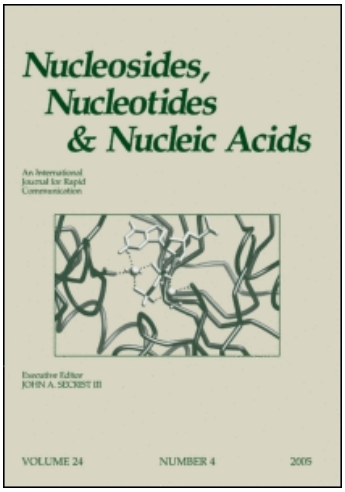

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Pyrimidine Deoxyribonucleosides Are Phosphorylated to Triphosphates at Low Temperatures Too, But Are Not Incorporated into DNA or Phospholipids.

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PYRIMIDINE DEOXYRIBONUCLEOSIDES ARE PHOSPHORYLATED TO TRIPHOSPHATES AT LOW TEMPERATURES TOO, BUT ARE NOT INCORPORATED INTO DNA OR PHOSPHOLIPIDS.

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ABSTRACT: Thymidine (Thd) was phosphorylated to dTTP also at 0°C, both in Ehrlich ascites tumor cells and human tonsillar lymphocytes, but was not incorporated into DNA. The uptake and phosphorylation of ^{14}C-Thd into the pool showed regular kinetics (Km 6,6 uM), and the main metabolite was dTTP (75-84%) both at 0°C, and 37°C. Similarly, deoxycytidine (dCyd) was also transported and phosphorylated to nucleotides (76%) at low temperature, but no incorporation into DNA and phospholipid precursor liponucleotides could be detected at 0°C. Under the same conditions, at 37°C, when lymphocytes were labeled with 5-^{3}H-dCyd, 51% of the total pool radioactivity was found in liponucleotides. Transport and phosphorylation of deoxynucleosides seem to be tightly coordinated at both temperatures, which processes are directly coupled to membrane-phospholipid and DNA biosynthesis, but only at physiological temperature while they seem "uncoupled" at low temperature. The fact that nucleoside phosphorylation occures also at low temperature has implications for several experimental techniques used in cell biology.

INTRODUCTION

The transport mechanisms for nucleosides are only partially identified (1,2), their phosphorylation has been suggested as rate limiting step in the salvage pathway of intact cells (3, 11). On the other hand, the inhibitory effect of arabinosyl-cytosine (araC) on cell growth correlated better with transport than with its phosphorylation (4) . The uptake of extracellular nucleosides is the dominant as compared to the de novo synthesis of DNA precursors in some tissues, like brain, lymphoid organs and polymorphonuclear cells (5). Selective uptake and phosphorylation of nucleoside analogues are key steps to their anti-viral or anti-cancer properties.

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The uptake of nucleosides by intact cells seemed to be coordinated with DNA synthesis (6,7,8,9). All processes, like transport, phosphorylation and incorporation of Thd into DNA were slowed down at 37°C by arabinosyl-cytosine (araC) in human tonsillar lymphocytes (9). It turned out that araC did not inhibit directly either the transport or the phosphorylation of Thd at low temperature, when DNA synthesis was arrested. Similar effect was observed in the presence of aphidicoline in activated Sea Urchin eggs (10).

It is generally accepted that no metabolism occures in cells at zero degree, e.g. ligand binding studies are usually performed at low temperature to avoid the metabolism of the ligand by the cells. However, the transport (11,12) and the conversion of nucleosides to nucleotides (13) even at low temperature have been reported in different mammalian cells.

The aim of the present work was to compare the uptake, phosphorylation and incorporation (into their endproducts: DNA and liponucleotides) of the two deoxypyrimidine nucleosides at low and at physiological temperatures. Both deoxypyrimidine nucleosides were transported and phosphorylated upto dNTPs at 0°C, while no trace amounts of radioactivity were detected in DNA of intact cells. No labeling occurred in phospholipid precursors from deoxycytidine (^{3}H-dCyd) at zero degree, while half of the labeling of the pool was found in this fraction at 37°C in lymphocytes.

