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CARNITINE INVOLVEMENT IN FUEL UTILIZATION
BY HUMAN PERIPHERAL BLOOD
MONONUCLEAR CELLS

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# CARNITINE INVOLVEMENT IN FUEL UTILIZATION BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

By

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ABSTRACT

CARNITINE INVOLVEMENT IN FUEL UTILIZATION BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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Carnitine is an essential cofactor in fatty acid oxidation and has proposed facilitative roles in metabolism of other fuels. Carnitine content and percentage of carnitine acylated were quantitated in lymphocytes and mononuclear phagocytic cells (MNP) (79 and 301 nmol total carnitine/$10^9$ cells with 29% and 69% acylation, respectively). Differences in carnitine status between these two cell types were postulated to reflect their metabolic and functional differences. The presence of acetyl-, propionyl- and long-chain acylcarnitines indicated carnitine's possible involvement in metabolism of carbohydrates, branched-chain amino acids and long chain fatty acids. Mitogenic stimulation of lymphocytes induced resting lymphocytes to proliferate and led to increased levels of carnitine per mg cellular DNA (from 10.6 to 22.5 nmol carnitine/mg DNA) and acylation of carnitine (from 16% to 38%). Thus, carnitine's involvement in fuel utilization was altered during lymphocyte activation suggesting that carnitine may be required to maintain an immune response.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | bovine serum albumin |
| CAT | carnitine acetyltransferase |
| CoA | Coenzyme A |
| Con A | Concanavalin A |
| CPT | carnitine palmitoyltransferase |
| DMEM | Dulbecco's Modified Eagle Medium |
| DTT | dithiothreitol |
| EDTA | ethylene-dinitrilo-tetraacetic acid |
| FBS | fetal bovine serum |
| HPLC | high performance liquid chromatography |
| $KP_i$ | potassium phosphate |
| LCAC | long-chain acylcarnitines |
| MNP | mononuclear phagocytic cells |
| NEM | N-ethylmaleimide |
| PBS | phosphate-buffered saline |
| PCA | perchloric acid |
| PHA | phytohemagglutinin |
| PMN | polymorphonuclear cells |
| SCAC | short-chain acylcarnitines |
| SD | standard deviation |
| SEM | standard error of the mean |

# INTRODUCTION

It is well established that the nutritional status of a mammalian organism can influence the ability of its immune system to combat infection. Diets deficient in protein (1), essential fatty acids (2), trace elements (3), specific amino acids (4) or vitamins (5) have resulted in impairment of immune function. Thus, it comes as no surprise that in many of the world's underdeveloped countries where nutrition is poor, diseases such as measles, small pox and viral infections are major causes of death (6). In spite of evidence which suggests that host defense is dependent upon available nutrients, few attempts have been made to carefully characterize the intermediary metabolism and metabolic requirements of cells comprising the immune system.

During the late 1950's through the 1960's reports began to appear in the literature with initial characterizations of fuel utilization in circulating leukocytes. At this time workers in the field of immunology knew little about the diversity of cells participating in the immune response and the technology required to isolate and characterize individual cell types had yet to be developed. Thus, studies were routinely performed on heterogeneous populations of white

blood cells given the general term leukocytes. Our present knowledge of cell biology of the immune system indicates that leukocyte populations are composed of many different cell types (macrophages, monocytes, polymorphonuclear cells and lymphocytes) which differ significantly in both their morphology and function. Further, within a specific cell subclass there may exist different stages of maturity and differentiation which effect function. Therefore, it is important to remember the limitations confronting the investigators of the 50's and 60's when interpreting their findings. Nevertheless, it was demonstrated that leukocytes contained small amounts of glycogen and were equipped with the metabolic machinery for both aerobic and anaerobic pathways (7, 8). It was also shown that the hexose monophosphate shunt represented less than 1% of metabolized carbohydrate (9) in white blood cells which had been depleted of phagocytic cells by adherence to glass beads. The ability of leukocytes to oxidize long-chain fatty acids was not investigated leading to an undocumented but widely held belief that glucose serves as the major metabolic fuel for white blood cells.

Advancements in the field of immunology and the technology of cell isolation were not accompanied by more careful metabolic characterizations of the individual cellular components of the immune system. Instead research efforts focused on the molecular aspects of the immune response

leaving what one investigator termed a "metabolic void" in the biochemistry of leukocytes (10). Recent research efforts in this field included the work of only a few investigative groups. The work of Suter and Weidemann challenged the belief that glucose catabolism is the major process for ATP production in lymphocytes. In rat spleen slices, glucose oxidation accounted for only 25% of $O_2$ consumed with 70% of glucose utilized being converted to lactate. This suggested that even at near saturating levels of glucose, endogenous triacylglycerols could contribute significantly as oxidative fuel (as much as 70%) (11). Ardawi and Newsholme reported a relatively lower rate of oleate utilization by resting lymphocytes in culture which accounted for less than 30% of oxygen consumed (12). This rate did not change when cells were isolated from starved rats. Only 1% of the oleate utilized was converted from [1-$^{14}$C]-oleate to $^{14}CO_2$. This low rate of fat utilization was probably not representative of fatty acid oxidation in these cells due to intracellular dilution of the radiolabelled fatty acid. About 50% of oleate utilized was recovered in an esterified form. Increasing the concentration of oleate from 0.5 mM to 1.5 mM did not increase the rate of fatty acid uptake or the rate of $O_2$ consumption. This contrasted with skeletal muscle (13), heart (14, 15) and kidney (16) where such an increase in fatty acid concentration was accompanied by an increase in ß-oxidation. In addition, muscle did not uptake fatty

acids below a concentration of 0.35 mM (13). Thus, the uptake and utilization of fats observed in lymphocytes at levels of around 0.5 mM indicated that they may oxidize fatty acids even in the fed state, since circulating levels of fatty acids are approximately 0.5 mM in the fed state and approach 2.0 mM during starvation (17).

Ardawi and Newsholme have also investigated the ability of lymphocytes to utilize other alternative fuel sources. In 1983 they reported that incubated lymphocytes isolated from rat mesenteric lymph nodes utilized glutamine at a rate of 2.7 μmol/min-g dry wt (18). Glutamine was found to be only partially oxidized to glutamate, aspartate and lactate, however, based upon formation of $^{14}CO_2$ from [$^{14}$C]-glutamine, this oxidation contributed up to 30% of $O_2$ consumed. In a later paper they characterized ketone body oxidation and found that incubated lymphocytes utilized acetoacetate and 3-hydroxybutyrate at an approximate rate of 0.5 μmol/min-g dry wt (12). They estimated that ketone bodies could account for up to 30% of $O_2$ consumption and may serve as an important fuel source.

Upon encounter with an appropriate immune stimulus, resting lymphocytes will replicate their DNA, differentiate and undergo cell division to become the effector cells involved in both cellular and humoral immune reactions. This process is termed lymphocyte activation, stimulation or blastogenesis. Although the metabolic pathways important in

this complex process have not been well characterized, it is known that increases in RNA, protein and DNA synthesis are dependent upon glycolysis (19). The effects of mitogenic stimulation on fuel utilization of lymphocytes were considered by Ardawi and Newsholme. Cells stimulated with concanavalin A (Con A) for 1 h demonstrated an increase in the rates of both glucose and glutamine utilization, while utilization of long-chain fatty acids and ketone bodies remained virtually unaltered (10). It is important to note that in these experiments cells were stimulated for only 1 h with mitogen, a time period which is inadequate to achieve actual stimulation of lymphocytes. Rates of protein and RNA synthesis are not increased until 2-4 h after stimulation by a mitogen and do not reach maximum rates until about 40 h, while DNA synthesis does not appear until 20 h, reaching its maximum rate at 50-69 h (20). Thus, the stimulation studies of Ardawi and Newsholme were probably not representative of transformed lymphocytes. While the work of Ardawi and Newsholme contributed much to our understanding of lymphocyte intermediary metabolism, it did not adequately describe potential changes in utilization during the immune response.

An important nutrient shown to be involved in fuel utilization of many tissues is L-carnitine (gamma-trimethyl-amino-ß-hydroxybutyrate). It is widely accepted that carnitine plays an important role in ß-oxidation of long-chain fatty acids by facilitating the transfer of activated

fatty acids across the acylCoA barrier imposed by the inner mitochondrial membrane (21, 22). With the discovery of three carnitine acyltransferases which differ in their acyl-chain length specificities and which can be found in several organelle locations, it became evident that carnitine had more than one role in intermediary metabolism (23). Recent findings demonstrated that carnitine plays a role in the metabolism of carbohydrates and short- and medium-chain fatty acids, although this has not been completely characterized (23). A summary of the roles of carnitine, additional to its primary role in ß-oxidation, was presented in a recent review (24) and are outlined here:

1. Elimination of selective acyl residues. Accumulation of nonmetabolized acylCoAs within the mitochondrial matrix is prevented by their conversion to carnitine esters which are then transported to the liver for catabolism or are eliminated in the urine (25). This detoxification mechanism of carnitine could be important in patients suffering from organic acidemias. For example, propionylcarnitine has been found to be excreted in the urine of patients diagnosed with propionic acidemia. This helps eliminate build up of toxic concentrations of propionylCoA within the mitochondria (26).

2. Reservoir of readily accessible activated acetyl groups in some insect flight muscle (27) and possibly mammalian sperm and heart (24). In the flight muscle of the blowfly, Phormia regina, initiation of flight leads to

generation of more pyruvate than can be utilized by the Krebs cycle (28, 27). To prevent accumulation of acetylCoA, which would inhibit pyruvate dehydrogenase, these activated acetyl groups are transferred to carnitine by the carnitine acetyltransferase. Reversal of this reaction quickly liberates acetylCoA which is then available for oxidation via the Krebs cycle.

3. Modulation of CoA/acylCoA ratio within the matrix of the mitochondria. The carnitine acetyltransferase (CAT) in the mammalian mitochondria appears to only be present on the matrix side of the inner membrane (22). Also, short-chain fatty acids ($C_2$-$C_{10}$) are activated in the mitochondrial matrix (29), so their oxidation does not require carnitine for transport of activated acylCoAs across the inner mitochondrial membrane. Instead carnitine's role in short-chain fatty acid oxidation appears to be facilitative rather than obligatory. Pearson and Tubbs suggested that carnitine is acting as a buffer of matrix short-chain acylCoA levels (30). The presence of carnitine in the matrix can lead to the regeneration of reduced CoA, which is required for oxidative processes, by conversion of acylCoAs to acylcarnitines. In heart and liver mitochondria, carnitine has been shown to increase pyruvate utilization accompanied by an increase in the production of acetylcarnitine. In contrast, relatively small amounts of acetylcarnitine were produced when octanoate was the oxidized substrate. These

data suggested that the role of carnitine in replenishing reduced CoA may be more important for oxidation of carbohydrate metabolites than of long-chain fatty acids (31). This was in direct opposition to the existing theory that acetylcarnitine is formed when excess fatty acids are oxidized (32).

4. Involvement in branched-chain keto acid metabolism. Free carnitine rapidly interacts with matrix short-chain acylCoAs derived from alpha keto acids of branched-chain amino acids (33). However, a role for carnitine in the metabolism of these amino acids remains to be elucidated.

5. Translocation of acetyl units into mitochondria of yeast for biosynthetic purposes (34). When the growth medium for the yeast Torulopsis bovina was supplemented with [$^{14}$C]acetyl-L-carnitine most of the radioactivity was incorporated into cell protein. Amino acid analysis of these radiolabelled proteins revealed that the label was incorporated into glutamate, arginine, proline, leucine and lysine. It was demonstrated that this was not the result of hydrolysis of the acetylcarnitine to acetate, since radiolabelled acetate labelled only leucine and lysine. Isolated mitochondria were shown to incorporate the [$^{14}$C]acetyl derived from acetylcarnitine into citrate and 2-oxoglutarate. Thus, carnitine was shown to transfer acetyl units into the mitochondria of yeast where they were used for synthesis of citrate and its metabolites.

6. A possible but not yet established role in shuttling of chain-shortened fatty acids out of peroxisomes. Liver peroxisomes have the ability to oxidize fatty acids (35). Carnitine may play a role in shuttling chain-shortened fatty acids out of peroxisomes. Their fate once outside the peroxisome is only speculative at this point, but perhaps these acylcarnitines are transported into the mitochondria to be further metabolized (23, 36).

Carnitine acyltransferases are the enzymes which catalyze the reversible reaction:

acylcarnitine + CoA <---> acylCoA + carnitine.

In mammals the number of transferases, their individual kinetic constants and subcellular organelle locations vary from tissue to tissue. For example, heart has been shown to contain two carnitine acyltransferases (37, 38, 39) while liver contains at least three (40, 41, 42, 43). Carnitine acetyltransferase (CAT) is the major acyltransferase in most tissues and catalyzes the reversible formation of short-chain acylcarnitines. In rat and pig liver CAT was present in mitochondria, peroxisomes and microsomes (40). Its function within these various organelles is still poorly understood.

Carnitine palmitoyltransferase (CPT) is required for tissue oxidation of long-chain fatty acids and is exclusively a mitochondrial enzyme (40). It appears to be a multisubunit enzyme exhibiting complex sigmoid kinetics which vary with

the assay system used. Control of CPT activity is highly controversial but the discovery that malonylCoA inhibits activity suggested that it is allosterically regulated (44, 45). CPT activity can change with an organism's physiological state, as demonstrated with diabetes, fasting or consumption of a high fat diet (46). Knowledge concerning the activities of specific transferases within a tissue, their organelle locations and changes in enzyme levels in response to different physiological states aids in elucidating the function(s) of carnitine within a specific tissue.

Although the levels and roles of carnitine and carnitine acyltransferases have been extensively studied in liver, heart and skeletal muscle, their importance in other tissues remains largely unknown. Recently, Katrib and coworkers measured levels of carnitine in leukocytes and found human polymorphonuclear (PMN) and mononuclear cells to contain high millimolar amounts of carnitine relative to other tissues, approximately six-fold that found in muscle (47). However, a reappraisal of that work demonstrated that the assay conditions employed led to several-fold overestimations in carnitine concentrations (48). Subsequent studies by the Katrib group showed increased levels of total carnitine and its degree of acylation in leukocytes isolated from patients with inflammatory disorders, when compared to healthy subjects (49). Although their assay system has been questioned, the increased levels in carnitine content and percentage of

carnitine acylated were probably real. This provides an in vivo example of an increased carnitine requirement for human leukocytes following inflammation.

As stated earlier, white blood cells do not constitute a homogenous tissue. Of these various cell types the mononuclear cell fraction will be the focus of this thesis investigation (Figure 1). Mononuclear cells constitute approximately 20% of circulating leukocytes and can be further subdivided into two cell types, lymphocytes and mononuclear phagocytic cells (MNP). Previous carnitine determinations (47, 48) did not separate these two cell types from one another prior to analysis in spite of the fact that they differ greatly in their cellular morphology and function. Lymphocytes are responsible for the adaptive immune response. Each lymphocyte carries a surface receptor specific for a particular antigen. Upon encounter with that antigen, a small population of lymphocytes will respond by clonal expansion and differentiation. There are two main types of lymphocytes, B cells which develop in the bone marrow or fetal liver and T cells which differentiate in the thymus. The primary responsibility of B cells is antibody production, while T cells are involved in regulation of the immune response through helper and suppressor functions and lymphokine production. In contrast to lymphocytes, MNP function quite differently. They engulf and digest particulate antigen and also play a role in presentation

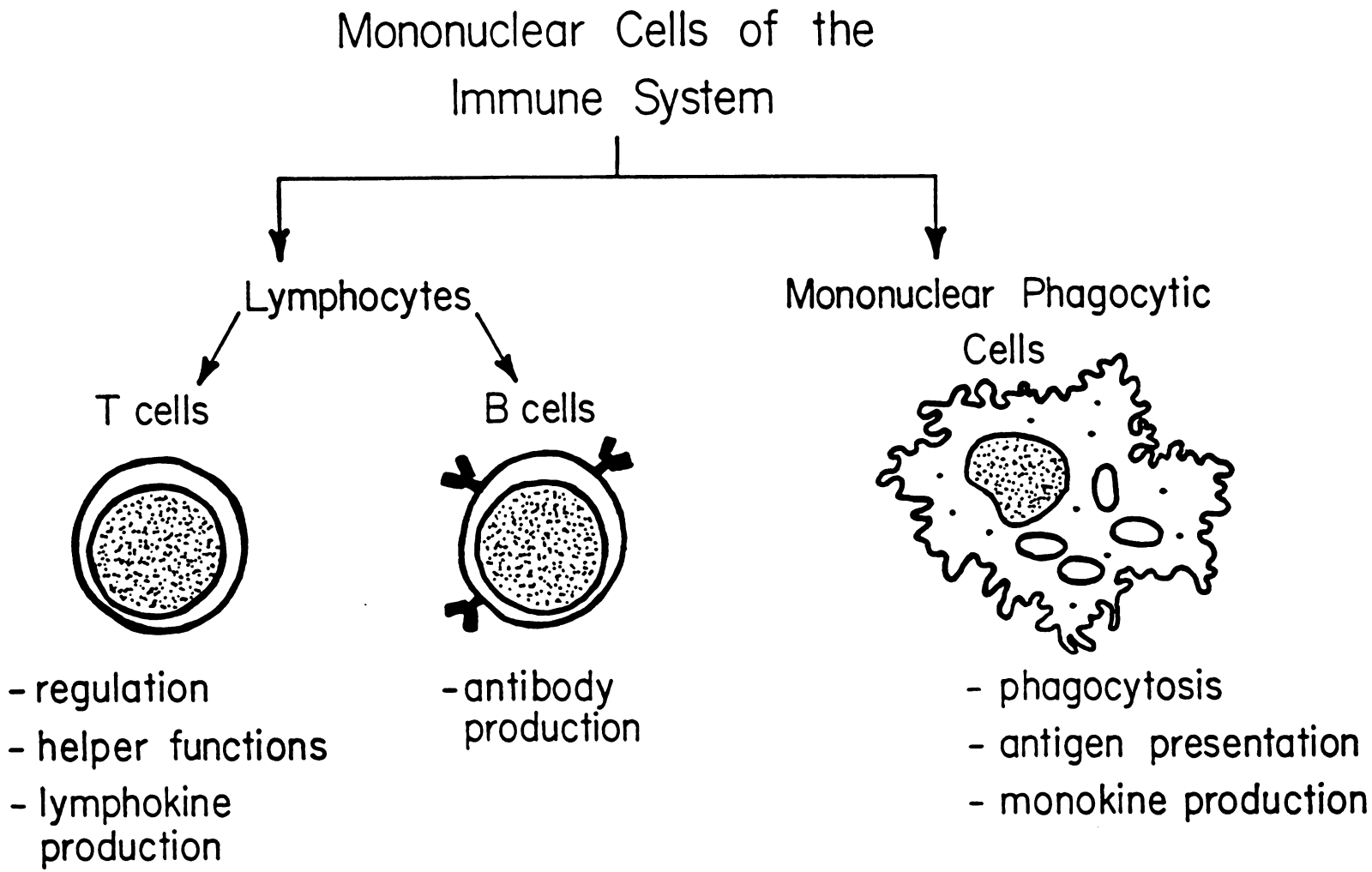


Figure 1. Cell types comprising the mononuclear cell fraction and their functions.

of antigen to lymphocytes and production of essential monokines. The blood MNP leaves the circulation after a few days and enters the tissue to become a macrophage.

