AGENTS AFFECTING CARNITINE UPTAKE IN THE ISOLATED CAPUT EPIDIDYMIDIS OF RATS *IN VITRO*

WIPAPORN PHATVEJ

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (TOXICOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-663-929-3

Thesis

entitled

AGENTS AFFECTING CARNITINE UPTAKE IN THE ISOLATED CAPUT EPIDIDYMIDIS OF RATS *IN VITRO*

Wipaporn Phatvej

Miss Wipaporn Phatvej
Candidate

Chumpol Pholpramool

Assoc. Prof. Chumpol Pholpramool, Ph.D.
Major-advisor

Pawinee Piyachaturawat

Assoc. Prof. Pawinee Piyachaturawat, Ph.D.
Co-advisor

Panas Chalermsanyakorn

Asst. Prof. Panas Chalermsanyakorn, M.D.
Co-advisor

G. Chaturapanich

Asst. Prof. Ganyapong Chaturapanich, Ph.D.
Co-advisor

Liangchai Limlomwongse

Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies

Jutamaad Satayavivad

Assoc. Prof. Jutamaad Satayavivad, Ph.D.
Chairman
Master of Science Programme in Toxicology
Faculty of Science

Thesis

entitled

AGENTS AFFECTING CARNITINE UPTAKE IN THE ISOLATED CAPUT EPIDIDYMIDIS OF RATS *IN VITRO*

was submitted to the Faculty of Graduate Studies, Mahidol University for the degree of Master of Science (Toxicology)

on

May 1, 2000

Wipaporn Phatvej

Miss Wipaporn Phatvej
Candidate

G. Chaturapanich

Asst. Prof. Ganyapong Chaturapanich, Ph.D.
Member

Chumpol Pholpramool

Assoc. Prof. Chumpol Pholpramool, Ph.D.
Chairman

Anant Srikhao

Assoc. Prof. Anant Srikhao, D.V.M., Ph.D.
Member

Pawinee Piyachaturawat

Assoc. Prof. Pawinee Piyachaturawat, Ph.D.
Member

Liangchai Limlomwongse

Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Amaret Bhumiratana

Prof. Amaret Bhumiratana, Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest appreciation and sincere gratitude to my advisor, Associated Professor Dr. Chumpol Pholpramool for his considerable guidance of basic research concept which I always remind throughout my thesis work. My advisor, whose experiences, knowledge and constant encouragements enabled me to obtain success in my studies and thesis research.

I would like to express my sincere gratitude and deep appreciation to Assistant Professor Dr. Ganyapong Chaturapanich, who kindly provided me one of the best opportunity to have laboratorial accessories. Her recommendation made me much more enthusiastic in research work. My deep appreciation is given to Associated Professor Dr. Anant Srikhao and Assistant Professor Dr. Panas Chalermsanyakorn for their helpful comments, guidance and valuable advises about this thesis. I also would like to express my heartful gratitude to Associated Professor Dr. Pawinee Piyachaturawat for her suggestions throughout the preparation of thesis. She never lacks her kindness and supports during my study.

I would like to thanks Miss Saithong Intraprasert for her excellent technical assistance in this studies.

I also would like to thanks my friends at the Faculty of Science, Mahidol University, and old friends for their understanding and moral supports throughout this study.

Finally, I would like to express my deepest appreciation to my dearest family for their infinite love and kindness throughout this study.

Wipaporn Phatvej

3936413 SCTX/M : MAJOR: TOXICOLOGY; M.Sc. (TOXICOLOGY)
KEY WORDS : TRANSPORT / CARNITINE / EPIDIDYMIS

WIPAPORN PHATVEJ: AGENTS AFFECTING CARNITINE UPTAKE IN THE ISOLATED CAPUT EPIDIDYMIDIS OF RATS *IN VITRO*. THESIS ADVISORS: CHUMPOL PHOLPRAMOOL, Ph.D., PAWINEE PIYACHATURAWAT, Ph.D., GANYAPONG CHATURAPANICH, Ph.D., PANAS CHALERMSANYAKORN, M.D. 79 p. ISBN 974-663-929-3

