during lymphocyte transformation, Cancer Res., 30: 2514.

79. Abell, C. W., Kamp, C. W. and Johnson, L. D. 1970 Effects of phytohemagglutinin and isoproterenol on DNA synthesis in lymphocytes from normal donors and patients with chronic lymphocytic leukemia, Cancer Res., 30: 717.

80. Havemann, K. and Rubin, A. D. 1968 The delayed response of chronic lymphocytic leukemia lymphocytes to phytohemagglutinin in vitro, Exp. Biol. Med., 127: 668.

81. Atkinson, D. E. 1966 Regulation of enzyme activity, Ann. Rev. Biochem., 35: 85.

82. Stadtman, E. R. 1966 Allosteric regulation of enzyme activity, Adv. Enzymol., 28: 41.

83. Blakley, R. L. and Vitols, E. 1968 The control of nucleotide biosynthesis, Ann. Rev. Biochem., 37: 201.

84. Murray, A. W., Elliot, D. C. and Atkinson, M. R. 1970 Nucleotide biosynthesis from preformed purines in mammalian cells: regulatory mechanisms and biological significance, Progr. Nucl. Acid Res., 10: 87.

85. Haga, S. E., Jones, M. E. 1965 Initial step in pyrimidine synthesis in Ehrlich ascites carcinoma in vitro, J. Biol. Chem., 240: 4556.

86. Mayfield, E. D. Jr., Lyman, K. and Brensnick, E. 1967 Incorporation of bicarbonate-^{14}C into pyrimidine and into ribonucleic acid of the Novikoff ascite tumor cell, Cancer Res., 27: 476.

87. Jones, M. E. and Hager, S. E. 1966 Source of carbamyl phosphate for pyrimidine biosynthesis in mouse Ehrlich ascites cells and rat liver, Science, 154: 422.

88. Tatibana, M. and Ito, K. 1967 Carbamyl phosphate synthetase of the hematopoietic mouse spleen and the control of pyrimidine biosynthesis, Res. Commun., 26: 221.

89. Ito, K. and Uchino, H. 1971 Control of pyrimidine biosynthesis in human lymphocytes: introduction of glutamine-utilizing carbamyl phosphate synthetase and operation of orotic acid pathway during blastogenesis, J. Biol. Chem., 246: 4060.

90. Smith, L. H. Jr., Baker, F. A. and Sullivan, M. 1960 Pyrimidine metabolism in man. II. Studies of leukemic cells, Blood, 15: 360.

91. Smith, L. H. Jr., Baker, F. A. 1960 Pyrimidine metabolism in man. III. Studies on leukocytes and erythrocytes in pernicious anemia, J. Clin. Invest., 39: 15.

92. Apple, S. H. and Pettis, P. 1967 Regulation of brain orotodylic decarboxylase activity, Fed. Proc., 26: 292.

93. McCollister, R. J., Gilbert, W. R. Jr., Ashton, D. M. and Wyngaarden, J. B. 1964 Pseudofeedback inhibition of purine synthesis by 6-mercaptopurine ribonucleotide and other purine analogues, J. Biol. Chem., 239: 1560.

94. Scott, J. L. 1962 Incorporation of adenine-8-^{14}C and formate-^{14}C into the nucleic acids of leukemia leukocytes, J. Clin. Invest., 41: 67.

95. Adams, R. L. P., Abrams, R. and Lieberman, I. 1966 Deoxycytidylate synthesis and entry into the period of deoxyribonucleic acid replication in rabbit kidney cells, J. Biol. Chem., 241: 903.

96. Fujioka, S. and Silber, R. 1971 Leukocyte ribonucleotide reductase: studies in normal subjects and in subjects with leukemia in pernicious anemia, J. Lab. Clin. Med., 77: 59.

97. Turner, M. K., Abrams, R. and Lieberman, I. 1966 Meso-α,ß-diphenylsuccinate and hydroxyurea as inhibitors of deoxycytidylate synthesis in extracts of Ehrlich ascites and L cells, J. Biol. Chem., 241: 5777.