The primary objective of this thesis was to help further define the metabolic requirements for lymphocytes and MNP by determination of the involvement of carnitine in their cellular metabolism. Lymphocytes and MNP were isolated from human peripheral blood of normal young adults and their levels of carnitine and its esters were quantitated. High performance liquid chromatography (HPLC) was used to characterize the individual acylcarnitines present. These data enabled us to postulate potential roles for carnitine within these cells. In addition, identification of the specific acylcarnitines indicated which acylCoAs in the cell were in equilibrium with intracellular carnitine pools giving some insight into possible fuels being utilized. Once carnitine profiles were established for resting mononuclear cells, they were examined in lymphocytes which had been mitogenically stimulated <u>in vitro</u> to simulate the metabolic state of activated lymphocytes during an immune response. The activities of the long- and short-chain acyltransferases in stimulated lymphocytes were compared to transferase levels in unstimulated cells maintained in culture under the same conditions. This provided additional insight into potential metabolic changes occurring in lymphocytes as they were transformed into effector cells and helped elucidate how

these enzymes may be involved in regulation of fuel utilization.

# CHAPTER 1

## CARNITINE AND ACYLCARNITINE LEVELS OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND MONONUCLEAR PHAGOCYTES


### ABSTRACT

Lymphocytes and mononuclear phagocytic cells are essential to host defense, yet little is known about their metabolic requirements. To determine the involvement of carnitine in the intermediary metabolism of these cells, the amounts of free carnitine and individual acylcarnitines were quantitated for human peripheral blood lymphocytes and mononuclear phagocytic cells. Lymphocytes from healthy young adults contained 79 and 56 nmol/$10^9$ cells total and free carnitine, respectively, showing 29% acylation. By comparison, mononuclear phagocytes contained approximately four-fold more total carnitine per cell (301 nmol/$10^9$ cells) and had a much higher level of acylation (69%). Acetylcarnitine was the predominant acylcarnitine in both lymphocytes (15.4 nmol/$10^9$ cells) and mononuclear phagocytes (98.3 nmol/$10^9$ cells) accounting for 72-73% of total acylcarnitines. The presence of propionyl- and (iso)butyryl-

carnitine in both cell types indicated they were oxidizing some branched-chain amino acids. Long-chain acylcarnitines constituted 3-4% of total acylcarnitines for both cell types. These data suggested that carnitine was involved in the metabolism of both short and long-chain acylCoAs within lymphocytes and mononuclear phagocytes, but its specific roles in these two cell types have not been determined (50). The addition of 5 mM glucose to the isolation medium resulted in an increase in the levels of acetylcarnitine in lymphocytes in 3 of the 6 donors tested suggesting that glucose may contribute to the acetylCoA pool in equilibrium with carnitine.

# INTRODUCTION

L-carnitine is required for efficient energy utilization of long-chain fatty acids (21, 22) and also participates in short- and medium-chain acylCoA metabolism (23). When cellular carnitine concentrations become limiting, energy metabolism is seriously impaired (51). While the involvement of carnitine in cellular metabolism has been extensively studied in tissues such as liver, heart and skeletal muscle (22), its functioning in cells of the immune system is unknown. The focus of this thesis chapter is the characterization of carnitine content in the lymphocytes and mononuclear phagocytic cells (MNP) which comprise the mononuclear cell fraction of blood and are integral to all antibody and cell-mediated responses.

There are many reasons to suspect that the immune system is a major user of nutrients and fuel molecules and thus should require carnitine for efficient metabolism. The immune system constitutes a sizable portion of the human body if one considers the bone marrow, primary and secondary lymphoid organs and peripheral blood (52). Its cells also have a high turnover rate, surviving only a few days before dying (53). In addition, stimulation of resting lymphocytes by a foreign antigen signals these cells to undergo rapid proliferation, maturation and production of mediators involved in the immune response. Mononuclear phagocytes are also

capable of acquiring a more activated metabolic state upon immunological challenge. Phagocytosis by human monocytes is accompanied by enhanced glucose oxidation, an increase in oxygen consumption, stimulation of the hexose monophosphate shunt, and an increase in production of hydrogen peroxide, superoxide anion and singlet oxygen (54). Thus, tissues involved in the immune response appear to be very metabolically active and offer diverse cell types in which to investigate carnitine metabolism.

In the present chapter human peripheral blood lymphocytes and MNP in the resting state, i.e. not immunologically activated, were isolated to determine whether carnitine and its esters were present. Care was taken to separate MNP from lymphocytes since the two are morphologically and functionally distinct. This is the first time the individual acylcarnitine species present within leukocytes have been characterized. The effect of glucose supplementation during cell isolation on mononuclear cell carnitine profiles was also assessed. These studies provide a first step towards understanding the involvement of carnitine in fuel utilization of cells of the immune system.

# MATERIALS AND METHODS

## Human Blood Collection

Approval for the use of human blood samples was obtained from the University Committee on Research Involving Human Subjects at Michigan State University. All donors were required to sign an informed consent form which outlined the purpose of the study and alerted them to possible risks associated with blood donation. Donor age ranged from 21 to 40 years with approximately equal numbers of males and females serving as volunteers. Blood samples were not collected from individuals who were ill or taking any type of medication. Venous blood (20 - 50 ml) was drawn by a registered nurse into Vacutainers (Becton Dickinson, Rutherford, NJ) coated with ethylenedinitrilo-tetraacetic acid. Blood was processed immediately.

## Lymphocyte and Mononuclear Phagocytic Cell Isolation

Anti-coagulated whole blood was mixed with an equal volume of a commercially available separation medium, Sepracell-MN (Sepratech Corp., Oklahoma City, OK). This mixture was centrifuged in a swinging bucket rotor (1500 x g, 20 min, room temperature) and yielded a continuous density gradient with a compact band of mononuclear cells just below the meniscus (Figure 1). This band contained both lymphocytes and MNP. Red blood cells and PMN pelleted at the bottom of the gradient. To separate lymphocytes from MNP, the band

# Isolation of Mononuclear Cells

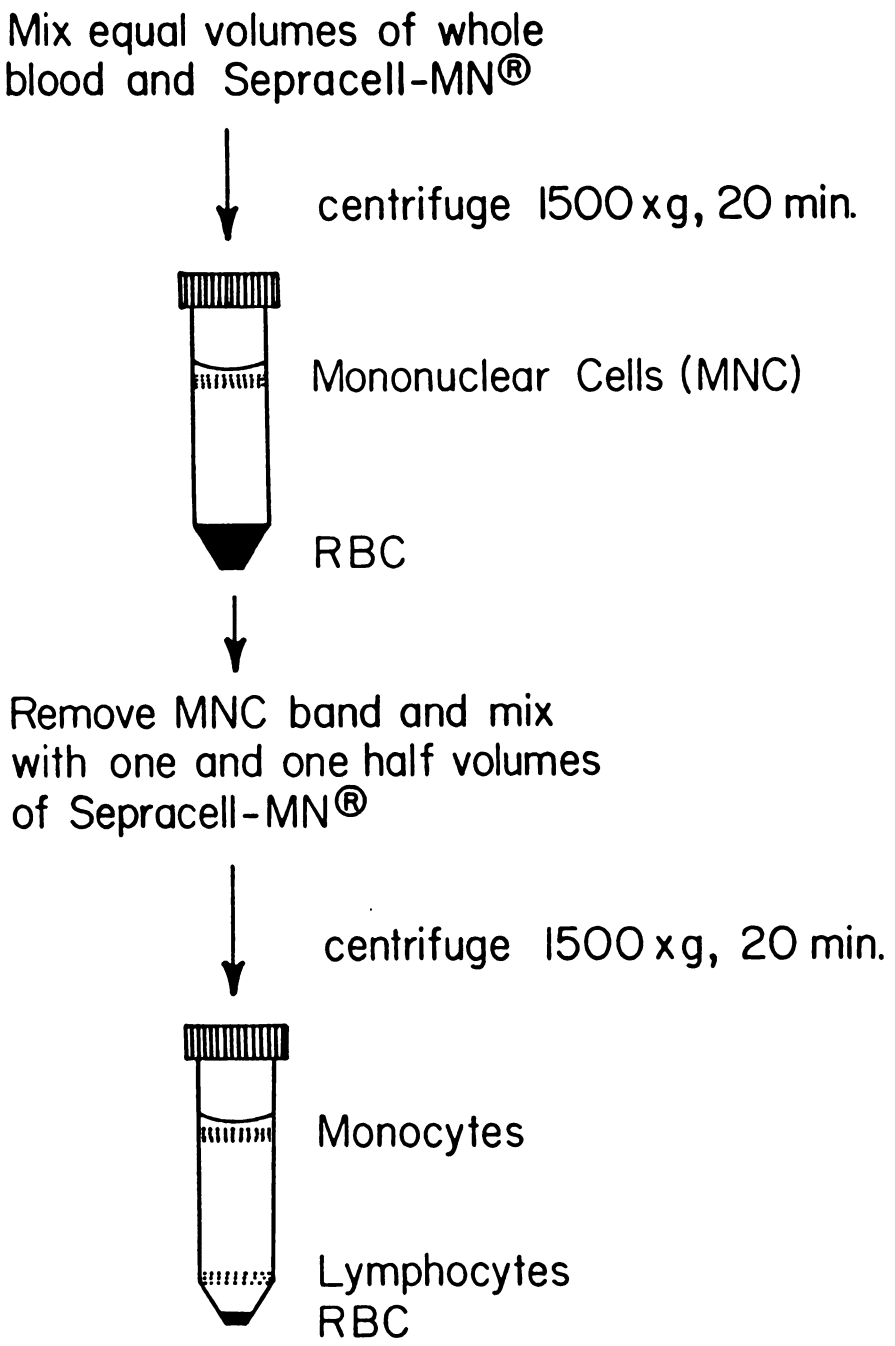


Figure 1. Isolation procedure for lymphocytes and mononuclear phagocytes.

containing mononuclear cells was collected and washed with phosphate-buffered saline (PBS-BSA) containing 0.1% (w/v) fatty acid-free bovine serum albumin, fraction V, (Calbiochem, LaJolla, CA), then mixed with one and one-half volumes of Sepracell-MN and centrifuged as before. Separate bands containing MNP and lymphocytes were removed and washed twice with PBS-BSA (final centrifugation at 50 x g to remove platelets). In one series of experiments the wash medium was supplemented with 5 mM glucose (Mallinckrodt, Paris, KY) to see if the addition of this exogenous fuel source altered acylcarnitine profiles of mononuclear cells.

Purity of the two cell populations was evaluated by microscopic examination of cell morphology. In addition, the presence or absence of MNP in each population was determined using the nonspecific esterase histochemical stain (55). Cell viabilities were determined by trypan blue exclusion (56). For carnitine analyses cells were suspended in 50 mM potassium phosphate buffer, pH 7.4, to a cell density of 1.0 x $10^7$ cells/ml and lysed by a 15 sec sonication at 0-4° C. Cell lysates could be stored frozen at -70° C. Protein concentrations of cell lysates were determined by a modified Lowry protein assay using BSA as standard (57).

Carnitine Analysis

Free carnitine (nonesterified) was quantitated by the radiochemical method (58) using modifications of Kerner and Bieber (59, 60). Perchloric acid (PCA) was added at a final

concentration of 2.1% to 100 µl aliquots of cell lysate. This was incubated on ice for 10 min and then centrifuged at 10,000 x g for 5 min, 4° C. The supernatant was removed and saved. The pellet was washed with 50 mM potassium phosphate buffer, pH 7.4, to remove any trapped carnitine. The PCA extract and wash supernatant were combined and the pellet discarded. This PCA extract was neutralized by 5 N KOH, incubated on ice for 1 h and then centrifuged and washed as before.

Neutralized perchloric acid extracts were incubated in the presence of 10 µM (0.25 µCi) [1-$^{14}$C] acetyl-coenzyme A (47 mCi/mmol, Research Products International, Mount Prospect, IL), 0.16 units carnitine acetyl-transferase (CAT) (EC 2.3.1.7) (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 5 mM N-ethylmaleimide (NEM) (Aldrich Chemical Comp., Milwaukee, WI), for 1 h in a final volume of 250 µl. Radioactive acetylcarnitine formed according to the reaction:

L-carnitine + $^{14}$C-acetyl-CoA --->

$^{14}$C-acetylcarnitine + CoA-NEM

was chromatographically separated from unreacted acetylCoA using minicolumns (30 x 5 mm) of anion exchange resin Dowex 1-X8 (acetate), 100-200 mesh (Bio-Rad, Richmond, CA). The above reaction was driven toward formation of acetylcarnitine by alkylation of free CoA using NEM (represented in the equation as CoA-NEM). Isotope content of column effluent was determined by liquid scintillation counting in a Delta

300 liquid scintillation counter (Tracor Analytic, Desplaines, IL). A standard curve from 20 to 400 pmoles carnitine yielded a linear response (Figure 2). The concentration of carnitine solutions used as standards was verified by a 4,4'dithio-dipyridine spectrophotometric assay (61).

Total carnitine (free plus esterified) was determined as free carnitine following alkaline hydrolysis of carnitine esters (0.5 N KOH, 1 h). Amounts of esterified carnitine were calculated by subtraction of the amount of free from total carnitine. All carnitine determinations were performed in triplicate with one replicate receiving an internal standard of carnitine (gift from Sigma Tau, Rome, Italy). Recoveries approached 100% and all values were corrected for losses.

Long-chain Acylcarnitine Analysis

Five-hundred µl aliquots of cell lysate were acidified with 150 g/l PCA, incubated on ice for 10 min then centrifuged (10,000 x g, 5 min, 4° C) to separate acid-insoluble long-chain acylcarnitines (LCAC) from free carnitine and short-chain acylcarnitines. The pellet was washed twice with 60 g/l PCA to remove any trapped carnitine (62). Long chain-acylcarnitine content in the pellet was assayed as free carnitine following alkaline hydrolysis (0.5 N KOH, 1 h, 50° C).