MATERIALS AND METHODS

Ehrlich ascites tumor cells were transplanted i.p. into Swiss H-Riop outbred mice with approximately 10^7 tumor cells per animal. Tumor-bearing mice were killed on the 4th day after transplantation, the ascites was removed and diluted approximately 1o-fold with a mixture of Na-citrate (3.3%) and Hank's solution (1:4). Cells washed twice at 0°C were resuspended in M-199 tissue culture medium, at about 10^7cells/ml. The incubation was carried out in the presence of the indicated concentrations of methyl-^{14}C-Thd (Amersham, 1,85 GBq/mmol) at 0°C or at 37°C. The incubation was stopped by 3-fold dilution of the reaction mixture with ice cold M-199 medium containing 2 mM unlabeled Thd. Cells were washed twice in ice-cold culture medium containing 0.5 mM Thd, than precipitated by 2 M perchloric acid (PCA) or by cold ethanol (70%) and kept overnight at -20°C. The precipitate was washed twice with 0,7 M PCA, than with cold ethanol-ether mixture (1:3) and finally with ether. The ether dried powder was dissolved in cc. formic acid. The radioactivity of the acid soluble (pool) and insoluble fractions (DNA) were measured separately in a toluene based liquid scintillation solution (14).

Lymphocytes were prepared from 3-6 years old children tonsils, radioactive labeling of cells and separation of cell fractions were done as described earlier (15-20). Briefly, lymphocytes ($2x10^6$ / ml) were labeled by 5-^{3}H-dCyd (740 GBq / mmol, Amersham), or by 5-methyl-^{3}H-Thd (888 GBq / mmol, Amersham) separately in Eagle's MEM solution as indicated in Figures. After incubation at 37°C or at 0°C cells were centrifuged and washed twice in culture medium, precipitated with 70% cold ethanol (-20°C) and kept overnight at the same temperature. Aliquotes of the ethanol soluble fraction were measured for **total pool** (intracellular radioactivity), DEAE-cellulose bound radioactivity i.e. **nucleotides.**

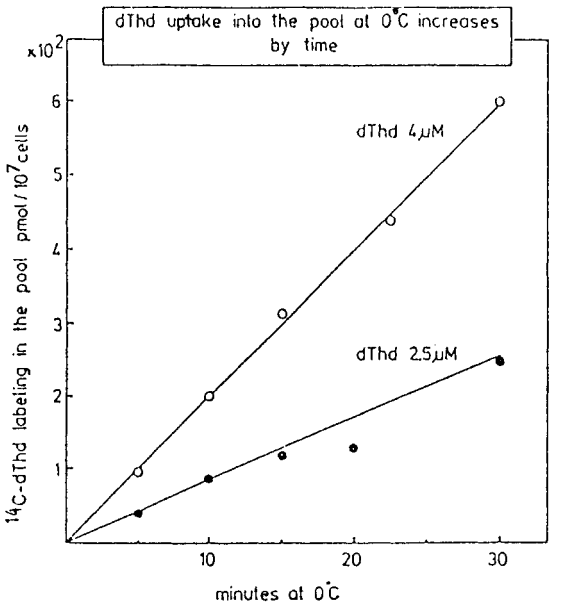


Figure 1.The time curve of Thd uptake into the pool of Ehrlich ascites cells at 0°C.
Cells were labeled by ^{14}C-Thd for indicated times in the presence of Thd. The radioactivity in the acid soluble fraction was calculated from the spec. activity of the extracellular Thd.