98. Littlefield, J. W. 1966 The periodic synthesis of thymidine kinase in mouse fibroblasts, Biochim. Biophys. Acta, 114: 398.

99. Eker, P. 1966 Studies on thymidine kinase of human liver cells in culture, J. Biol. Chem., 241: 659.

100. Rabinowitz, Y. and Wilhite, B. A. 1969 Thymidine salvage pathway in normal and leukemic leukocytes with effects of ATP on enzyme control, Blood, 33: 759.

101. Davidson, J. D. and Winter, T. S. 1964 Purine Nucleotide Pyrophosphorylases in 6-mercaptopurine-sensitive and -resistant human leukemias, Cancer Res., 24: 261.

102. Smith, J. L., Omura, G. A., Krakoff, I. H. and Balis, M. E. 1971 IMP: and AMP: Pyrophosphate phosphoribosyltransferase in leukemic and normal human leukocytes, Proc. Soc. Exp. Biol. Med., 136: 1299.

103. Seegmiller, J. E., Rosenbloom, F. M. and Kelly, W. N. 1967 Enzyme defect associated with a sex-linked human neurological disorder and excessive purine synthesis, Science, 155: 1682.

104. Rabinowitz, Y., Farmer, R. and Czebotar, V. 1971 Deoxycytidine pathway in separate normal and leukemic leukocytes with effects of cell culture, Blood, 38: 312

105. Lorenson, M. Y., Maley, G. F. and Maley, F. 1967 The purification of properties of thymidylate synthetase from chick embryo extracts, J. Biol. Chem., 242: 3332.

106. Maley, F. and Maley, G. F. 1960 Nucleotide Interconversion, J. Biol. Chem., 235: 2968.

107. Brown, S. S., Neal, G. E. and Williams, D. C. 1965 Subcellular distribution of some folic acid-linked enzymes in rat liver, Biochem. J., 97: 34c.

108. Maley, F. and Maley, G. F. 1961 Nucleotide interconversions. IV. Activities of deoxycytidylate deaminase and thymidylate synthetase in normal rat liver and hepatomas, Cancer Res., 21: 1421.

109. Maley, F. and Maley, G. F. 1962 On the nature of a sparing effect by thymidine on the utilization of deoxycytidine, Biochemistry, 1: 847.

110. Fiala, S. and Fiala, A. E. 1965 Deoxycytidylic acid deaminase in Ehrlich ascites tumor cells, Cancer Res., 25: 922.

111. Silber, R. 1967 Regulatory mechanisms in the human leukocyte. I. The feedback control of deoxycytidylate deaminase, Blood, 29: 896.

112. Sutherland, E. W. and Rall, T. W. 1960 The relation of adenosine-3', 5'-phosphate and phosphorylase to the actions of catecholamines and other hormones, Pharmacol. Rev., 12: 256.

113. Sutherland, E. W., Øye, I. and Butcher, R. W. 1965 Action of epinephrine and the role of the adenyl cyclase system in hormone action, Recent Progr. Hormone Res., 21: 623.

114. Role of Cyclic AMP in Cell Function. 1970 Editors: Greengard, P. and Costa, E. Raven Press, New York.

115. Cyclic AMP and Cell Function. 1971 Editors: Robison, G. A., Nahas, G. G. and Triner, L. New York Acad. Sci., New York.

116. Robison, G. A., Butcher, R. W. and Sutherland, E. W. 1972 Cyclic AMP. Academic Press, New York.

117. Barka, T. 1965 Stimulation of DNA synthesis by isoproterenol in the salivary gland, Exp. Cell Res., 39: 355.

118. MacManus, J. P. and Whitfield, J. F. 1969 Stimulation of DNA synthesis and mitotic activity of thymic lymphocytes by cyclic adenosine-3',5'-monophosphate, Exp. Cell Res., 58: 188.

119. Ryan, W. L. and Heidrick, M. L. 1968 Inhibition of cell growth in vitro by adenosine-3',5'-monophosphate, Science, 162: 1484.