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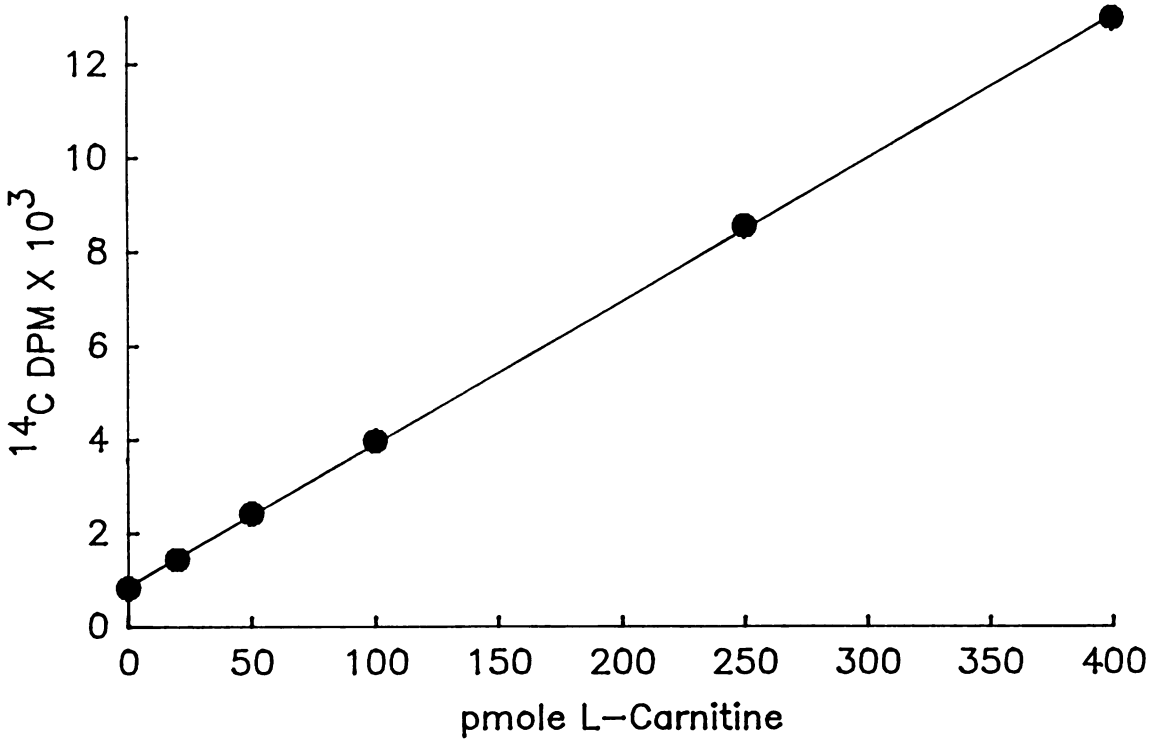



Figure 2. Standard curve for radiochemical determinations of carnitine content.

Sample Preparation for Short-chain Acylcarnitine Analysis

Sample preparation for short-chain acylcarnitines (SCAC) was modified from the procedure of Kerner and Bieber (59) for work with lymphocytes and MNP. Cell lysates containing 0.2-5.0 nmole total carnitine were deproteinized by addition of absolute methanol to a final concentration of 80%, incubated on ice for 30 min and centrifuged at 10,000 x g for 5 min, 4° C. Supernatants were dried by rotary evaporation under vacuum and reconstituted in 250 µl double distilled water. Samples were then applied to Dowex 1-X8 (acetate) minicolumns (30 x 5 mm) and eluted with 1.0 ml double distilled water to remove short-chain acylCoAs and dicarboxylic acids which could interfere with the exchange reaction. Recoveries of acylcarnitines from the minicolumns exceeded 95%. Column effluents were dried by rotary evaporation and redissolved in 25 µl of double distilled water; 18 µl was isotopically labelled for HPLC analysis and the remainder was used for carnitine determinations.

Purification of Radiolabelled Carnitine for Short-chain Acylcarnitine Analysis

Tritiated carnitine used in the exchange was purified before use to remove a metabolically inert impurity according to the procedure of Ramsay and Tubbs (63). L-[methyl-$^{3}$H]carnitine (72 Ci/mmol, Amersham, Arlington Heights, IL), 125 nmoles (0.5 mCi), was incubated with 1 mM acetylCoA, 5 mM Tris-HCl buffer, pH 7.5, and 25 µg of undialyzed

carnitine acetyltransferase in a final volume of 1.0 ml for 2 h. The mixture was then acidified (pH 2-3) with PCA and applied to a column (50 x 1.0 cm) of Dowex 50W-X8, 200-400 mesh, previously equilibrated with 0.15 M formic acid, pH 4.5 with trimethylamine. Acetyl-$^{3}$H-carnitine was eluted from the column with the same buffer and radioactivity in 0.5 ml fractions was monitored. Pooled fractions containing the acetylcarnitine peak were lyophilized. The residue was reconstituted in 2 ml of 2 N $NH_4OH$ for 2 h at 50° C to hydrolyze the ester linkages, lyophilized to dryness, redissolved into 0.5 ml double distilled water and then desalted on a column (12 x 2 cm) of P-2 Biogel (Bio-Rad, Richmond, CA). Aliquots of purified carnitine were concentrated by rotary evaporation and stored at -20° C.

Isotopic Labelling of Short-chain Acylcarnitines

Short-chain acylcarnitines were analyzed by the radioisotopic-exchange labelling procedure of Kerner and Bieber (59). Carnitine acetyl-transferase was utilized to incorporate [$^{3}$H]carnitine into the mixture of SCAC in the sample. The 50 µl reaction mixture contained: 25 mM potassium phosphate buffer, pH 7.4; 4-100 µM total carnitine; 0.213 µCi L-[methyl-$^{3}$H]carnitine; 5 µM CoA and 1 mM dithiothreitol (DTT) (Sigma, St.Louis, MO). The CAT enzyme was dialyzed against 25 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT using a microdialysis unit to remove $(NH_4)_2SO_4$. The exchange reaction was initiated by addition

of 1.5 units of dialyzed CAT to the reaction mixture followed by a 1 h incubation at room temperature. N-ethylmaleimide was then added at a final concentration of 2.8 mM to ensure conversion of acylCoAs to acylcarnitines. The reaction was terminated by addition of absolute methanol to a final concentration of 80%, incubated for 30 min on ice and centrifuged (10,000 x g, 5 min., 4° C) to pellet the precipitated protein. Supernatants were filtered through a 0.4-µm-pore-diameter nylon filters and 150 µl aliquots of supernatant were used for HPLC separation. Standard acylcarnitines (gift from Sigma Tau, Rome, Italy) were used as controls for determination of the efficiency of the exchange.

High Performance Liquid Chromatography

Radioisotopically labelled SCAC were separated and quantitated by reversed-phase HPLC. A flow-through ß-counter was used to monitor the amount of radioactivity in the column effluent. Carnitine and SCAC were separated on a microBondapak C-18, 250 x 4.6 mm column (Waters Assoc., Milford, MA) equilibrated with 100% 5 mM butanesulfonic acid, 5 mM ammonium acetate, pH 3.4. The gradient used for separation was: 0-15 min, linear increase to 21% absolute methanol; 15-25 min, linear increase to 50% methanol; 25-35 min, linear increase to 100% methanol; 35-45 min, isocratic at 100% methanol to wash off any residual radioactivity off the column (59). To prepare for another run, the column was

reequilibrated with 100% buffer for 15 min. Radioactivity of the column effluent was monitored by a FLO-ONE Model HS radioactivity flow detector using ScintiVerse™ LC (Fischer Scientific Comp., Fairlawn, NJ) as scintillation cocktail. Counting efficiency of $^{3}H$ was 34% throughout the elution gradient. The amounts of individual acylcarnitines were determined from the specific radioactivity of carnitine. Standard acylcarnitines were run to determine characteristic retention times to allow identification of sample peaks.

# RESULTS

## Lymphocyte and Mononuclear Phagocytic Cell Isolation

The isolation procedure outlined in Figure 1 efficiently removed red blood cells and polymorphonuclear cells from the mononuclear cell fraction and separated lymphocytes and MNP from one another. Microscopic examination and nonspecific esterase staining of isolated cell populations revealed a purity for lymphocyte and MNP fractions of about 95% and 90%, respectively. Cell viabilities exceeded 98% for all preparations as determined by trypan blue exclusion.

Previously we had attempted to use a Ficoll-Paque density gradient (64), a commonly used procedure to isolate the mononuclear cell fraction followed by further separation of MNP from lymphocytes by adherence of the phagocytic MNP to a plastic petri dish (65). This separation scheme did not yield highly purified cell populations. Lymphocytes were only about 80% pure when evaluated by microscopic examination and histochemical staining. Removal of adherent MNP from the dish surface resulted in low cell recoveries, poor viabilities (less than 50% by trypan blue exclusion) and only slightly enriched populations (40% positive cells by the nonspecific esterase stain). Thus, the superior Sepracell-MN procedure was adopted as the routine method for cellular isolation.

Carnitine Content in Lymphocytes and Mononuclear Phagocytes

Table 1 shows the amounts of total and free carnitine in human peripheral blood mononuclear cells isolated from 10-14 healthy young adults. Mononuclear phagocytes contained approximately four-fold more total carnitine than lymphocytes when expressed on a per cell basis (301 nmol/$10^9$ MNP vs. 79 nmol/$10^9$ lymphocytes) or two-fold more when expressed as total carnitine per mg cell protein (5.78 nmol/mg MNP protein vs 3.41 nmol/mg lymphocyte protein). The larger cell size of the MNP gave it a larger protein content per cell than the lymphocyte; this may account for the discrepancy observed when comparing data expressed per cell number versus per mg cell protein. Twenty-nine percent of the total carnitine in lymphocytes was acylated; in contrast, the carnitine pool of MNP was 69% acylated. It should be noted that the acylcarnitine to carnitine ratio could have been altered during isolation and processing procedures. However, when freshly isolated lymphocytes were incubated in serum-supplemented tissue culture medium under standard culture conditions (37° C, 5% $CO_2$), no significant changes in the levels of total carnitine acylated were seen. Therefore, the levels of acylation originally measured were believed to be accurate.

Identification of Acylcarnitines

Partial purification of the SCAC prior to analysis was required to remove protein, acylCoAs and dicarboxylic acids

TABLE 1

Carnitine Levels of Lymphocytes and Mononuclear Phagocytes

| Cell Type[a] | Total | Free | % Acylation |
|---|---|---|---|
| | nmol carnitine/$10^9$ cells | | |
| Lymphocytes | 79 ± 6[b] | 56 ± 5 | 28% |
| Mononuclear Phagocytes | 301 ± 23 | 93 ± 10 | 69% |
| | nmol carnitine/mg protein | | |
| Lymphocytes | 3.41 ± 0.54 | 2.44 ± 0.40 | 28% |
| Mononuclear Phagocytes | 5.78 ± 1.08 | 1.67 ± 0.37 | 71% |

[a] Cells were isolated from fresh human peripheral blood from equal numbers of males and females of ages ranging from 22-37 years. No differences were noted for sex or age in this group.

[b] Mean ± SEM, n = 10-14.

acylCoAs and dicarboxylic acids which could interfere with the radioisotopic labelling procedures. Due to the limited availability of human tissue, the sample preparation procedure of Kerner and Bieber (59) had to be modified to be made technically feasible for small amounts of tissue (Figure 3). A first attempt to modify the procedure (Figure 3, Prep. #1) involved concentration of the Dowex 1-X8 column effluents by lyophilization. However, high salt concentrations remaining in residues inhibited the radioisotopic exchange. Methanol extraction of the residue did not eliminate this complication. Another problem encountered was hydrolysis of alkaline labile acylcarnitines during neutralization of the PCA extract following deproteinization. To eliminate the neutralization step, protein was precipitated by a final concentration of 80% methanol (Figure 3, Prep. #2). The methanol could then be evaporated, the entire sample reconstituted in water and applied to a Dowex minicolumn. The resulting effluent volume could be easily concentrated in a 1.5 ml eppendorf minifuge tube by rotary evaporation. Thus, Prep. #2 provided a successful way to purify SCAC from human mononuclear cells prior to exchange labelling procedures.

Carnitine acetyltransferase catalyzes the reversible conversion of acylcarnitines to acylCoAs which can then react with radiolabelled carnitine as shown:

$$\begin{array}{ccc} \text{acylcarnitine} & \text{CAT} & {}^{3}\text{H-acylcarnitine} \\ + & \langle\text{----------}\rangle & + \\ \text{3H-carnitine} & \text{CoA} & {}^{3}\text{H-carnitine} \end{array}$$

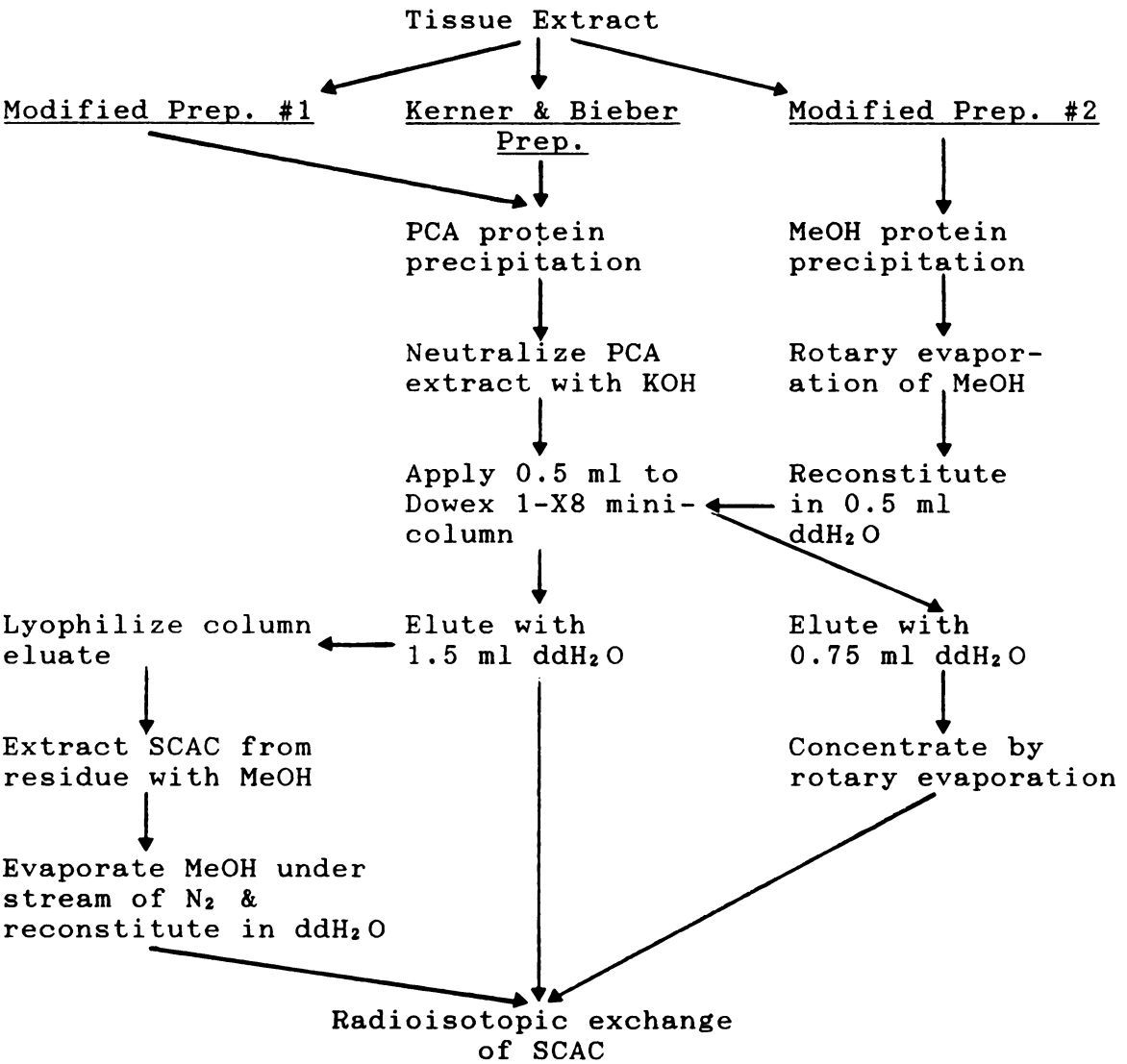



Figure 3. Flow chart of sample work-up for short-chain acylcarnitine analysis.

The rate of labelling depends on the amount of each acylcarnitine present and the individual kinetic constants. For quantitative purposes isotopic equilibrium must be attained. Sixty minutes was the suggested reaction time necessary to attain isotopic equilibrium for all SCAC (59). This was verified for these experiments with standard acylcarnitines (greater than 95% of valerylcarnitine was exchanged by 60 min).

High performance liquid chromatography analysis demonstrated that acetylcarnitine accounts for 73% of the total acylcarnitines in lymphocytes (Figure 4, Table 2) and 72% for MNP (Figure 4, Table 2). Propionylcarnitine represented 21% of the total acylcarnitine pool of lymphocytes and 5% for MNP. Small amounts of butyryl and/or isobutyrylcarnitine were detected in each cell type. Mononuclear phagocytes contained a six-carbon carnitine ester (Figure 4, Table 2) which was not detected in lymphocytes.