Liponucleotides (LN) were calculated as the difference between total and DEAE-cellulose bound radioactivity. LN fraction contained dCDP-choline, dCDP-ethanolamine as identified by HPLC earlier (17, 19), and trace amount of dCDP-diacylglycerol identified on borate impregnated silica TLC (18). Incorporation of radioactivity into DNA was determined in the precipitate hydrolised for 30 min at 90°C in 0.5 N perchloric acid.

Separation of ^{14}C-Thd labeled metabolites were performed both on DEAE-cellulose paper (Watman DE-81) and on PEI-cellulose thin layers (evaluated in 1.0 M LiCl). The ethanol soluble extracts, labeled from 5-^{3}H-dCyd were analysed on Kieselgel 60 F254 silica (Merck) TLC developed in butanol:acidic acid:water (2:1:1 v/v) for 14-15 hrs. Unlabelled nucleotides and nucleosides (Sigma) were used as markers, as indicated on the Figures.

Data were calculated in picomoles in case of Ehrlich ascites cells from the measured dpm and from the spec. radioactivity of the extracellular Thd applied. In case of lymphocytes and in the separation experiments the directly measured cpm values are given.

RESULTS

Thymidine was transported and phosphorylated to dTTP at 0°C but not incorporated into DNA by Ehrlich ascites cells or human lymphocytes.

As it was shown, thymidine and deoxycytidine are metabolised by different pathways in cells (17-19,22-23). While Thd after phosphorylation directly incorporated into DNA, dCyd is

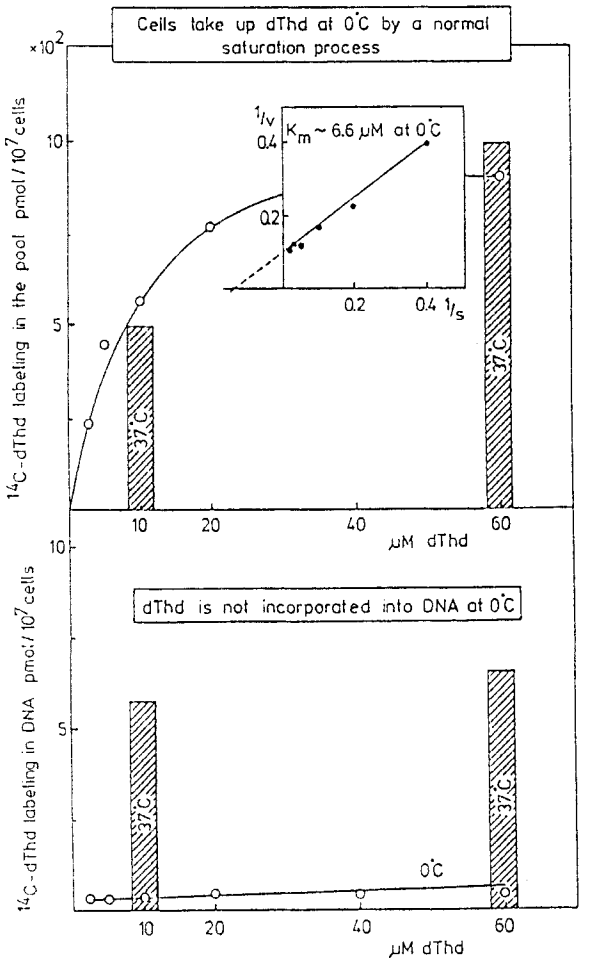


Figure 2. The concertration dependence of Thd uptake into pool and DNA at 0°C and 37°C in Ehrlich ascites cells. Cells were labeled with increasing concentrations of ^{14}C-Thd at 0°C (o-o), reciprocal plot of the saturation curve is presented as insert. The uptake and incorporation at 37° were measured only at two concentrations indicated by bars.