Carnitine is accumulated against a very high concentration gradient in the luminal fluid of epididymis in most species. However, the mechanism of transport in this tissue is largely unknown in spite of the implication of carnitine in the regulation of sperm motility. This study, therefore, investigated the tubular uptake of ^{3}H-L-carnitine in sperm-free, isolated caput epididymidis of rats *in vitro*. Uptake of labeled carnitine showed saturation kinetics with an estimated Michaelis-Menton's Km of 95.1 μM and Vmax of 11.9 mmole/mg-60 min. The transport system exhibited stereospecificity for L-carnitine. The uptake was inhibited by a structurally related compound with a three-carbon backbone containing a terminal carboxyl group such as γ-butyrobetaine, acetylcarnitine and octanoylcarnitine. On the other hand, glycine enhanced the uptake, but trimethyllysine and γ-aminobutyrate failed to compete with carnitine. In addition, substrates which have been shown to interact with organic cation or anion transporters of many tissues, i.e. tetraethylammonium, N′-methylnicotinamide and cepharolidine, had virtually no effect on carnitine uptake in the epididymis. The uptake is highly temperature and Na-dependent. It was suppressed by the respiratory inhibitor, KCN, but not in the absence of glucose. Both sulfapyridine and sulfanilamides which are male antifertility agent, did not alter carnitine uptake. These results suggest that transport of carnitine across the basolateral membrane requires a carrier which is Na-dependent and stereospecific. The carrier is probably distinct from the organic cation or anion transporters. The source of energy for the transport system is primarily from the oxidative phosphorylation. The antifertility activity of some sulfonamides is not associated with their interferences with carnitine uptake in the caput epididymidis.

3936413 SCTX/M : สาขาวิชา : พิษวิทยา; วท.ม. (พิษวิทยา)

วิภาพร พัฒน์เวช : สารที่มีผลต่อการขนส่งสารคาร์นิตินในท่อพักเชื้ออสุจิของหนูพุกขาว (AGENTS AFFECTING CARNITINE UPTAKE IN THE ISOLATED CAPUT EPIDIDYMIDIS OF RATS *IN VITRO*) คณะกรรมการควบคุมวิทยานิพนธ์: ชุมพล ผลประมูล, Ph.D., ภาวิณี ปิยะจตุรวัฒน์, Ph.D., กัลยพงษ์ จตุรพาณิชย์, Ph.D., พนัส เฉลิมแสนยากร, M.D. 79 หน้า. ISBN 974-663-929-3

คาร์นิตินถูกสะสมแบบต้านความเข้มข้นในท่อพักเชื้ออสุจิของสัตว์เกือบทุกชนิด อย่างไรก็ตามกลไกการขนส่งนี้ยังไม่ทราบแน่ชัดทั้งๆที่คาร์นิตินมีส่วนในการควบคุมการเคลื่อนไหวของเชื้ออสุจิ ได้ทำการศึกษากลไกและฤทธิ์ของสารต่างๆต่อการขนส่งของแอล-คาร์นิตินติดฉลากตริเตรียม ในท่อพักเชื้ออสุจิส่วนต้น (caput epididymidis) ของหนูพุกขาวที่แยกออกจากตัวสัตว์ทดลอง พบว่าการขนส่งของคาร์นิตินแสดง saturation kinetics และจาก Michaelis-Menton's หาค่า Km และ Vmax เท่ากับ 95.1 μM และ 11.9 mmol/mg-60 min ระบบการขนส่งนี้มีความจำเพาะต่อแอล-คาร์นิตินเท่านั้นและถูกยับยั้งโดยสารคาร์บอนอะตอม 3 หน่วยเป็นองค์ประกอบหลักและมีกลุ่มคาร์บอกซิล 1 กลุ่ม เช่น γ-butyrobetaine, acetylcarnitine และ octanoylcarnitine ขณะที่ betaine, choline, γ-aminobutyrate และ trimethylyine ไม่มีผล ในทางกลับกัน glycine ช่วยทำให้การขนส่งของคาร์นิตินเพิ่มขึ้น นอกจากนี้สารที่ถูกขนส่งโดยระบบขนส่งสารประเภท organic cation หรือ organic anion ในเนื้อเยื่อต่างๆ เช่น tetraethylammonium, N′-methylnicotinamide และ cepharolidine ไม่มีผลต่อการขนส่งของคาร์นิตินในท่อพักเชื้ออสุจิ อุณหภูมิ และ Na มีผลต่อการขนส่งของคาร์นิตินด้วย การขนส่งนี้จะถูกยับยั้งโดย KCN แต่ไม่ถูกยับยั้งหากขาดกลูโคส สาร sulfapyridine และ sulfanilamides ซึ่งเป็นสารที่ยับยั้งการสืบพันธุ์ในเพศชายก็ไม่มีผลต่อการขนส่งของคาร์นิตินเช่นกัน