The amounts of individual SCAC were calculated from the specific radioactivity of the $^{3}$H-carnitine in the reaction mixture and are given in Table 2. Mononuclear phagocytes contained approximately six-fold more acetylcarnitine (98.3 nmol/$10^{9}$ cell vs. 15.9 nmol/$10^{9}$ cells) and ten-fold more butyryl and/or isobutyryl carnitine (4.0 nmol/$10^{9}$ cells vs. 0.3 nmol/$10^{9}$ cells) than lymphocytes. Both cell types contained similar amounts of propionlycarnitine. Levels of

Figure 4. HPLC separation of short-chain acylcarnitines of lymphocytes and mononuclear phagocytes. Radio-isotopically labelled short-chain acylcarnitines from lymphocytes (a) and MNP (b) were separated using reversed-phase HPLC coupled to a flow-through ß-counter as described in experimental section. Peaks are denoted as: I is free carnitine, II is acetyl-, III is propionyl-, IV is isobutyryl-, and V is $C_6$-acylcarnitine.

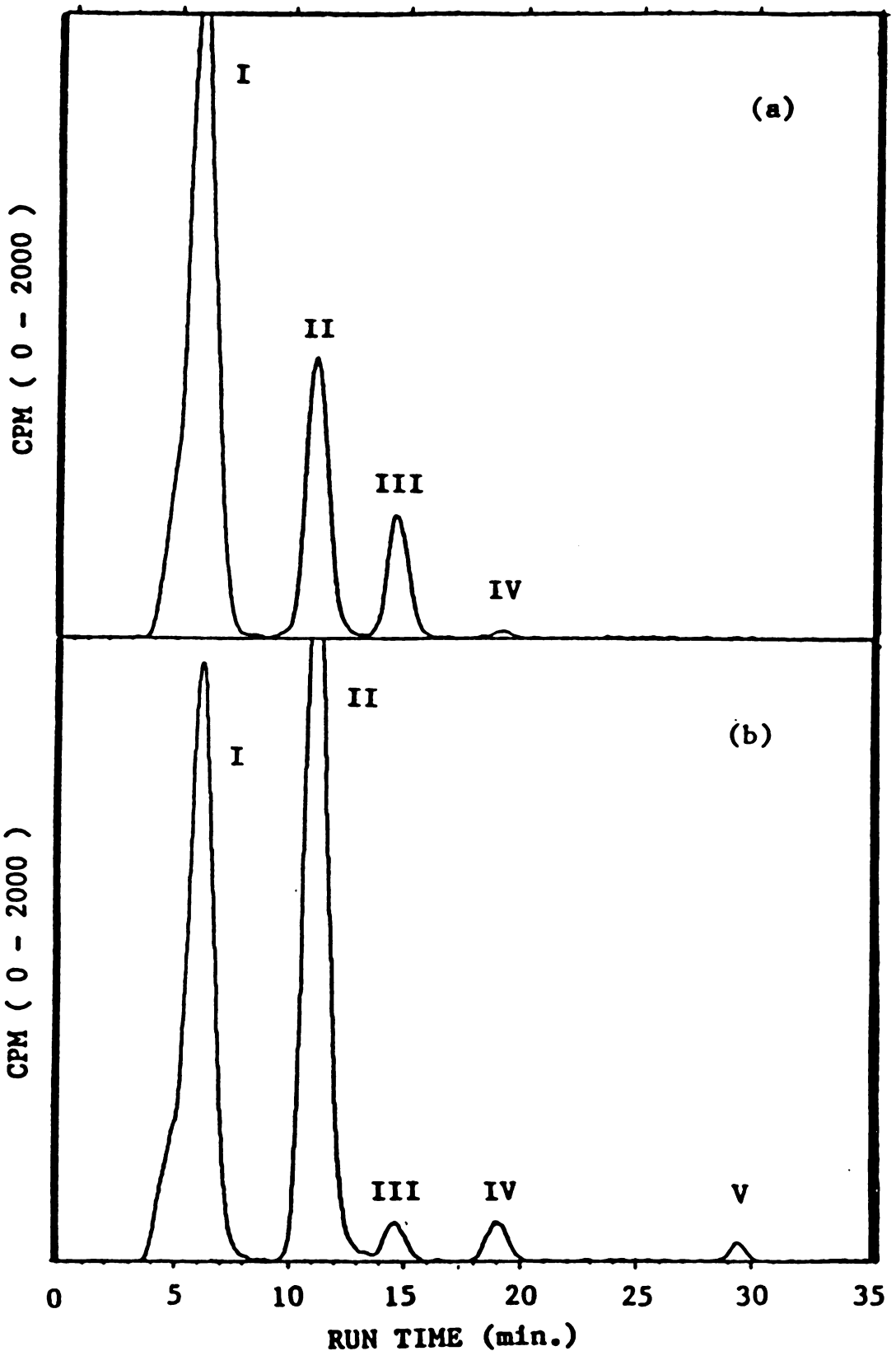

I
(a)
II
III
IV
CPM ( 0 - 2000 )
II
I
(b)
III
IV
V
CPM ( 0 - 2000 )
0
5
10
15
20
25
30
35
RUN TIME (min.)

TABLE 2

Amounts of Individual Acylcarnitines of Lymphocytes and Mononuclear Phagocytes

| Acyl Group | Lymphocytes (nmol/$10^9$ cells) | Mononuclear Phagocytes (nmol/$10^9$ cells) |
|---|---|---|
| Total Acylcarnitine | 21.1 ± 1.2[a] | 137.0 ± 21.5 |
| $C_2$[b] | 15.4 ± 2.2 | 98.3 ± 10.5 |
| $C_3$ | 4.4 ± 0.7 | 6.9 ± 0.6 |
| $C_4$ | 0.3 ± 0.1 | 4.0 ± 3.8 |
| $C_6$ | below detection | 0.8 ± 0.3 |
| Long Chain | 0.7 ± 0.2 | 4.0 ± 1.6 |
| % Recovery of Material | 98% | 83% |

[a] Mean ± SEM, n = 6. Donors included approximately equal numbers of males and females of ages ranging from 22-37 years.

[b] $C_n$, where n = number of carbons in acyl chain.

long-chain acylcarnitines represented a small percentage of total acylcarnitines, 3%-4% (Table 2).

Total recovery of acylcarnitines was 99% for lymphocytes and 83% for MNP. This lower recovery for MNP suggested that unidentified carnitine esters were present in MNP samples which are not detected by the analytical procedures employed.

Effect of Glucose Supplementation on Acylcarnitine Levels

Mononuclear cells from 6 donors were isolated in the absence and presence of 5 mM glucose to determine if availability of this substrate altered their carnitine profiles. These were paired experiments in which cells isolated from the same donor were subjected to the two different treatments (+/- glucose). Table 3 and Figure 5 show that in experiment #1 the levels of acetylcarnitine increased two-fold when lymphocytes were supplemented with glucose. Levels of propionylcarnitine remained the same for both treatments. In experiment #2 donor C demonstrated similar increases in acetylcarnitine levels, while donor D showed no effect of glucose supplementation. In experiment #3 acylcarnitine profiles from donors E and F were unaltered by the presence of glucose in the isolation medium. Mean values for acetylcarnitine levels show an increase with glucose supplementation (from 12.8 to 18.7 nmol/$10^9$ lymphocytes). However, this increase is not statistically significant when analyzed by a Paired t Test ($p<.05$). Propionylcarnitine levels of glucose-supplemented cells remained the same as

TABLE 3

Effect of Glucose Supplementation on Levels of Short-chain Acylcarnitines of Lymphocytes

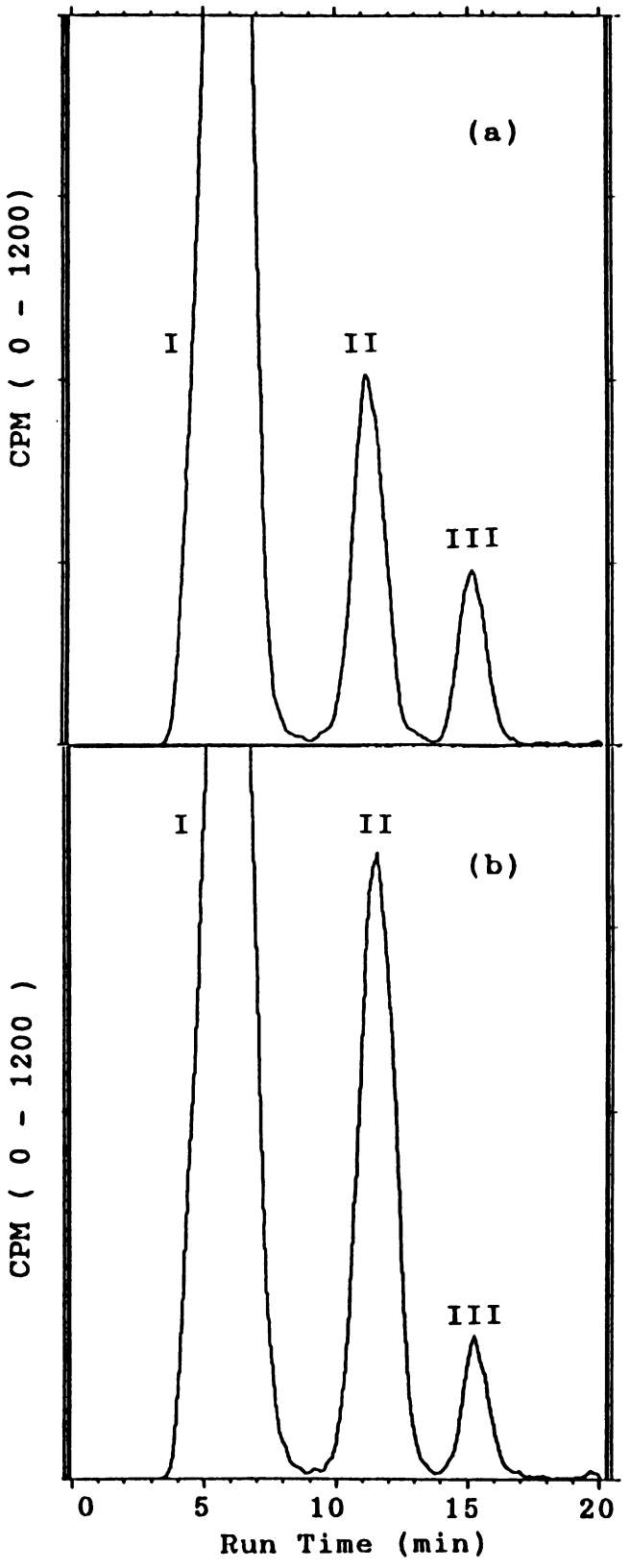
| Donor[a] / Experiment# | (nmol/$10^9$ cells) Acetylcarnitine | | Propionylcarnitine | |
|---|---|---|---|---|
| | (- glc)[b] | (+ glc) | (- glc) | (+ glc) |
| A/1 | 14.9 | 27.0 | 6.4 | 6.6 |
| B/1 | 15.3 | 35.0 | 7.0 | 7.6 |
| C/2 | 10.0 | 18.5 | 3.9 | 3.9 |
| D/2 | 13.2 | 10.4 | 2.7 | 1.5 |
| E/3 | 14.9 | 12.6 | 3.4 | 2.3 |
| F/3 | 8.4 | 8.7 | 3.2 | 2.6 |
| $\bar{x}$ ± SD | 12.8 ± 2.9 | 18.7 ± 10.4[c] | 4.4 ± 1.8 | 4.1 ± 2.5[c] |

[a] Donors included 3 males and 3 females between the ages of 22-30 years.

[b] Lymphocytes from 6 donors were isolated in the absence (- glc) and presence (+ glc) of 5.0 mM glucose.

[c] No statistical difference from (- glc) treatment when statistically analyzed by Paired t Test ($p < 0.05$).

Figure 5. HPLC analysis of the effect of glucose supplementation on short-chain acylcarnitines of lymphocytes. Cells from the same donor were isolated in the absence (a) and presence (b) of exogenously added glucose and then subjected to HPLC analysis as described in experimental section. Peaks are denoted as: I is free carnitine, II is acetyl-, III is propionylcarnitine.


(a)
I
II
III
CPM ( 0 - 1200)
(b)
I
II
III
CPM ( 0 - 1200 )
0
5
10
15
20
Run Time (min)

unsupplemented cells. No changes were observed in acylcarnitine levels of MNP when isolated in the presence of glucose (data not shown).

# DISCUSSION

Human peripheral blood lymphocytes and MNP isolated from healthy young adults contained moderate amounts of carnitine and acylcarnitines suggesting a metabolic function for carnitine in these cells. When compared to human skeletal muscle, a tissue known to contain high concentrations of carnitine (20.5 nmol total carnitine/mg noncollagen protein) (Engel, 1986), carnitine levels of MNP and lymphocytes were several-fold lower. Concentrations reported here were considerably lower than those reported by Katrib, et. al. (47) for the mononuclear cell fraction. However, they were in close agreement with the results of Furst, et. al. (48), who measured 220 nmol/$10^9$ cells for mononuclear cells. Furst postulated that the presence of a Tris-buffer in the spectrophotometric assay used by Katrib was an acetyl acceptor. Carnitine acetyltransferase can transfer acetyl moieties to Tris which could produce high values for carnitine. In our experiments, samples were spiked with known amounts of carnitine which served as an internal standard for each preparation. This increased the validity of carnitine values we obtained.

Separation of MNP from lymphocytes revealed differences in carnitine content between these two cell types. This separation of the individual cell types comprising the mononuclear cell fraction was not done in previous studies

where carnitine values were reported (47, 48). Carnitine concentrations in MNP were higher than in lymphocytes suggesting a larger carnitine requirement for metabolic processes of MNP. It is possible that carnitine serves different roles within these 2 cell types, since it is known that they differ greatly in both their morphology and function.

The most notable difference between the two cell types was their degree of acylation. The carnitine pool in MNP was 69% acylated, which was very high when compared to amounts found in lymphocytes and other tissues. Analysis of the individual acylcarnitines revealed that acetylcarnitine was the major acylcarnitine present in both cell types, with MNP containing levels six-fold that of lymphocytes. In most tissues acetylcarnitine represents about 20 to 30% of the total carnitine pool and the amount of free carnitine exceeds that of the acylcarnitine pool (51, 66). The very high levels of acetylcarnitine in MNP suggested an accumulation of acetyl groups. Studies of others have shown that acetylcarnitine may have several roles in metabolism. In some tissues it is known to modulate the CoA/acylCoA ratio (30). In yeast, carnitine can be used for biosynthetic purposes (34). High levels of acetylcarnitine have been observed in insect flight muscle where it functions as a source of readily available acetyl groups for oxidation during the transition from rest to rapid contraction (27).

From our analyses we were unable to determine which of these functions was important in mononuclear cells but it was apparent that carnitine was in equilibrium with cellular acetylCoA pools. The high levels of acetylcarnitine present in MNP may suggest that carnitine plays a role similar to that observed in insect flight muscle. Since MNP constitute part of the first line of defense of the immune response, it is necessary that they can quickly shift from a resting to a more activated metabolic state during phagocytosis. Carnitine may be important in facilitating the oxidation of substrate by acting as a reservoir for acetyl groups. When levels of acetylCoA exceed that which can be accommodated by the TCA cycle, the acetyl group can be transferred to carnitine to form acetylcarnitine and liberate free CoA. This would prevent acetyl groups from sequestering all available reduced CoA, which is necessary for continuation of oxidative processes within the mitochondrial matrix. Investigation into possible changes in carnitine profiles of MNP upon activation would help elucidate the reason(s) for this unusually high degree of acylation.

The precursor acetylCoA could be derived from either carbohydrate (31) and/or fatty acid substrates (32). Recent studies suggest that in rat heart much of the acetylcarnitine is derived from glucose (67). Glucose is thought to be the major catabolic fuel for leukocytes, although the catabolic metabolism of these cells has not been well characterized

(68, 69). Further investigation is required to determine the fuel source(s) contributing the acetyl group. Isolation of mononuclear cells in the presence of glucose did not statistically alter the carnitine profiles in lymphocytes or MNP. However, an approximate two-fold increase in the levels of acetylcarnitine was observed in 3 individuals when their lymphocytes had access to exogenous glucose during isolation. We believe that these increases were real since the propionylcarnitine levels in these same cells did not change providing a convenient internal standard. These data suggested that glucose could be serving as a precursor for acetylcarnitine in lymphocytes, in spite of the widely held belief that the acetylCoA pool in equilibrium with carnitine is derived solely from fatty acid oxidation. Increases in acetylcarnitine levels in the presence of glucose also indicated that our cells did not become anaerobic during isolation procedures, since oxygen is required for pyruvate dehydrogenase activity which catalyzes the conversion of pyruvate to acetylCoA. Why similar increases were not observed in the other 3 donors analyzed is unknown. The large standard deviations accompanying the acetylcarnitine levels of cells supplemented with glucose demonstrated the variability of this glucose effect. The effect appeared to be dependent upon different cell preparations and not on the analytical procedures, since propionylcarnitine levels were unaltered in donors which showed increases in acetylcarnitine

levels. Perhaps the ability of lymphocytes to demonstrate this effect was dependent upon the physiological state of the individual when the blood was drawn. No correlation between this glucose effect and donor sex or age were noted.