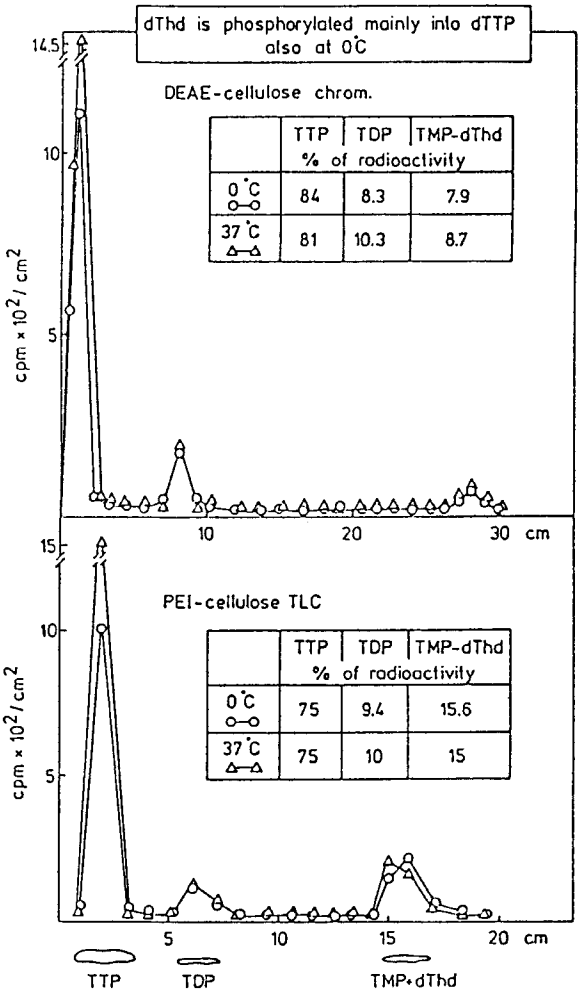


Figure 3.DEAE-cellulose (acid soluble) and PEI-cellulose (ethanol soluble) separation of pools from Ehrlich ascites cells. Cells were labeled for 30 min with ^{14}C-Thd at 0°C (o-o) or at 37°C (Δ-Δ), precipitated with cold PCA or with ethanol. Aliquots were run with standards.

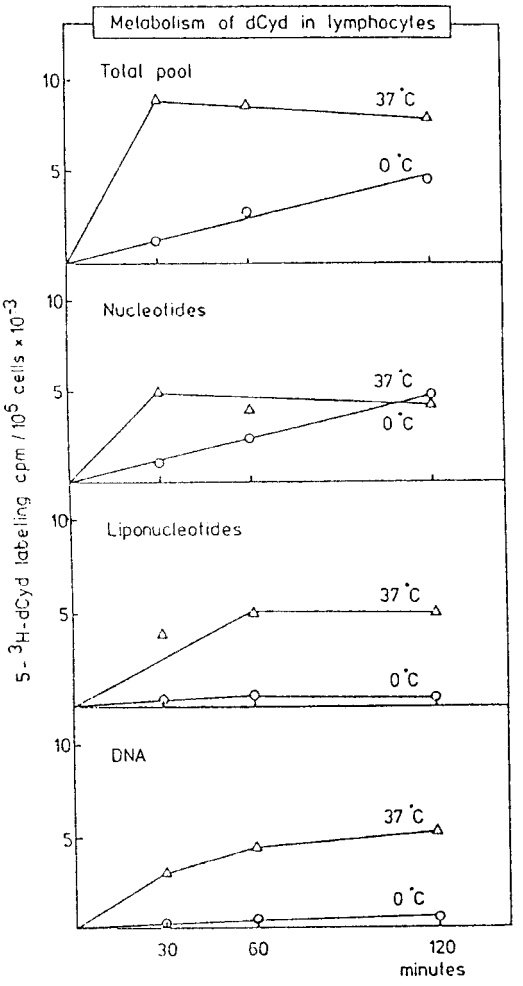
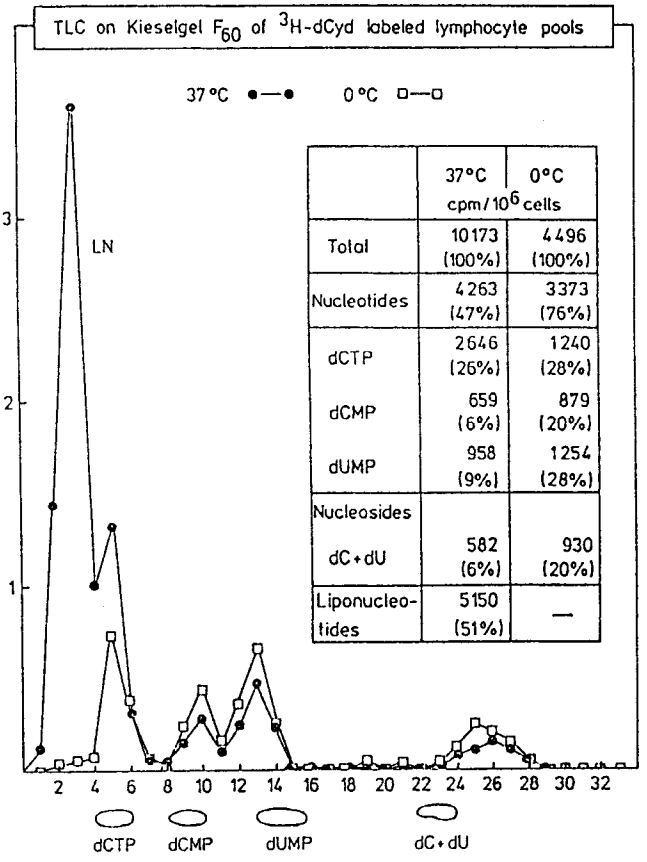