จากผลการทดลองสรุปได้ว่าการขนส่งของคาร์นิตินผ่าน basolateral membrane ต้องการตัวพาซึ่งต้องอาศัย Na และขนส่งเฉพาะแอล-คาร์นิติน ตัวพานี้แตกต่างจากตัวพาที่ขนส่ง organic cation หรือ organic anion แหล่งของพลังงานในการขนส่งของระบบนี้ได้มาจาก oxidative phosphorylation ส่วนยาซัลฟาที่ยับยั้งการสืบพันธุ์ในเพศชายไม่มีผลต่อการขนส่งของคาร์นิติน

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRIVATIONS

γ-	gamma
ATP	adenosine triphosphate
ANOVA	analysis of variance
cm	centimeter
Ci	curies
°C	degree celsius
DPM	disintegration per minute
et al.	et alii (and others)
g	gram
^3H	tritium
i.e.	id est
Km	constant for Michaelis-Menten kinetics
L	liter
µl	microliter
µM	micromolar
mg	miligram
mM	milimolar
min	minute
M	mole per liter
n	number

Na^+	sodium ion
pmol	picomole
SEM	standard error of mean
Vmax	transport maximum

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

The epididymis is the site of accumulation, maturation and storage of spermatozoa. When spermatozoa leave the testis, they are physiologically immature. However, they acquire motility and capacity to fertilize ova. The epithelium lining of this organ is believed to contribute to their maturation (1). Sulfonamides (salicylazo sulfapyridine), a compound formed by azo linkage between sulfapyridine and 5-amino salicylic acid, was first introduced to treat rheumatoid arthritis in 1930 (2). More recent evidence indicates that the sulfapyridine moiety, which is a metabolic breakdown product of sulfasalazine by bacteria in the gut (3) or its metabolite is responsible for the antifertility effect in man (4) and in rat (5). In rat sulfasalazine and sulfapyridine do not alter testicular weight and histology, gonadotrophin and testosterone concentration in the epididymis, or daily sperm production rate and sperm concentration in the epididymis (5). They have studied the antifertility effect of several type of sulfonamides and related compounds in the rats and found that sulfapyridine was the most effective (6). The effectiveness of their sulfonamides seem to be related to the ability to pass into the epididymal fluid, again indicating that the site of action of sulfonamide drugs in the epididymis but a mechanism is not known.

Carnitine ((-)-3 hydroxy-4-N-dimethylaminobutyrate) is present in the lumen of the epididymis in high concentration. The physiological significance of the high

carnitine concentration in the epididymis is unknown, but metabolic and osmotic roles have been proposed and it may be associated with the acquisition of flagella activities by maturing spermatozoa (7). Extensive investigations have been conducted on the transport of carnitine in various organs including liver, kidneys, intestine, and muscles. However, little is known in the epididymis. In the rat, carnitine is accumulated by the epithelial of the epididymal caput and corpus and then secreted into the fluid (8). Microperfusion studies showed that the rate of carnitine secretion into the luminal fluid was highest in the caput epididymidis (9). The aims of this study are to determine the characteristics of carnitine transport in the epididymis and to test whether sulfapyridine and other sulfonamide compounds interfere with carnitine transport in the epididymis. Uptake of ^{3}H-L-carnitine into the isolated segment of caput epididymal (tubule) of rats, whose luminal contents was replaced by water saturated paraffin oil was investigated *in vitro* after incubation at various times or in the presence of various substances.

OBJECTIVES

The main objectives of this investigation are:

1. To study the characteristics of carnitine uptake in the rat epididymis with an emphasis on the basolateral membrane of the epithelium.

2. To test whether some sulfonamides such as sulfapyridine interfere with carnitine transport through the basolateral membrane of the caput epididymidis.