Other aliphatic SCAC (propionyl and (iso)butyryl carnitine) were found in both lymphocytes and MNP indicating that these cells were oxidizing some branched-chain amino acids (23). The other important acylcarnitines measured were the long-chain acylcarnitines. Although they constituted only a small percentage of the total acylcarnitine pool, this low amount does not preclude a role for carnitine in mitochondrial ß-oxidation of long chain fatty acids by lymphocytes or MNP. The steady state concentrations of SCAC are much higher than the LCAC in most cell types including those that readily utilize fatty acids as their primary fuel source.

These series of experiments have provided valuable insight into the role of carnitine in two important cells involved in the immune response. It was demonstrated that both lymphocytes and MNP isolated from human peripheral blood contained carnitine at moderate concentrations relative to skeletal muscle. This carnitine was found to be acylated indicating it was metabolically active within these cells. Percentage of total carnitine acylated in lymphocytes was similar to that seen in most other body tissues. Of considerable interest was the unusually high degree of

acylation observed in MNP. The possibility of carnitine facilitating short-chain fatty acylCoA metabolism within MNP was discussed. Quantitation of the individual acylcarnitine species allowed identification of specific acylCoAs in equilibrium with cellular carnitine pools. Carnitine appeared to be involved in both short and long-chain acylCoA metabolism in both cell types. These studies also illustrated the importance of careful separation of MNP from lymphocytes because their carnitine profiles differed dramatically indicating their intermediary metabolism could be quite different.

# CHAPTER 2

## COMPARISON OF CARNITINE CONTENT AND CARNITINE ACYLTRANSFERASE ACTIVITY IN RESTING AND STIMULATED LYMPHOCYTES


### ABSTRACT

Activation of lymphocytes by a mitogen dramatically alters lymphocyte intermediary metabolism signalling the cells to differentiate and proliferate. Since carnitine is required for efficient energy utilization it was postulated that monitoring carnitine content and/or its degree of acylation would reflect some of the metabolic changes occurring in resting versus activated cells. For this purpose, lymphocytes isolated from fresh human peripheral blood were either stimulated in culture by phytohemagglutinin (PHA) for 66 h or maintained in the resting state under identical conditions without a mitogen. Measurement of incorporation of [$^3$H]-thymidine into DNA verified that stimulated cells were undergoing proliferation while resting cells remained relatively quiescent. Quantitation of carnitine in resting and activated cells revealed a significant increase in total carnitine content in cell

lysates of PHA-stimulated lymphocytes (22.51 nmol/mg DNA) as compared to unstimulated controls (10.63 nmol/mg DNA). However, no increase was observed when carnitine concentrations were calculated on a per mg cell protein basis. Free carnitine levels were also altered in response to stimulation (it increased from 8.85 nmol/mg resting lymphocyte DNA to 14.24 nmol/mg stimulated lymphocyte DNA). Stimulation doubled the percentage of total carnitine acylated indicating a shift in metabolic state. The enzymes responsible for carnitine metabolism in lymphocytes were measured for the first time. Carnitine acetyltransferase activity remained unchanged in response to stimulation, while carnitine palmitoyltransferase activity increased per mg cellular DNA. From these results it was concluded that carnitine content and percentage of carnitine acylated differed significantly between resting and activated cells. These changes in carnitine metabolism were postulated to reflect alterations in one or more of carnitine's roles in fuel utilization which may be required to maintain the immune response.

# INTRODUCTION

In order to further understand the involvement of carnitine in the intermediary metabolism of lymphocytes, the effect of lymphocyte activation upon carnitine metabolism was investigated. Resting lymphocytes contained carnitine and acylcarnitines as described in Chapter 1 of this thesis (50). The carnitine content and degree of acylation of activated cells remained to be determined. Since activated lymphocytes are morphologically and functionally distinct from resting cells, it was postulated that carnitine profiles would change with the developmental stage of the lymphocyte. Study of activated cells was important since these are the cells which participate in antibody and cell-mediated responses essential to host defense.

Resting lymphocytes have cell surface receptors, immunoglobulins on B cells and the T cell receptor on T cells, specific for one particular antigenic determinant. When antigen binds directly to these receptors or is presented by accessory cells in association with major histocompatibility complex products, resting cells are signalled to differentiate and proliferate (53). Binding of antigen stimulates only a small proportion of the lymphocyte population making measurements of biochemical changes associated with activation difficult (70). Therefore, most existing knowledge concerning lymphocyte activation came

from studies performed using mitogens, molecules which bind to and nonspecifically activate a high proportion of lymphocytes. For experiments reported in this chapter lymphocytes were mitogenically-stimulated in culture to better facilitate assessment of potential changes in carnitine metabolism associated with activation.

Activation involves a dramatic shift in cellular metabolism which enables lymphocytes to engage in multiple rounds of division and synthesize numerous immune modulators involved in the immune response such as antibodies and lymphokines. Changes in intermediary metabolism known to be associated with stimulation are increases in rates of RNA, protein and DNA synthesis (70). Accompanying these changes are increases in rates of glucose and glutamine utilization (10). However fuel utilization throughout the proliferative response has not been adequately characterized. Since carnitine is required for oxidation of long-chain fatty acids (21, 22) and appears to be involved in short- and medium-chain fatty acid metabolism as well (23), it was of interest to relate the increased metabolic activity associated with lymphocyte activation to the carnitine status in these cells. If carnitine plays important roles in fuel utilization of activated lymphocytes then one expects changes in carnitine content and/or its degree of acylation during an immune response.

Changes in physiological states are frequently accompanied by changes in carnitine content within tissues. For example, when rats were fasted for several days their percentage of total carnitine acylated in the liver increased (71). This was presumed to reflect alterations in steady-state levels of acyl-CoA pools in equilibrium with carnitine. The work of Adlouni and coworkers demonstrated an increase in the carnitine content of polymorphonuclear and mononuclear cells isolated from patients suffering from inflammatory disorders (49). This in vivo example suggested that shifts in white blood cell carnitine profiles might occur with an immunological challenge.

The experimental approach for studies reported in this chapter was to optimize the mitogenic response of lymphocytes isolated from human peripheral blood using a defined culture system. Once appropriate culture conditions were established, the levels of carnitine and its degree of acylation were determined in unstimulated and stimulated lymphocytes. In addition, amounts of CAT and CPT were measured for the first time in unstimulated and mitogenically-stimulated lymphocytes.

# MATERIALS AND METHODS

## Preparation of Cell Culture Reagents

Methods for cell culture were modified slightly from those described by Mishell and Dutton for spleen cell cultures from mice (72). The culture medium was Dulbecco's Modified Eagle Medium (DMEM) (Whittaker M.A. Bioproducts, Walkersville, MD) buffered with 10 mM Hepes buffer, pH 7.4, 0.06% w/v sodium bicarbonate and supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 µg/ml gentamicin (Whittaker M.A. Bioproducts), 100 units/ml penicillin and 100 µg/ml streptomycin (all from Gibco, Grand Island, NY), $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, MO) and 0.1 mM sodium pyruvate (Whittaker M.A. Bioproducts). This medium was referred to as DMEM-Incomplete (DMEM-I). When DMEM-I was supplemented with 5 or 10% fetal bovine serum (FBS) (Whittaker M.A. Bioproducts) it was called DMEM-Complete (DMEM-C). Where indicated, medium was supplemented with 0.1 mM L-carnitine (gift from Sigma Tau, Rome).

## Lymphocyte Isolation and Preparation for Cell Culture

Lymphocytes were isolated from fresh human peripheral blood from healthy adults, ranging from 22-40 years, using a continuous density gradient, Sepracell-MN, as described in Chapter 1; mononuclear phagocytes were not used in these studies and were discarded. After the final wash step, lymphocytes were resuspended in desired culture medium to a

final density of 2.0 x $10^6$ cells/ml as determined by trypan blue exclusion (56). Purity of isolated lymphocytes was evaluated by microscopic examination and histochemical staining as described in Chapter 1.

Mitogenic Stimulation of Lymphocytes

To determine optimal culture conditions for mitogenic stimulation, 100 µl aliquots containing 2.0 x $10^5$ viable lymphocytes were cultured in flat-bottom 96-well cell culture cluster dishes (Gibco). Where indicated, round-bottom 96-well dishes were used. Mitogens tested were phytohemagglutinin P (PHA) (5.0, 10.0, 20.0 µg/ml) and concanavalin A (Con A) (5.0, 10.0, 20.0 µg/ml) both purchased from Sigma. Plates were incubated at 37° C in a humidified atmosphere of 5% $CO_2$ and ambient air. Eighteen hours prior to cell harvest, cultures were pulsed with 10 µl of DMEM-I containing 1.0 µCi of methyl-[$^3$H]-thymidine (2 Ci/mmol, ICN Radiochemicals, Irvine, CA). Cells were harvested onto glass fiber filters using a multiple sample harvester (Otto Hiller Co., Madison, WI). Cold 10% (w/v) trichloroacetic acid precipitated DNA onto the filters. The incorporation of isotope into cellular DNA was measured by scintillation counting.

To obtain stimulated lymphocytes for carnitine determinations, cells were resuspended to a final density of 2 x $10^6$ cells/ml in DMEM-C (10% FBS) supplemented with 0.1 mM carnitine. Four ml aliquots of cell suspensions were cultured in 60 mm tissue culture dishes (Corning, Corning, NY) using

incubation conditions described above. Phytohemagglutinin was added at final concentration of 10 μg/ml. Unstimulated cells were cultured under these same conditions without a mitogen present. Lymphocytes isolated from the same donors were divided into unstimulated and stimulated groups, thus, all experiments were paired. This eliminated variability in carnitine content between different donors. After 66 h in culture, lymphocytes were removed from the plate using a Pasteur pipet and then plates were washed with 4 ml cold PBS-BSA to remove any remaining cells. Cells were pelleted from suspension by centrifugation (300 x g, 7 min.), washed twice in PBS to remove any extracellular carnitine and resuspended in 50 mM potassium phosphate buffer, pH 7.4, to an approximate final density of $1.0 \times 10^7$ cells/ml. Cellular membranes were disrupted by sonication for 30 sec on ice prior to carnitine analysis.

Carnitine Analysis of Resting and PHA-Stimulated Lymphocytes

Levels of total and free carnitine were determined for unstimulated and PHA-stimulated cell lysates using the radiochemical carnitine assay as described in detail in Chapter 1 of this thesis (58). Colonization and aggregation of mitogen-stimulated cells made it impossible to obtain accurate cell counts, so carnitine levels could not be expressed on a per cell basis. Instead data were expressed as nmol carnitine per mg DNA or per mg cell protein. Deoxyribonucleic acid content of 20 μl aliquots of cell

lysate was quantitated by the fluorescent Hoechst 33258 dye method (73) using salmon sperm DNA as standard (Sigma).

Assay of Carnitine Acyltransferases

Levels of CAT and CPT were assayed using discontinuous (end point) assays which measure the formation of a specific radiolabelled acylcarnitine (46). To measure CAT activity 50 µl aliquots of cell lysate were incubated for 0, 1, 2, or 3 minutes in the presence of 25 mM $KP_i$, pH 7.4, 1 mM NEM, 0.1 mM [1-$^{14}$C] acetylCoA (0.14 µCi) and 5 mM carnitine in a final volume of 100 µl. The zero time point was assessed in two ways: either cell lysate was present and carnitine was omitted from the reaction mix or cell lysate was replaced by buffer and carnitine was present. Identical results were obtained for both. Reactions were terminated by application of the entire incubation mixture onto a column (30 x 5 mm) of Dowex 1-X8 (acetate) followed by elution with 1.0 ml distilled water. Amounts of radioactivity in column effluents were measured by scintillation counting for 10 min.

Carnitine palmitoyltransferase activity was assayed as described for CAT with these exceptions: 1) Incubation mixtures contained 40 µM decanoylCoA (2.3 mCi/mmol) in place of acetylCoA and the concentration of carnitine was increased to 10 mM. [1-$^{14}$C]-DecanoylCoA was prepared by Janos Kerner of this lab (74). 2) The reaction was terminated by addition of absolute methanol to a final concentration of 80% and the mixture placed over a Dowex minicolumn and eluted with 1.0 ml

80% methanol. Enzyme activities were expressed as nmol of acylcarnitine formed per min per mg cell protein or per mg DNA.

To demonstrate formation of the specific acylcarnitine, aliquots of Dowex column eluates were analyzed by HPLC. For acetylcarnitine analysis, Dowex minicolumn eluates were filtered through 0.4 μm cellulose filters and 150-200 μl aliquots were analyzed by HPLC using the gradient described in Chapter 1 for SCAC (59). For decanoylcarnitine analysis, eluates were evaporated to dryness under stream of $N_2$, reconstituted in 175 μl distilled water, filtered as above and 150 μl analyzed by HPLC. A Bondapak C-18 column (Waters Assoc., Milford, MA) was equilibrated with 70% of absolute methanol and 30% of 5 mM butanesulfonic acid, 5 mM ammonium acetate, pH 3.4. The gradient used for separation was: 0-2 min, linear increase to 80% of methanol; 2-7 min, linear increase to 90% of methanol; 7-10 min, linear increase to 100% of methanol. Methanol was then continued for 10 min to wash off any residual radioactivity and the column was reequilibrated with a 10 min linear increase to 30% of buffer, followed by an additional 15 min. The flow rate was 1.0 ml/min.

Statistical Methods

The mean and standard deviation or standard error of the mean were calculated for both control and mitogen stimulated groups. Levels of thymidine incorporation between

two independent groups were examined by a Student's t Test. Carnitine values were examined by a Paired t Test ($p<0.05$). Percentages were analyzed by a Signed Rank Test ($p<0.05$) for nonparametric data.

# RESULTS

## Mitogenic Stimulation of Lymphocytes

The proliferative response of human peripheral blood lymphocytes to the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) is shown in Figure 1. For both mitogens the optimal dose was 10 μg/ml. PHA was a more effective mitogen than Con A, giving twice the levels of thymidine incorporation at the three mitogen concentrations tested (5, 10 and 20 μg/ml) (Figure 1).

Use of DMEM supplemented with 10% FBS resulted in better mitogenic responses for both PHA and Con A than supplementation with 5% or no serum (Figure 2). Low levels of proliferation in DMEM-I revealed that serum supplementation was required to achieve stimulation suggesting that serum contributed essential nutrients, hormones, factors, etc. to the culture medium. Supplementation with 5% FBS gave approximately 75% the levels of thymidine incorporation seen with 10% FBS (a significant difference when analyzed by the Student's t test, $p < 0.001$). The addition of exogenous carnitine to the three culture mediums did not alter thymidine incorporation at 10 μg/ml of PHA (Figure 3) or Con A (data not shown).

Kinetics of the PHA-induced mitogenic response were determined by measurement of thymidine incorporation at various time points (24, 48, 66 and 72 h) following

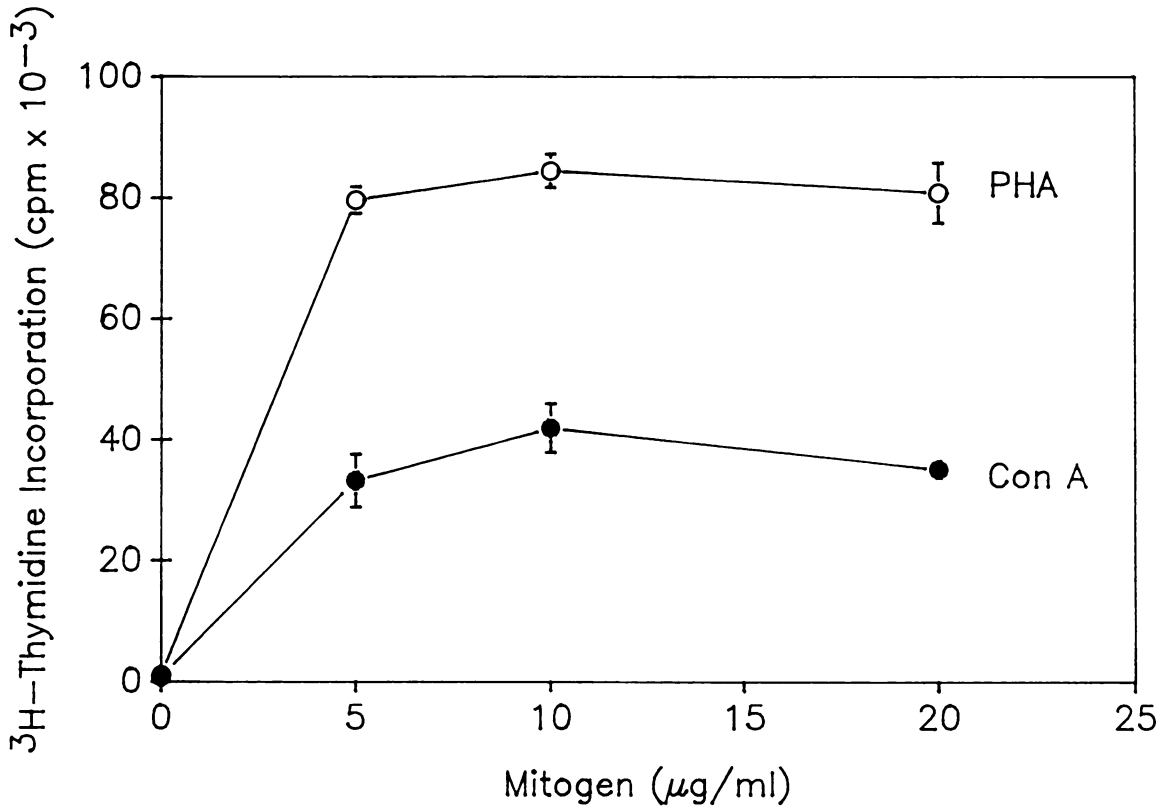



Figure 1. Dose response curve to PHA and Con A by lymphocytes after 66 h in culture. Cells were maintained in DMEM-C (10% FBS) as described in experimental section. After 48 h cells were pulsed with $^3$H-thymidine and harvested 18 h later. Incorporation of $^3$H-thymidine into DNA was determined. Each point represents the mean ± SD, n = 4. Standard deviation bars were to small to plot for several data points.