Figure 4.The metabolism of dCyd in different factions of human lymphocytes at 0°C and 37°C. Lymphocytes were labeled with 5-^{3}H-dCyd at the two indicated temperatures for 60 min. as described in Methods. After washing, cells were precipitated by 70% cold ethanol. The radioactivity of the different fractions were measured.



	37°C	0°C
	cpm/10^6 cells	
Total	10173 (100%)	4496 (100%)
Nucleotides	4263 (47%)	3373 (76%)
dCTP	2646 (26%)	1240 (28%)
dCMP	659 (6%)	879 (20%)
dUMP	958 (9%)	1254 (28%)
Nucleosides		
dC+dU	582 (6%)	930 (20%)
Liponucleotides	5150 (51%)	—

Figure 5. TLC separation on silica plates of the ethanol soluble fraction of the 5-^{3}H-dCyd labeled lymphocytes. Lymphocytes were labeled for 60 min as described in Methods. Aliquotes of the ethanol soluble fraction was separated on TLC. The distribution of radioactivity in the metabolites can be seen in the insert, the place of standards are indicated.

Table 1. Metabolism of ^{3}H-dThd in lymphocytes

	37 °C cpm/10^6 cell/hr	O °C cpm/10^6 cell/hr
Total uptake	3500	3520
Nucleotides	3450	3460
DNA	10150	95

The metabolism of Thd in lymphocytes at 0°C and 37°C.
Lymphocytes were labeled for 60 min with ^{3}H-Thd as described and the radioactivity was measured in the ethanol soluble (Total uptake, Nucleotides) and insoluble (DNA) fractions.

salvaged not only into DNA but also into phospholipid precursors (16-19,23). Ehrlich ascites tumor cells were incubated in the presence of ^{14}C-Thd and the labeling of the acid soluble pool was measured as described in Methods. As shown in Fig.1., the uptake of the nucleoside was linear upto 30 min and was proportional to the concentration of Thd at 0°C. The saturation curve of Thd followed Michaelis-Menten kinetics with an apparent Km for Thd of 6,6 uM (Fig.2. insert). A similar saturation curve was obtained at 37°C, with the same Km value (data not shown). There was no difference in the labeling of the pool at low and higher temperature, while there was a pronounced difference in the radioactivity of the acid insoluble fraction (DNA). No radioactivity could be detected in the DNA at zero degree, in contrast to the high incorporation detected in the same fraction at 37°C (Fig.2).