120. Heidrick, M. L. and Ryan, W. L. 1970 Cyclic nucleotides on cell growth in vitro, Cancer Res., 30: 376.

121. Gericke, D. and Chandra, P. 1969 Inhibition of tumor growth by nucleoside cyclic 3',5'-monophosphate, Hoppe-Seyler Z. Physiol. Chem., 350: 1469.

122. Hirschhorn, R., Grossman, J. and Weissman, G. 1970 Effect of cyclic 3',5'-adenosine monophosphate and theophylline on lymphocyte transformation, Proc. Soc. Exp. Biol. Med., 133: 1361.

123. Johnson, L. D. and Abell, C. W. 1970 The effect of isoproterenol and cyclic adenosine-3',5'-phosphate on phytohemagglutinin stimulated DNA synthesis in lymphocytes obtained from patients with chronic lymphocytic leukemia, Cancer Res., 30: 2718.

124. Lichtenstein, L. M. and Margolis, S. 1968 Histamine release in vitro: inhibition by catecholamines and methylxanthines, Science, 161: 962.

125. Butcher, R. W. and Sutherland, E. W. 1962 Adenosine 3',5'-monophosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine, J. Biol. Chem., 237: 1244.

126. Nair, K. G. 1966 Purification and properties of 3',5'-cyclic nucleotide phosphodiesterase from dog heart, Biochemistry, 5: 150.

127. Cheung, W. Y. 1967 Properties of cyclic 3',5'-nucleotide phosphodiesterase from rat brain, Biochemistry, 6: 1079.

128. Cheung, W. Y. 1966 Inhibition of cyclic nucleotide phosphodiesterase by adenosine 5'-triphosphate and inorganic pyrophosphate, Biochem. Biophys. Res. Commun., 23: 214.

129. Appleman, M. M. and Kemp, R. G. 1966 A potent metabolic effect independent of protein synthesis, Biochem. Biophys. Res. Commun., 24: 564.

130. O'Dea, R. F., Haddox, M. K. and Goldberg, N. D. 1970 Kinetic analysis of a soluble rat brain cyclic nucleotide phosphodiesterase, Fed. Proc., 29: 473 abs.

131. Brooker, G., Thomas, L. J. Jr. and Appleman, M. M. 1968 The assay of adenosine 3',5'-cyclic monophosphate in biological materials by enzymatic radioisotopic displacement, Biochemistry, 7: 4177.

132. Blecher, M., Merlino, N. S. and Ro'Ane, J. T. 1968 Control of the metabolic and lipolytic effects of cyclic 3',5'-adenosine monophosphate in adipose tissue by insulin, methyl xanthine and nicotinic acid, J. Biol. Chem., 243: 3973.

133. Thompson, W. J. and Appleman, M. M. 1970 Separation and characterization of multiple forms of 3',5'-cyclic AMP phosphodiesterase, Fed. Proc., 29: 602 abs.

134. Monn, E. and Christiansen, R. O. 1971 Adenosine 3',5'-monophosphate phosphodiesterase multiple molecular forms, Science 173: 540.

135. Goren, E. N., Hirsch, A. H. and Rosen, O. M. 1971 Activity stain for the detection of cyclic nucleotide phosphodiesterase separated by polyacrylamide gel electrophoresis and its application to the cyclic nucleotide phosphodiesterase of beef heart, Anal. Biochem., 43: 156.

136. Johnson, L. D. 1971 The Biochemistry of Lymphocytes from Patients with Chronic Lymphocytic Leukemia. Doctoral Dissertation, University of Oklahoma, Oklahoma City, Oklahoma.

137. Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter, Anal. Biochem., 1: 279.

138. Burton, K. A. 1956 A study of the conditions and mechanism of the diphenylamine reaction of the colorimetric estimation of deoxyribonucleic acid, Biochem. J., 62: 315.

139. Fallon, H. J., Frei, E. III., Davidson, J. D., Trier, J. S. and Burk, D. 1962 Leukocyte preparation from human blood: evaluation of their morphologic and metabolic state, J. Lab. Clin. Med., 59: 779.

140. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951 Protein measurement with the Folin phenol reagent, J. Biol. Chem., 193: 265.