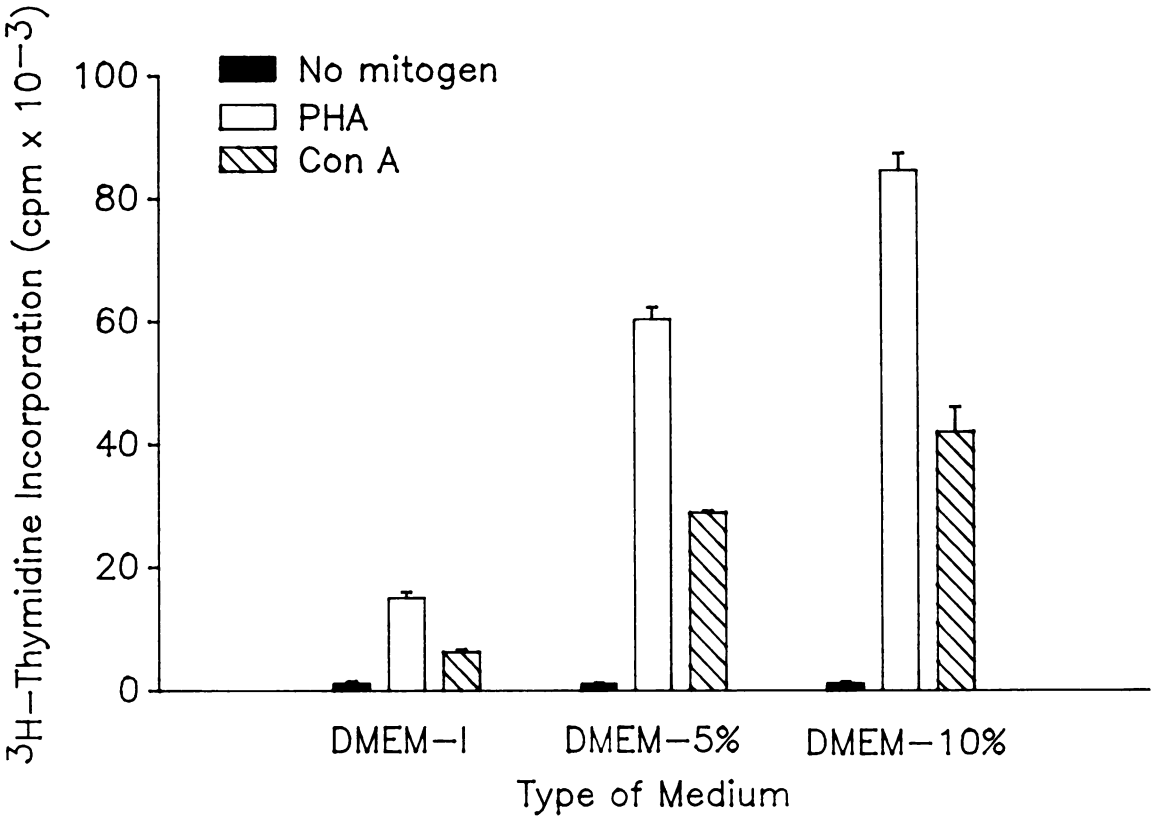



Figure 2. Proliferative response to PHA and Con A by lymphocytes cultured in three mediums. Culture mediums tested were DMEM with no serum (DMEM-I), with 5% FBS (DMEM-5%) or with 10% FBS (DMEM-10%) supplementation. Concentration of both mitogens was 10 µg/ml. Cells were pulsed with $^3$H-thymidine after 48 h in culture and were harvested 18 h later to determine incorporation of thymidine into DNA. Each bar represents the mean ± SD, n = 4.

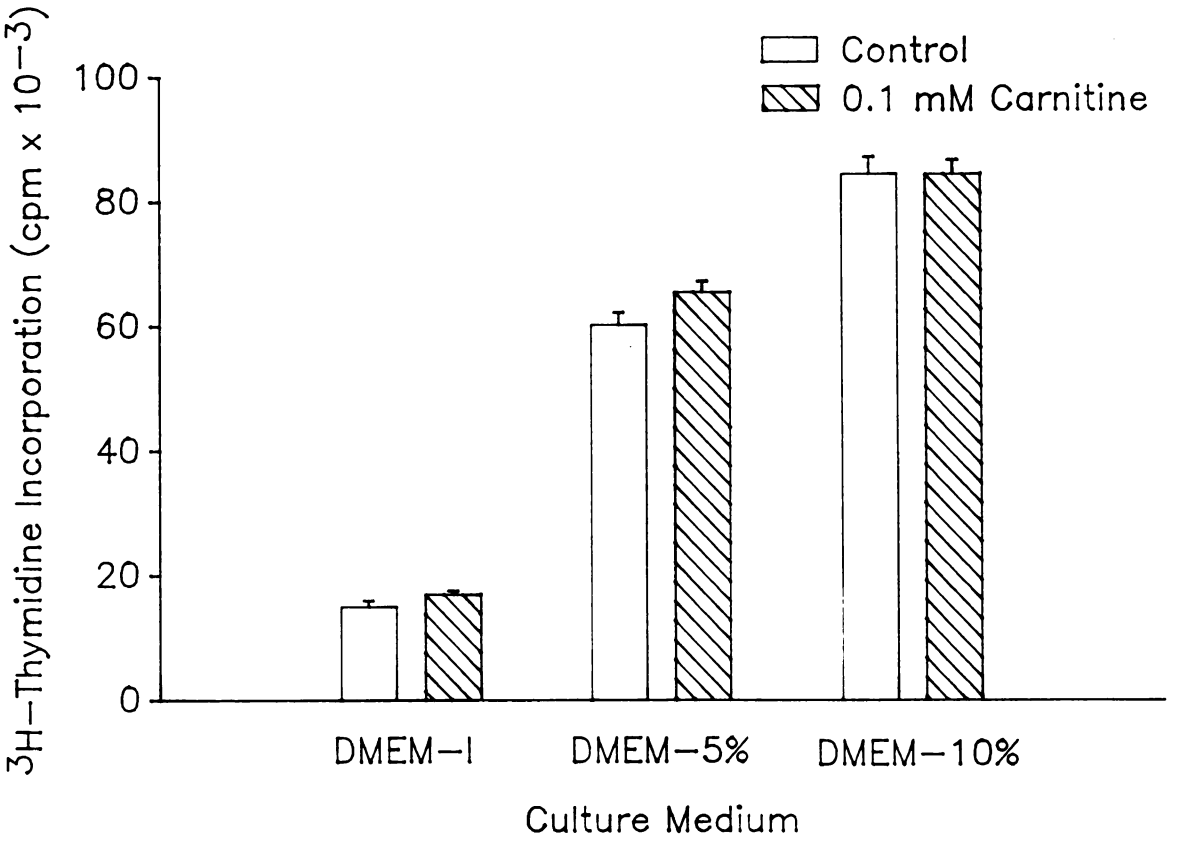



Figure 3. Effect of carnitine supplementation of medium on the proliferative response to PHA by lymphocytes cultured in three mediums. Carnitine was added at 0.1 mM final concentration. Both control and carnitine supplemented cultures were stimulated by PHA at a final concentration of 10 µg/ml. Mediums were the same as in Figure 2. Cells were pulsed with $^3$H-thymidine after 48 h in culture and then harvested to determine incorporation of thymidine into DNA. Each bar represents the mean ± SD, n = 4.

stimulation (Figure 4). Virtually no thymidine was incorporated at 24 h. By 48 h the response had dramatically increased having peaked at about 66 h. Incorporation began to subside at 72 h. Thus, 66 h was chosen to be the time point at which to harvest cells for carnitine analysis.

Comparison of two types of tissue culture plates demonstrated a higher proliferative response for lymphocytes cultured in plates with flat-bottom wells than those with round-bottom wells (Figure 5). There were significant differences in thymidine incorporation between the two types of tissue culture dishes at PHA concentrations of 10 and 20 μg/ml (Student's t Test, $p < 0.05$). Although it is generally accepted that round-bottom well culture plates are required to achieve a proliferative response with human blood lymphocytes, these results demonstrated flat-bottom wells to be superior.

Carnitine Analysis of PHA-Stimulated Lymphocytes

Table 1 shows the effects of mitogenic stimulation on the amounts of total and free carnitine found in human peripheral blood lymphocytes from 5 healthy adults. Activated lymphocytes contained approximately two-fold more total carnitine than controls when expressed on a per mg DNA basis (10.63 nmol/mg control DNA vs. 22.51 nmol/mg stimulated DNA). However, carnitine content was constant when data were expressed as total carnitine per mg cell protein (2.35 nmol/mg

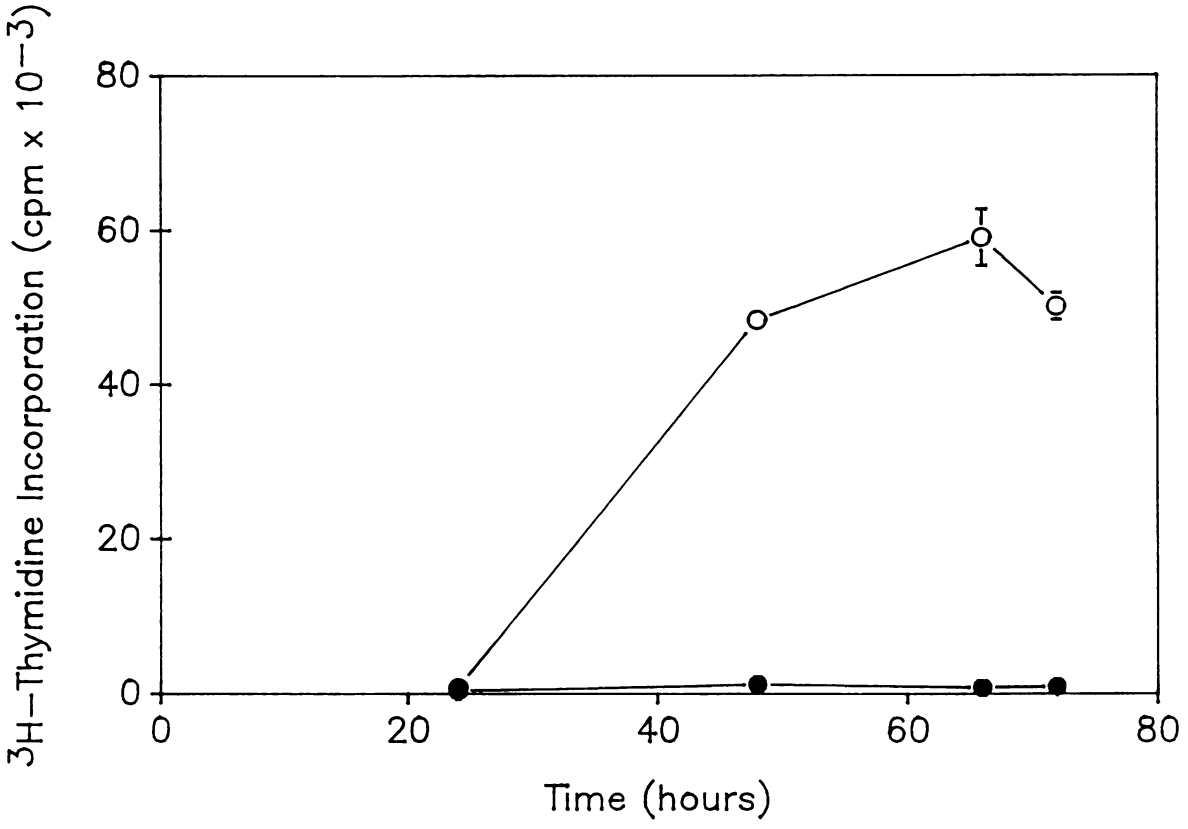



Figure 4. Kinetics of the proliferative response to PHA by lymphocytes. Cells were cultured in DMEM-10% FBS and were either stimulated with 10 μg/ml PHA (open circles) or received no mitogen (closed circles). Cells were pulsed with $^3$H-thymidine after 6, 30, 48 and 54 h in culture and harvested 18 h later to determine incorporation of thymidine into DNA. Each point represents the mean ± SD, n = 4. Standard deviation bars were to small to plot for several data points.

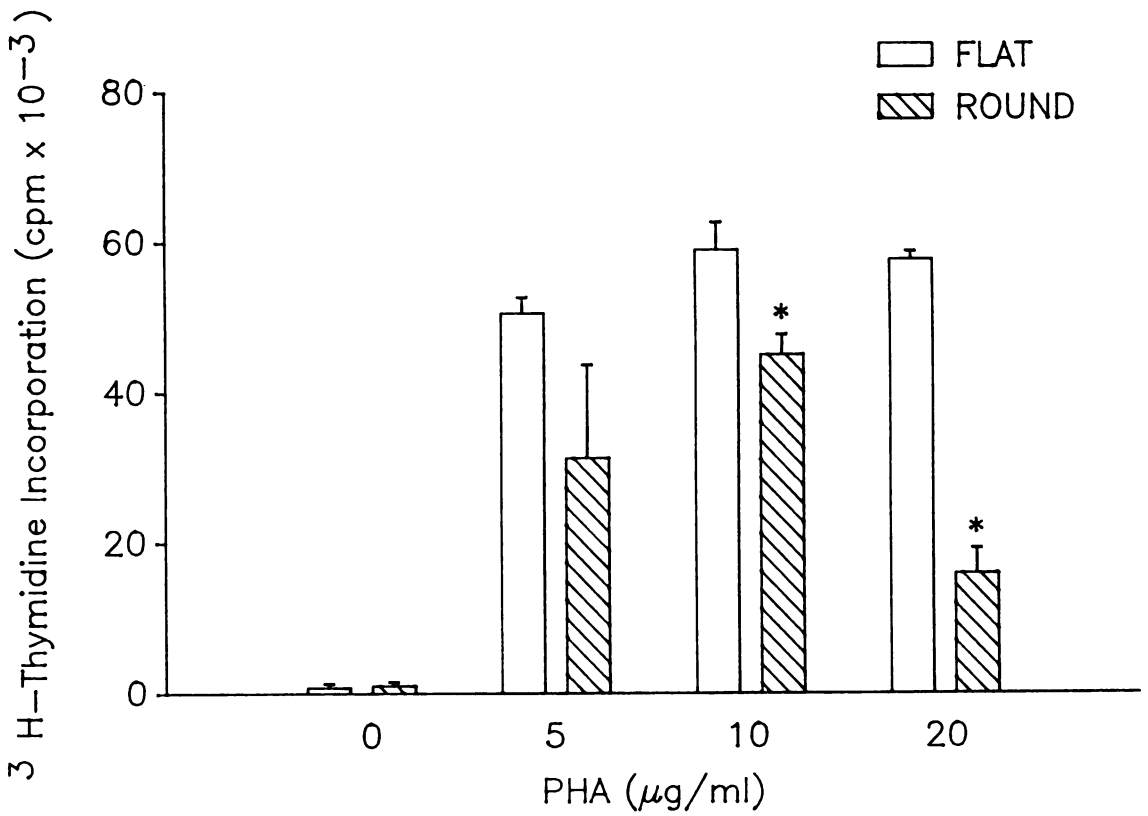



Figure 5. Comparison of the proliferative response by lymphocytes cultured in two types of tissue culture plates. Lymphocytes were cultured in DMEM-10% FBS in either flat- or round-bottom well plates and stimulated with PHA at 10 μg/ml. Cells were pulsed with $^{3}$H-thymidine after 48 h in culture and harvested 18 h later to measure incorporation of thymidine into DNA. Asterisk (*) indicates statistical difference from thymidine incorporation by cells cultured in flat-wells at the same mitogen concentration when analyzed by a Student's t Test ($p < 0.01$). Each bar represents the mean ± SD, $n = 4$.