The influence of the temperature on the phosphorylation and metabolism on Thd was also tested in other cells . Human tonsillar lymphocytes were isolated from palatinal tonsils of children. The cells contain in vivo activated lymphocytes incorporating thymidine, have elevated level of DNA polymerase alfa and thymidine kinase-1 (TK1) (9,16). ^{3}H-Thd was also phosphorylated at 0°C in tonsillar lymphocytes, and no incorporation was detected in DNA at low temperature, similarly to ascites tumor cells (Table I.).

The labeling of intracellular nucleotides in ascites tumor cells was analysed on DEAE-cellulose paper and on PEI-cellulose sheets. There was no difference in the distribution of the radioactivity in the Thd nucleotides in cells incubated at 0°C or at 37°C (Fig.3). Most of the radioactivity appeared in the dTTP peak (84% and 75 %) separated either on DEAE-cellulose or on PEI-cellulose, respectively. Similar distribution of the dThd nucleotides were obtained also in lymphocytes (data not presented).

At low temperature dCyd was phosphorylated to nucleotides but was not incorporated into DNA and membrane phospholipids by lymphocytes.

The salvage of dCyd is more pronounced in lymphoid cells than in others. As it has been shown in tonsillar lymphocytes, the main part of dCyd (55-7o%) salvaged by the cells is deaminated by

dCMP-deaminase to dUMP and consequently methylated by thymidylate synthase to dTMP and incorporated finally as dThd nucleotide into DNA. The smaller part of dCyd after phosphorylation to dCTP goes directly into DNA (14-15%) and about the same fraction participates in phospholipid biosynthesis (16-19). Similar pathways and two different dCTP pools were found also in other cells (22, 23).

The labeling of the pool and nucleotides at zero degree was lower than at 37°C, and it already reached its equilibrium after 3o min incubation or even sooner. At zero degree the lymphocytes need four times longer incubation with labeled dCyd to reach the same radioactivity as at 37°C (Fig.4). Half of labeling in the pool was identified as nucleotides of dCyd , and the other half of the radioactivity in the pool were shown to be liponucleotides (dCDP-choline, dCDP-ethanolamine, dCDP-diacylglycerol, 16-20), at physiological temperature.

It was surprising that at low temperature not only the incorporation of dCyd into DNA was abolished, like in case of dThd, but no labeling was detected in the phospholipid precursor fraction (Fig. 4 liponucleotides). An aliquot of the 3H-dCyd labeled ethanol soluble fraction was analysed on silica gel TLC, as described in Methods (Fig.5). The liponucleotide peak (LN) contained 51% of the total radioactivity of the pool at 37°C and nothing at O°C. The labeling of the dCyd-nucleotides (Nucleotides) represented 47% of the total radioactivity at 37°C and 76% at 0°C measured in the ethanol soluble fraction. It is interesting that the labeling of dUMP is significantly higher at 0°C (28%) than at 37°C (9%).

Cell free extracts of both cells were tested for thymidine (TK) and deoxycytidine kinase (dCK) enzyme activities at the two different temperatures. At zero degree no dCK activity could be detected during 2 hr incubation, while at 37°C the same activity was measured as published earlier (21). TK catalysed the phosphorylation of dThd also at 0°C but about 4-5 times slower than at 37°C, in both cells, which is in good agreement with results in CHO cells (24). TS activity in cell free extracts was also measured at both temperatures, no activity could be detected at 0°C (data not presented).