CHAPTER II

LITERATURE REVIEW

Following the process of spermatogenesis, the spermatozoa, which are almost completely immotile, are transported passively from the seminiferous tubules to the rete testis. The rat rete testis is a branched reservoir into which both ends of each seminiferous tubule open. The rete testis is linked to the epididymis by the 10 to 20 vasa efferentia that are located near the upper pole of the testis. These efferent ducts become highly convoluted as they reach the epididymis. The epididymis is a single, long, highly convoluted duct on the posterior border of the testis. It has usually been divided into three regions, the head, the body, and the tail (caput, corpus and cauda) (10). The following sections describe in more details the structure and function of mammalian epididymis (Figure 1).

EPIDIDYMIS

1. Structure

The epididymis is generally considered to be of mesonephric (Wolffian) duct in origin, arising from the upper segment of the duct. This tissue develops during gestation under the influence of testosterone, not dihydrotestosterone. The location of the epididymis together with the testis in the scrotum results in the maintenance of epididymal temperature several degrees below that of core body temperature (11). The epididymis comprises a duct system leading spermatozoa and fluid from the testis

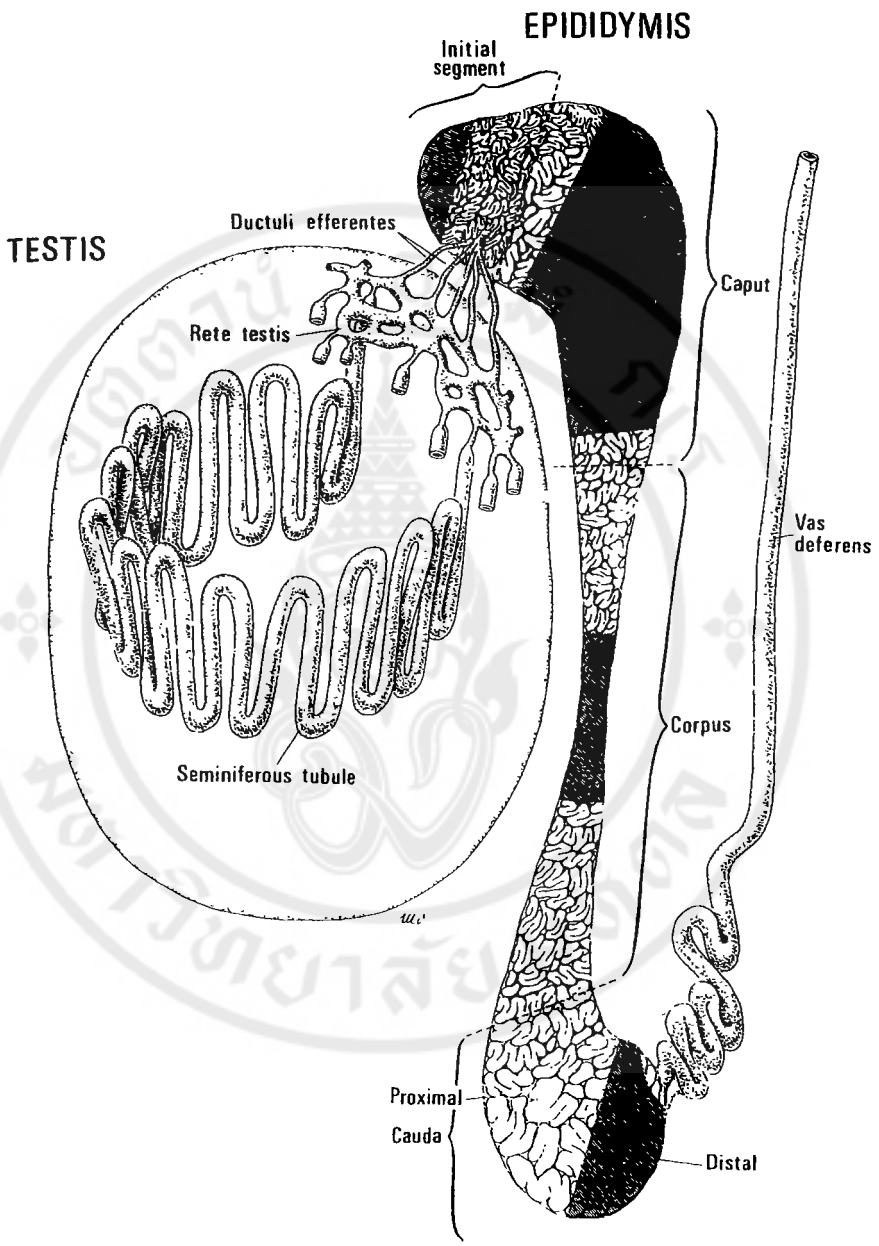


Figure 1. The testis showing a seminiferous tubule and the rete testis, the ductuli efferents, the epididymis, and vasdeferens. The shaded regions indicate areas of the epididymis, i.e., the initial segment, caput, corpus, and proximal and distal cauda.