TABLE 1

Carnitine Content for Unstimulated and PHA-Stimulated Lymphocytes

| Cell State[a] | Total | Free | Acyl-carnitine | % Acylation |
|---|---|---|---|---|
| | nmol carnitine/mg DNA: | | | |
| Unstimulated | 10.63 ± 0.66[b] | 8.95 ± 0.71 | 1.68 ±0.38 | 16% |
| Stimulated | 22.51[c] ± 3.02 | 14.24[c] ± 2.22 | 8.27[c] ± 0.93 | 37%[d] |
| | nmol carnitine/mg protein: | | | |
| Unstimulated | 2.86 ± 0.42 | 2.40 ± 0.34 | 0.46 ± 0.14 | 16% |
| Stimulated | 2.35 ± 0.24 | 1.46[c] ± 0.14 | 0.89 ± 0.11 | 38%[d] |

[a] Lymphocytes were cultured in DMEM-C (10% FBS) supplemented with 0.1 mM carnitine for 66 h. Stimulated cell cultures were incubated in the presence of PHA (10 μg/ml) while unstimulated cells did not receive mitogen.

[b] Mean ± SEM of 5 healthy adults between the ages of 21-40 years.

[c] statistically different from unstimulated cells when analyzed by a Paired t Test ($p < 0.05$)

[d] statistically different from unstimulated cells when analyzed by a Signed Rank Test ($p < 0.05$)

stimulated protein vs 2.86 nmol/mg control protein). Free carnitine also increased when expressed on a per mg DNA basis (8.95 nmol/mg control DNA vs 14.24 nmol/mg but it decreased when expressed on a per mg cell protein basis (2.40 nmol/mg control protein vs. 1.46 nmol/mg stimulated protein). Total acylcarnitine levels increased approximately 5-fold in response to stimulation when expressed as nmol per mg DNA (1.68 nmol/mg control DNA vs. 8.27 nmol/mg stimulated DNA).

Percentage of carnitine found to be acylated increased two-fold as a result of stimulation (Table 1). This shift in the degree of acylation was evident regardless of how the data was expressed, either per mg DNA or per mg protein. When analyzed by a nonparametric statistical test for paired data, percentage of total carnitine acylated in stimulated cells differed significantly from unstimulated cells.

Carnitine Acyltransferase Analysis

Carnitine acetyl- and palmitoyltransferase activities were measured for the first time in unstimulated and PHA-stimulated lymphocytes. The presence of these activities provided additional evidence for carnitine's involvement in short-and long-chain acylCoA metabolism in lymphocytes. Discontinuous (end point) enzyme assays were used which measured the formation of radioactive acylcarnitines when cell lysates were incubated in the presence of radiolabelled acylCoA substrates. Dowex 1-X8 minicolumns were used to

separate substrates from products by retention of acylCoAs but not acylcarnitines. Figure 6 is a representative CAT enzymatic rate measured in stimulated lymphocytes, linear rates were also measured in unstimulated cells (not shown). Figure 7 is a CPT enzymatic rate measured in unstimulated lymphocytes; linear rates for CPT activity were also measured in stimulated cells, data not shown. Formation of product appeared to be linear during the reaction time for both CAT and CPT indicating that substrate concentrations were maintained at saturating levels throughout the assay.

To demonstrate formation of the desired product (acetylcarnitine for CAT and decanoylcarnitine for CPT) during the enzyme assay, aliquots of Dowex minicolumn eluates were analyzed by HPLC. When cell lysates were incubated in the presence of labelled acetylCoA for 0, 1, 2 and 3 min, a peak was observed on HPLC chromatographs with a retention time corresponding to acetylcarnitine (Figure 8). Likewise, a decanoylcarnitine peak appeared when radiolabelled decanoylCoA served as substrate (Figure 9). The small peak with a retention time at 4-5 min in Figure 9 was probably decanoate resulting from hydrolysis of decanoylcarnitine during HPLC sample preparation. Since the zero time point did not contain this peak, it was unlikely that this decanoate represented leakage from the Dowex resin which should retain this carboxylate.

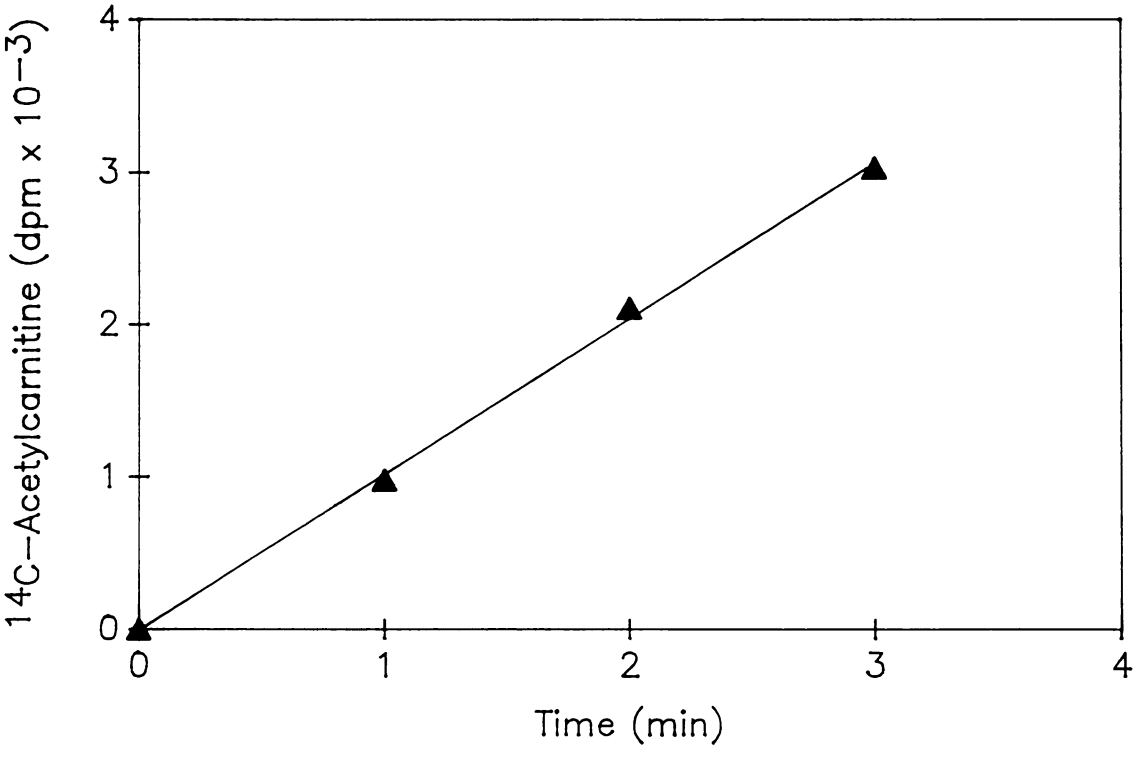



Figure 6. CAT activity in PHA-stimulated lymphocytes. Cells were stimulated in culture by PHA (10 µg/ml) in DMEM-C (10% FBS) for 66 h. Enzyme assay conditions were 25 mM potassium phosphate buffer, pH 7.4, 0.1 mM $^{14}$C-acetylCoA, 5 mM carnitine and 1 mM N-ethylmaleimide. Amounts of $^{14}$C-acetylcarnitine formed in 0, 1, 2 and 3 min were determined. This rate represents activity of lymphocytes from one individual.

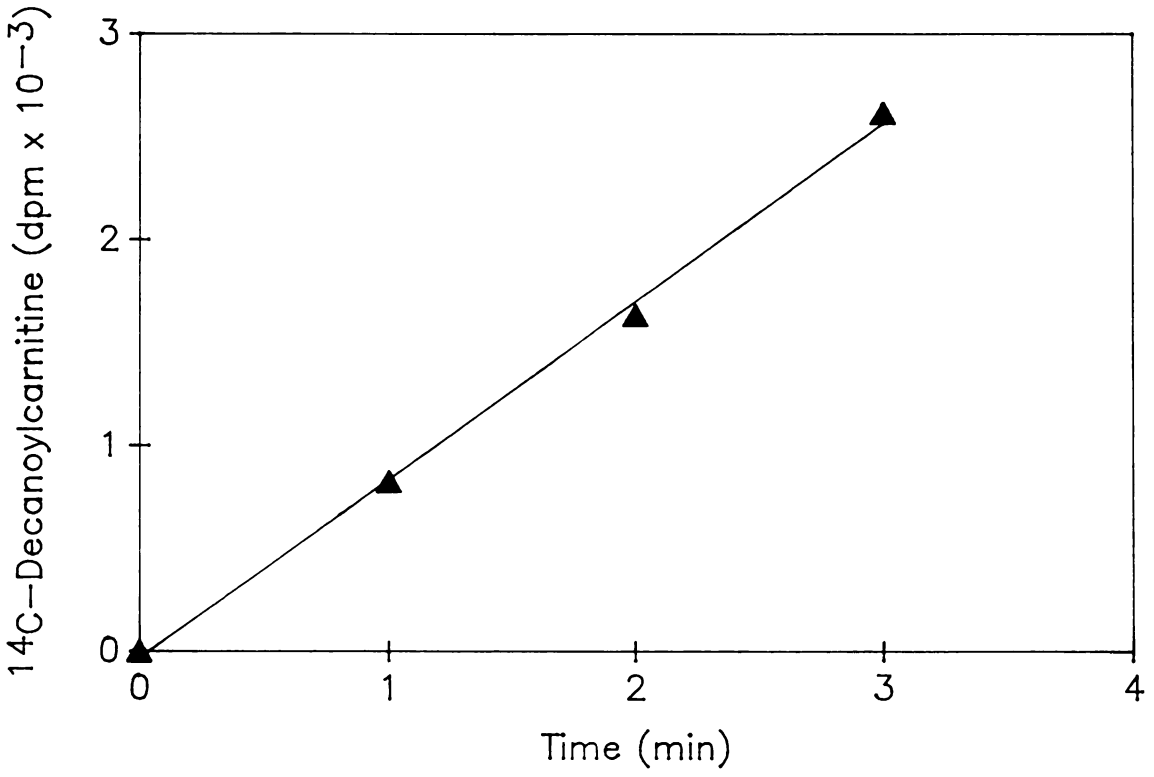



Figure 7. CPT activity in resting lymphocytes. Cells were maintained in DMEM-C (10% FBS) for 66 h. Enzyme assay conditions were 25 mM potassium phosphate, pH 7.4, 40 μM $^{14}$C-decanoylCoA, 10 mM carnitine and 1 mM N-ethylmaleimide. Amounts of $^{14}$C-decanoylcarnitine formed in 0, 1, 2 and 3 min were determined. This rate represents activity of lymphocytes from one individual.

Figure 8. HPLC analysis of acetylcarnitine formation by PHA-stimulated lymphocytes. Cell homogenates were incubated for 0 (a), 1 (b), 2 (c) and 3 min (d) in the presence of radiolabelled acetylCoA and then analyzed by HPLC as described in experimental section. Peak areas represent amounts of radioactivity detected by the flow-through ß-counter which monitors HPLC effluent.

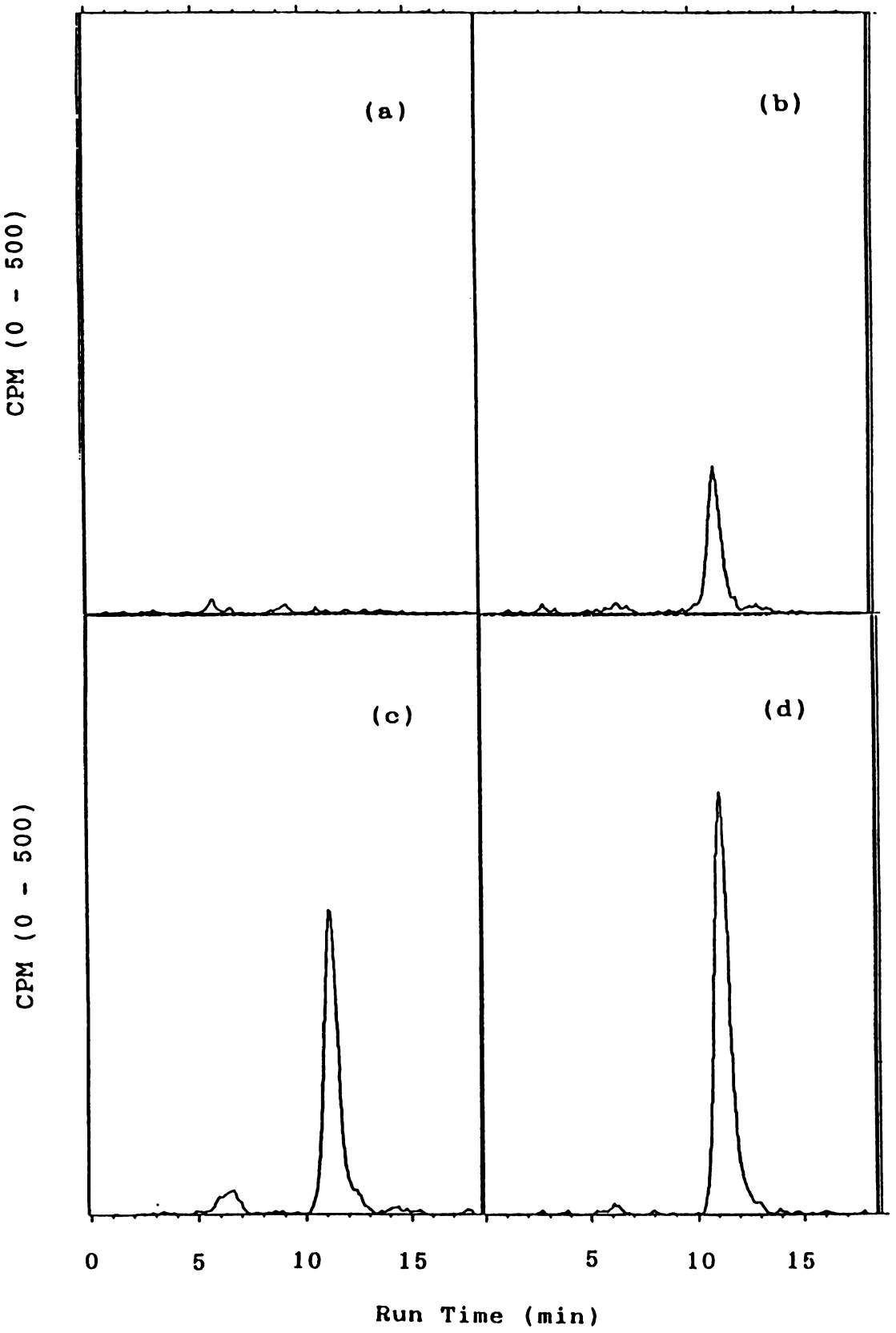
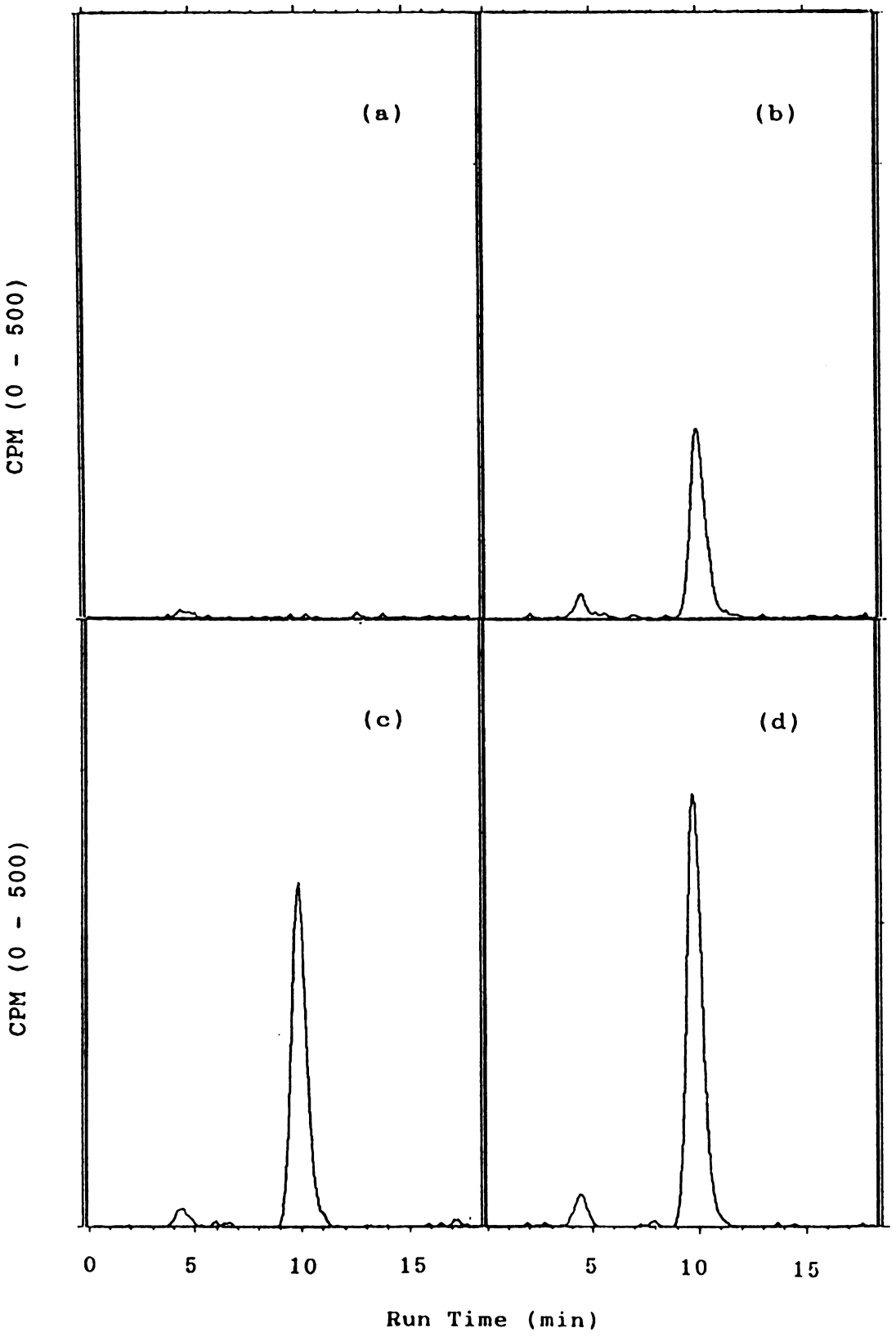

(a)
(b)
(c)
(d)
CPM (0 - 500)
CPM (0 - 500)
0
5
10
15
5
10
15
Run Time (min)

Figure 9. HPLC analysis of decanoylcarnitine formation by PHA-stimulated lymphocytes. Cell homogenates were incubated for 0 (a), 1 (b), 2 (c) and 3 min (d) in the presence of radiolabelled decanoylCoA as described in experimental section. Peak areas represent amounts of radioactivity detected by the flow-through ß-counter which monitors HPLC effluent.