DISCUSSION

It is widely accepted that chemical and even more enzyme-catalysed reactions including the metabolism is negligable at low temperatures in the warm-blooded organisms. Binding kinetics of ligands to their specific receptors on cells are usually measured at low temperatures to avoid metabolism. In our earlier experiments it was shown, that at low temperature Thd was phosphorylated but not incorporated into DNA (9), and phosphorylation was not inhibited by araC in human tonsillar lymphocytes. At the same time, Schaer and Maurer have investigated the cell cycle dependence of Thd metabolism in CHO cells (24) and they found also phosphorylation at zero degree, but no incorporation into DNA. An active transport at low temperature of dCyd and araC (11) and adenosine (12) were also found. Recently, the nucleoside transport of an anaerobic protozoan cell, which lack de novo synthesis of all nucleotides, was investigated, and no phosphorylation of dCyd was observed at 0°C (25).

In this paper the salvage of dThd and dCyd were compared at 0°C and at 37°C in Ehrlich

ascites tumor cells and in human tonsillar lymphocytes. The phosphorylation of Thd at 0°C followed regular substrate saturation kinetics (Fig.1, 2) . The apparent Km values were similar at both temperatures suggesting that the transport and phosphorylation must be tightly coupled molecular events, and can not be easily separated from each other as also observed in E.coli (3,25,26). TLC analysis of the intracellular pools have revealed that 75-85% of the radioactivity after ^{14}C-Thd labeling appeared as dTTP both at zero and at 37°C. No incorporation was found in DNA at 0°C (Fig.3), while most of labeling was found in DNA at 37°C. Similar distribution of the radioactivity was found after ^{3}H-Thd labeling in human lymphnode lymphocytes (Table I, 9).

The rate of DNA synthesis in cells is usually characterised by the incorporation of Thd and considered as S phase cells. Schaer and Maurer (24) have found, that G_1 phase CHO cells were also labeled at 0°C, but almost all the radioactivity of the nucleotide fraction was released into the medium after increasing the temperature to 37°C, whereas in S phase cells nearly all of the labeled nucleotide fraction was incorporated into DNA. The phosphorylation and dephosphorylation of nucleotides should keep the balance between extracellular and intracellular environment of the cells, regulated by "substrate cycles" (27).

An other interesting correlation between DNA synthesis and membrane phospholipid synthesis was shown in lymphocytes following the metabolism of dCyd. As shown in Fig.4 and 5, the ^{3}H-dCyd was phosphorylated at 0°C like dThd, and no radioactivity was detected in DNA either from Thd or from dCyd. Even more, the metabolism of dCyd into the membrane phospholipids (LN) was also abolished at 0°C. The liponucleotide fraction (Fig.5. LN) contained dCDP-choline, dCDP-ethanolamine and trace amounts of dCDP-diacylglycerol identified earlier by HPLC (16-20), and borate impregnated silica plates (28). The synthesis of membranes seems to bo coupled to the synthesis of DNA not only in microorganisms (29) but also in eukaryotic cells, where the inhibition of phospholipid synthesis by chloropromasine rapidly decreased also the synthesis of DNA as shown in our earlier experiments (18). Transport and phosphorylation of nucleosides at low temperature need intact cells as shown in different laboratories, while cell free extracts had very low (TK) or even no nucleoside kinase activities (dCK) at low temperature. The explantion of the cold-transport and -phosphorylation of nucleosides could be as followes: i. The mobility of loaded nucleoside carrier protein might be increased by decreasing the temperature as shown in erythrocytes (30), which allowes the transport and the tightly coordinated phosphorylation (31). ii. The cold-transport-phosphorylation of nucleosides might be a cosequence of a non-carrier mediated permeation as shown at normal temperature also for adenine (32). Further steps in the metabolism: dCyd-Thd interconversion, incorporation into DNA and into phospholipids are under coordinated control with nucleoside transport-phosphorylation at physiological temperature, which control is disrupted at low temperature. Further experiments have to declaire the primary effects of the low temperature: is it the condition of the membrane or the dissotiation of metabolic complexes in the cells.

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