141. Cheung, W. Y. 1970 Role of Cyclic AMP in Cell Function. Editors: Greengard, P. and Costa, E. p. 51, Raven Press, New York.

142. Krishna, G., Weiss, B. and Brodie, B. B. 1968 A simple, sensitive method for the assay of adenyl cyclase, J. Pharmacol. Exp. Ther., 163: 379.

143. Posternak, Th., Sutherland, E. W. and Henion, W. F. 1962 Derivatives of cyclic 3',5'-adenosine monophosphate, Biochim. Biophys. Acta, 65: 558.

144. Butcher, R. W., Ho, R. J., Meng, H. C. and Sutherland, E. W. 1965 Adenosine 3',5'-monophosphate in biological materials. II. The measurement of adenosine 3',5'-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine, J. Biol. Chem., 240: 4515.

145. Bdolah, A. and Schramm, M. 1965 The function of 3',5'-cyclic AMP in enzyme secretion, Biochem. Biophys. Res. Commun., 18: 452.

146. Kay, J. E., Leventhal, B. G. and Cooper, H. L. 1969 Effects of inhibition of ribosomal RNA synthesis on the stimulation of lymphocytes by phytohemagglutinin, Exp. Cell Res., 54: 94.

147. Cohen, R. M. and Wolfenden, R. 1971 Cytidine deaminase from Escherichia Coli, J. Biol. Chem. 246: 7561.

148. Wang, T. P., Sable, H. Z. and Lampen, J. O. 1950 Enzymatic deamination of cytosine nucleosides, J. Biol. Chem., 184: 17.

149. Conway, E. J. and Cooke, R. 1939 Blood ammonia, Biochem. J., 33: 457.

150. Greenstein, J. P., Carter, C. G. and Chalkley, H. W. 1947 Enzymatic degradation of ribonucleic and deoxyribonucleic acids with an addendum on the effect of nucleates on the heat stability of proteins, Cold Spring Harbor Symp., Quant. Biol., 12: 64.

151. Orengo, A. 1969 Regulation of enzymic activity by metabolites. I. Uridine-cytidine kinase of Novikoff ascites rat tumor, J. Biol. Chem., 244: 2204.

152. Goren, E., Erlichman, J., Rosen, O. M. and Rosen, S. M. 1970 A possible role for cyclic nucleotide phosphodiesterase in the regulation of the intracellular concentration of cyclic 3',5'-AMP, Fed. Proc., 29: 602 abs.

153. Franks, D. J. and MacManus, J. P. 1971 Cyclic GMP stimulation and inhibition of cyclic AMP phosphodiesterase from thymic lymphocytes, Biochem. Biophys. Res. Commun., 42: 844.

154. Murad, F., Manganiello, V. and Vaughan, M. 1970 Effects of guanosine 3',5'-monophosphate on glycerol production and accumulation of adenosine 3',5'-monophosphate by fat cells, J. Biol. Chem., 245: 3352.

155. Rosen, O. M. 1970 Preparation and properties of a cyclic 3',5'-nucleotide phosphodiesterase isolated from frog erythrocytes, Arch. Biochem. Biophys., 137: 435.

156. Beavo, J. A., Hardman, J. G. and Sutherland, E. W. 1970 Hydrolysis of cyclic guanosine and adenosine 3',5'-monophosphates by rat and bovine tissues, J. Biol. Chem., 245: 5649.

157. Ramsden, E. N. 1970 A kinetic study of the enzymic hydrolysis of adenosine 3',5'-cyclic monophosphate, Biochem. J., 120: 12p.

158. Yip, M. C. M. and Knox, W. E. 1970 Glutamine-dependent carbamyl phosphate synthetase, J. Biol. Chem., 245: 2199.

159. Bresnick, E. and Mosse, H. 1966 Aspartate carbamyltransferase from rat liver, Biochem. J., 101: 63.

160. Crui, M. R. and Donachie. W. D. 1964 An attempt to find pyrimidine inhibitors of mammalian aspartate carbamyltransferase, Biochim. Biophys. Acta, 85: 338.