(a)
(b)
(c)
(d)
CPM (0 - 500)
CPM (0 - 500)
0
5
10
15
5
10
15
Run Time (min)

The specific activities of CAT and CPT measured in unstimulated and stimulated lymphocytes are shown in Table 2. Short-chain acyltransferase (CAT) activity in unstimulated cells was 2.3 nmol/min-mg protein or 9.2 nmol/min-mg DNA. Mitogen-treated cells contained similar levels (1.3 and 10.5 nmol/min per mg protein and per mg DNA, respectively). Long-chain acyltransferase (CPT) activity was dependent upon assay conditions employed. For example, CPT activity for unstimulated cells assayed in the presence of KCL/Hepes buffer (25.9 nmol/min-mg DNA) was three-fold greater than activity assayed in phosphate buffer (8.2 nmol/min-mg DNA). However, in both buffer systems CPT activity was greater in PHA-stimulated cells than unstimulated cells when expressed per mg DNA (Table 2). These data were not analyzed for statistical differences due to small numbers of individuals tested (n= 1-3).

TABLE 2

Carnitine Acyltransferase Activities in Unstimulated and PHA-Stimulated Lymphocytes

I. Carnitine Acetyl Transferase Activity:

| Cell State[a] | nmol/min | |
|---|---|---|
| | Per mg DNA | Per mg protein |
| Unstimulated | 9.2 ± 1.3[b] | 2.3 ± 1.4 |
| PHA-Stimulated | 10.5 ± 2.3 | 1.3 ± 0.3 |

II. Carnitine Palmitoyl Transferase Activity:

| Cell State | nmol/min | |
|---|---|---|
| | Per mg DNA | Per mg protein |
| Unstimulated | 8.2[c] | 2.8[c] |
| | 25.9[d] | 2.8[d] |
| PHA-Stimulated | 16.4[c] | 2.2[c] |
| | 57.7[d] | 4.6[d] |

[a] Lymphocytes were cultured in DMEM-C (10% FBS) supplemented with 0.1 mM carnitine for 66 h. Stimulated cells were incubated in the presence of PHA (10μg/ml) while unstimulated cells did not receive mitogen.

[b] CPT activity assayed in 25 mM $KP_i$ buffer, pH 7.5 (n=1).

[c] CPT activity assayed in 120 mM KCL, 20 mM Hepes, 1 mM EGTA, pH 7.5, 1% octylglucoside (n=1).

# DISCUSSION

Results of the experiments reported in this chapter demonstrated the effects of activation of human peripheral blood lymphocytes upon carnitine levels, its degree of acylation and acyltranferase activities. Cells were stimulated in culture prior to carnitine analysis using the mitogen PHA. Measurement of $^{3}$H-thymidine incorporation into cellular DNA allowed assessment of degree of lymphocyte proliferation in response to the mitogen. In this way the relative metabolic state of cells could be verified for carnitine studies; mitogen-treated cells were shown to be rapidly undergoing cell proliferation while unstimulated cells remained in the resting state. Thymidine incorporation was indicative of rates of DNA synthesis and was not a measure of actual numbers or proportions of cells stimulated (75).

Mitogenic stimulation resulted in several changes in carnitine content. Total, free and acylcarnitine content per mg of lymphocyte DNA increased in response to activation suggesting an increased carnitine requirement for stimulated cells. It was unlikely that carnitine was synthesized endogenously by lymphocytes since only liver, kidney and brain possess the enzyme necessary for the final reaction in the carnitine biosynthetic pathway (76, 77), however activity of this enzyme in lymphocytes was not investigated. It is more probable that the increased carnitine content observed

in lymphocytes upon activation was the result of enhanced carnitine uptake.

These increased levels of carnitine were in agreement with the work of Adlouni, et. al. who studied patients with inflammatory disorders and found that the presence of infection in these patients increased the free and acylcarnitine content in mononuclear cells when expressed on a per cell basis (49). Unfortunately they did not separate lymphocytes from the mononuclear cell fraction, preventing direct comparisons with our data. Nevertheless, their results provided additional support for increased carnitine content within lymphocytes during an activated immune state.

In contrast to results observed per mg DNA, expression of this same data per mg cell protein revealed no significant changes in total carnitine content. The reason for this discrepancy between the two methods of data expression may be due to the increased cell volume of mitogen-treated cells giving stimulated cells a greater protein content than unstimulated cells. The work of Poulton and coworkers showed that twenty-four hours following addition of PHA 82% of T cells had increased in volume (78). The fact that carnitine concentration remained relatively constant with respect to protein content suggested that increased cellular protein associated with activation must have been accompanied by increased carnitine uptake. This was supported by the finding that carnitine content increased per mg DNA, indicative of

uptake by stimulated cells. Thus, increased carnitine levels within the cell (i.e. per mg DNA) were paralleled by increased cellular protein content. This may suggest that the greater size of stimulated lymphocytes necessitated an increase in carnitine levels to accommodate the oxidative needs of this larger cell. Another less likely possibility was that increased carnitine levels in stimulated cells reflected a greater requirement for increased fuel utilization. However, normal metabolic changes such as increased fuel utilization are usually marked by alterations in the percentage of carnitine acylated not changes in total carnitine content per mg protein (30, 71).

Probably the most significant effect of lymphocyte activation upon carnitine metabolism was a doubling of the percentage of total carnitine acylated. It was already known that during activation rates of RNA, protein and DNA synthesis were dramatically increased accompanied by increased levels of fuel utilization (70). Increased carnitine acylation suggested that carnitine was involved in this shift in the intermediary metabolism of activated cells. This increase in acylation could not be interpreted as increased flux through a particular pathway, since both increased and decreased flux can result in increased acylation of carnitine.

A possibility for the increased degree of acylation may be related to carnitine's role in modulation of the

acylCoA/CoA ratio. Isolated rat mitochondria oxidizing pyruvate increased their production of acetylcarnitine as levels of acetylCoA increased (Lysiak, 79). This served to replenish reduced CoA for continuation of oxidative processes within the matrix. Levels of CoA were limiting in the matrix of the mitochondria making this function of carnitine potentially important for efficient utilization of carbohydrate, branched-chain amino acids and long-chain fatty acids (23). Increased acylation in activated lymphocytes suggested a higher level of acylCoAs in equilibrium with cellular carnitine pools. If this increased acylation reflected an increase in the acylCoA/CoA ratio within the mitochondrial matrix, then carnitine may be very important in short-term metabolite control. In previous studies most acylCoAs examined exerted allosteric effects upon oxidative enzymes within the matrix, such as inhibition of pyruvate dehydrogenase by acetyl CoA (80, 81), propionylCoA and isobutyrylCoA (81). Thus, carnitine is potentially very important for proper functioning of the immune system if it facilitates fuel utilization required to maintain the immune response.

Quantitation of the specific acylcarnitine species in resting and activated cells using techniques described in Chapter 1 of this thesis would show whether this increased percentage of acylation was due to formation of long or short-chain acylcarnitines. This would help elucidate which role

of carnitine was affected by lymphocyte activation, its obligatory role in fatty acid oxidation or its facilitative role in the metabolism of short-chain acylCoAs derived from either carbohydrates, amino acids and/or fatty acids. These studies were not performed in experiments included in this chapter.

Addition of exogenous carnitine to lymphocyte cultures at a final concentration of 0.1 mM did not enhance or inhibit the proliferative response. Cultures which did not receive supplementation still contained approximately 1-2 μM carnitine due to addition of fetal bovine serum to medium. This concentration was much lower than normal serum levels (50 μM), however, no differences in lymphocyte function were observed at these low carnitine concentrations as compared to carnitine supplemented cultures. This does not necessarily mean that carnitine was not important for lymphocyte metabolism during activation. Turnover time for carnitine within a tissue usually takes several days, approximately 8 days for skeletal muscle and 65 days for whole body (83). Thus, it was unlikely that cellular carnitine levels were severely depleted during our 66 h culture period. In addition, carnitine uptake has been shown to take place against a concentration gradient by a saturable active transport process in a variety of cell types from several species (83), so even these low levels of carnitine in the surrounding medium may have been sufficient to satisfy carnitine requirements during our short culture

period. Thus, carnitine levels present in FBS supplemented medium may have been sufficient to supply the carnitine requirements of lymphocytes during a short 3 day culture period.

Our investigations into carnitine involvement in lymphocyte metabolism included measurement of enzyme activities of the acyltransferases responsible for acylcarnitine formation. Lymphocytes possessed both short- and long-chain acyltransferase activity when analyzed by the radiochemical end point assay described. Levels of CAT remained unchanged upon mitogenic-stimulation of lymphocytes. This was consistent with it's postulated role in buffering against rapid changes in tissue content of acylCoAs (30). To act as a buffer the enzyme activity must exceed maximum rates of other enzymes involved in formation and breakdown of acylCoAs. Therefore, increased buffering requirements do not require increased CAT activity.

Work of several investigators had suggested that lymphocytes oxidize fatty acids as an important fuel source (10). The presence of CPT activity in lymphocytes provided additional evidence for this, since activated long-chain fatty acids must first be converted to acylcarnitines before entering the mitochondrial matrix for ß-oxidation. CPT levels increased as a result of lymphocyte activation when expressed on a per mg DNA basis. This increased activity was not necessarily the result of increased amount of enzyme.

Norum found that CPT activity increased in rat liver during fasting and this increase was not prevented by inhibitors of protein synthesis (84). Although regulation of CPT activity is poorly understood, some investigators believe that CPT is closely involved in regulation of fatty acid oxidation by controlling substrate entry into the matrix. There are numerous examples in the literature of alterations in levels of CPT in response to a change in physiological state (84, 85, 86). For example, during fasting CPT levels were shown to be elevated in response to increased demands upon fatty acid oxidation to supply energy needs (46, 84, 87, 88). These data suggested that CPT activity present within a tissue may be rate-limiting to allow CPT to serve as a metabolic regulatory point. In contrast, studies on CPT activity and fatty acid oxidation in liver mitochondria of neonatal pigs suggested that CPT levels did not necessarily limit the rate of fatty acid oxidation (89). Whether or not CPT was involved in regulation of fatty acid oxidation during the proliferative response, it was clear that activation resulted in an increase in activity within the cells indicating that it must be important to accommodate the oxidative needs of activated lymphocytes. In conclusion, human peripheral blood lymphocytes were cultured for 66 h under defined culture conditions and were stimulated by a mitogen to simulate the immune response in culture. This was done to determine the effects of activation upon carnitine

metabolism within lymphocytes. These series of experiments showed that the change in metabolism associated with activation was accompanied by subsequent changes in carnitine status. Total carnitine content increased along with the percentage of that carnitine found to be acylated. The doubling in degree of carnitine acylated provided evidence for a dramatic shift in the metabolic state during the proliferative response. Acyltransferase enzymes were also found to be present within control and stimulated lymphocytes. Short-chain acyltransferase activity was not affected by stimulation providing support for its role as a buffer of acylCoA levels within the mitochondrial matrix. In contrast, CPT activity increased in response to activation suggesting that fatty acid oxidation was important during proliferation. Our results provided considerable evidence for the involvement of carnitine in the intermediary metabolism of the lymphocyte both in resting and activated states and demonstrated that the shift in the metabolic state during lymphocyte activation was reflected in carnitine metabolism.

# SUMMARY AND CONCLUSIONS

Lymphocytes and MNP, two cellular components of the immune system, are central to humoral and cell-mediated immunity. In spite of their importance to host defense, little is known about their metabolic requirements. The purpose of this thesis was to further characterize the intermediary metabolism of these cells by investigating the involvement of carnitine in their fuel utilization. Carnitine is required for oxidation of long-chain fatty acids (21, 22) and in some tissues facilitates utilization of other fuels such as glucose and amino acids (23).

In Chapter 1 it was reported that human peripheral blood lymphocytes and MNP isolated from healthy adults contained moderate amounts of carnitine and acylcarnitines relative to other body tissues such as skeletal muscle which contains high carnitine levels. Mononuclear phagocytic cells contained higher levels of carnitine than resting lymphocytes suggesting a greater carnitine requirement for MNP metabolic processes. The percentage of carnitine acylated measured in resting lymphocytes was similar to that observed in most other body tissues, while MNP demonstrated a much higher percentage. This large degree of acylation suggested an alternative role for carnitine in MNP. It may reflect metabolic differences between these two cell types.

Analysis of the individual acylcarnitines provided insight into possible roles for carnitine in lymphocytes and MNP. Acetylcarnitine was the predominant acylcarnitine in both cell types. Its formation was postulated to be the result of carnitine's role in modulation of the acylCoA/CoA ratio within the mitochondrial matrix. This function of carnitine facilitates efficient energy utilization because formation of acetylcarnitine from acetylCoA replenishes reduced CoA which is required for continuation of oxidative processes. Unusually high levels of acetylcarnitine were observed in MNP in comparison to lymphocytes, approximately six-fold greater. In MNP acetylcarnitine may function as a source of readily available activated acetyl groups to serve as an energy source upon immunological challenge.

In addition to acetylcarnitine, other aliphatic SCAC were detected in lymphocytes and MNP. The presence of propionyl- and (iso)butyrylcarnitine indicated that cells were oxidizing some branched-chain amino acids. Long-chain acylcarnitines were also detected in both MNP and lymphocytes demonstrating carnitine's involvement in ß-oxidation of long-chain fatty acids. Therefore, fats may serve as an important fuel source necessary to maintain an immune response. In summary, these experiments clearly demonstrated carnitine's involvement in both long and short-chain acylCoA metabolism within lymphocytes and MNP. Differences in their

carnitine profiles may indicate that carnitine's role varies with the individual cell type.

Experiments reported in Chapter 2 demonstrated that mitogenic stimulation of lymphocytes induced resting lymphocytes to proliferate and led to increased levels of carnitine per mg DNA. However, no increases were observed when carnitine values were expressed on basis of protein. It was concluded that the increased volume of stimulated cells resulted in increased uptake of carnitine to accommodate the oxidative needs of this larger more metabolically active cell.

Lymphocyte activation also resulted in a dramatic shift in percentage of carnitine acylated indicative of a metabolic change upon stimulation. This shift was probably the result of an increase in the steady-state concentrations of acylCoA pools in equilibrium with carnitine, thus carnitine may be serving to replenish reduced CoA for continuation of oxidative processes. This facilitative function of carnitine may be important during the proliferative response.

The carnitine acyltransferase enzymes responsible for acylcarnitine formation were measured for the first time in cell lysates from resting and activated lymphocytes. Both short and long-chain acyltransferase activities were present which reaffirmed carnitine's involvement in both short and long-chain fatty acid metabolism within lymphocytes. CPT activity increased as a result of stimulation suggesting

that oxidation of fatty acids was important during lymphocyte activation.

Studies presented in this thesis aided in elucidation of the function(s) of carnitine in lymphocytes and MNP and helped to further define the intermediary metabolism of these cells. Clearly, these cells offer a readily accessible human tissue capable of diverse metabolic states in which to study the ß-oxidative and non-ß-oxidative roles of carnitine. Further investigations are needed to explain the unusually high degree of acylation observed in the phagocytic MNP and to further characterize changes in the levels of acylcarnitines associated with lymphocyte activation. In view of the critical role lymphocytes and MNP play in host defense, further characterization of their metabolic requirements is essential if researchers hope to understand compromised immune function when fuels are limited such as in nutritional deficiencies and various disease states.

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