161. Gerhart, I. C. and Pardee, A. B. 1962 The enzymology of control by feedback inhibition, J. Biol. Chem., 237: 891.

162. Jordan, D. O. 1960 The Chemistry of Nucleic Acids. Butterworths, Washington, D. C.

163. Shapiro, R. and Klein, R. S. 1966 The deamination of cytidine and cytosine by acidic buffer solutions: mutagenic implications, Biochemistry, 5: 2358.

164. Cohen, R. M. and Wolfenden, R. 1971 The equilibrium of hydrolytic deamination of cytidine and N^4-methylcytidine, J. Biol. Chem., 246: 7566.

165. Camiener, G. W. and Smith, C. G. 1965 Studies of the enzymatic deamination of cytosine arabinoside. I. Enzyme distribution and species specificity, Biochem. Pharmacol., 14: 1405.

166. Kornfeld, S. 1969 Decreased phytohemagglutinin receptor sites in chronic lymphocytic leukemia, Biochim. Biophys. Acta, 192: 542.

167. Chu, M. Y. and Fisher, G. A. 1965 Comparative studies of leukemic cells sensitive and persistant to cytosine arabinoside, Biochem. Pharmacol., 14: 333.

168. Creasey, W. A., Papac, R. J., Martin, M. E., Calabresi, P. and Welch, A. D. 1966 Biochemical and pharmacological studies with 1-β-D-arabinofuranosyl cytosine in man, Biochem. Pharmacol., 15: 1417.

169. Steuart, C. D. and Burke, P. J. 1971 Cytidine deaminase and the development of resistance to arabinosyl cytosine, Nature New Biol., 233: 109.

170. Steck, T. L., Nakata, Y. and Bader, J. P. 1969 The uptake of nucleosides by cells in culture. I. Inhibition by heterologous nucleosides, Biochim. Biophys. Acta, 190: 237.

171. Abell, C. W. and Marchand, N. W. Unpublished data.

172. Wislocki, G. B., Rheingold, J. J. and Dempsey, E. W. 1949 The occurence of the periodic acid-Schiff reaction in various normal cells of blood and connective tissue, Blood, 4: 562.

173. Astaldi, G. and Verga, L. 1957 The glycogen content of the cells of lymphatic leukaemia, Acta Haemat., 17: 129.

174. Quaglino, D. and Cowling, D. G. 1964 Cytochemical studies on cells from chronic lymphocytic leukemia and lymphosarcoma cultured with phytohemagglutinin, Brit. J. Haemat., 10: 358.

175. Abell, C. W. and Kamp, C. W. Unpublished data.

176. Sutherland, E. W. and Rall, T. W. 1960 The relationship of adenosine-3',5'-phosphate and phosphorylase to the actions of catecholamines and other hormones, Pharmacol. Rev., 12: 265.

177. Krebs, E. G. and Fischer, E. H. 1960 The role of metals in the activation of muscle phosphorylase, Ann. N. Y. Acad. Sci., 88: 378.

178. Krebs, E. G., Huston, R. B. and Hunkeler, F. L. 1968 Properties of phosphorylase kinase and its control in skeletal muscle, Adv. Enzyme Regul., 6: 245.

179. Song, S. Y. and Cheung, W. Y. 1971 Cyclic 3',5'-nucleotide phosphodiesterase properties of the enzyme of human blood platelets, Biochim. Biophys. Acta, 242: 593.

180. Weiss, B., Lehne, R. and Strada, S. 1972 Rapid microassay of adenosine 3',5'-monophosphate phosphodiesterase activity, Anal. Biochem., 45: 222.

181. Schroder, J. and Plagemann, P. W. W. 1972 Cyclic 3',5'-nucleotide phosphodiesterase of Novikoff rat hepatoma, mouse L and HeLa cells growing in suspension culture, Cancer Res., 32: 1082.

182. Thompson, W. J. and Appleman, M. M. 1971 Multiple cyclic nucleotide phosphodiesterase activity from rat brain, Biochemistry, 10: 311.