to the vasdeferens (12). The tubule is a single highly convoluted duct, closely applied to the surface of the testis extending from the anterior to the posterior pole of that organ by connective tissue. The duct is coiled into segments determined by connective tissue septula, and the organ is contained within a fibrous tissue capsule. The segment into which the ductuli efferents empty is usually referred to as the initial segment and the less of the epididymis is loosely defined into three parts termed the caput, corpus and cauda epididymidis. The first part of the epididymis or initial segment is characterized by a high epithelium with long straight stereocilia almost obliterate the lumen which is sparsely populated with spermatozoa. The middle segment in which supranuclear vacuoles are prominent in the epithelium, has a wider lumen and the stereocilia are usually bent and sometimes branched. The terminal segment has a lower epithelium; stereocilia are shorter and less dense, and the lumen of the tubule is wider and densely packed with sperm. The epididymal tubule is surrounded by connective tissue which contain fibroblasts, collagen, elastic fibers, blood vessels, lymphatic vessels, nerve fibers, macrophages, wandering leucocytes, and concentric layers of smooth muscle fibers (11). The epithelial lining of epididymal tubule is pseudostratified columnar and consists of principal and basal cells (13).

The lumen of epididymal duct system contains water, ions, small organic molecule, proteins and glycoproteins, spermatozoa and other particulate matters. The rete testis fluid (RTF), which passes to the epididymis by way of the ductuli efferentes, is the initial source of luminal fluid for the epididymis. The composition of the RTF is modified when its progress through the epididymal duct. Sodium ions are passively transported from the luminal fluid at the luminal surface of the epithelial cells and

actively transported at the serosal surface to establish a standing osmotic gradient which draws water and chloride ions from the luminal fluid. Potassium and phosphate ions are retained or secreted into the luminal fluid with the result that their concentrations increase as the fluid travels down the epididymal duct. Another major constituent of epididymal fluid, except in the bull, is carnitine. This compound is transported into the epididymal lumen against a concentration gradient of over 2000:1 by a saturable uptake process (11).

2. Functions

When spermatozoa leave the testis, they are physiologically immature. When they pass through the epididymal duct (a period of about two weeks in the rat) they acquire the capacity to fertilize eggs (12). The epithelium of this organ is believed to contribute to their maturation (1). Spermatozoa are in contact with a specialized microenvironment which has been provided by the epithelial secretions of the seminiferous tubules and epididymal duct. This microenvironment is probably very important for their maturation (14). The fluid of the epididymal duct of mammals provides an appropriate environment for post testicular maturation of spermatozoa (15). Products concentrated from blood into the lumen and proteins secreted by the epididymal epithelium together with testicular factors contribute to the progressive motility and fertility capacity of spermatozoa. The epididymis plays a crucial role in male fertility and defects in its function are responsible for several causes of human infertility. It is considered a preferential target for antifertility programs. Functions of the epididymis may be classified into:

2.1 Absorption

The epithelia of epididymis can absorb both fluid and particulate matters. Fluid, ions and small organic molecules are absorbed by the caput epididymidis. Both sodium and chloride are absorbed in all regions of the epididymis. The absorption of the organic molecule, L-carnitine, from the proximal caput epididymis was found to be saturable and primarily sodium-independent, whereas this compound was passively absorbed in the cauda (16).

2.2 Secretion

The epididymis can synthesize and secrete small molecules such as glyceryl phosphorylcholine as well as array of glycoproteins. Both potassium and phosphorus are also secreted by this tissue. The three small organic molecules secreted by the epididymis are carnitine, inositol, and glycerylphosphorylcholine (16).

2.3 Transport of spermatozoa

The duration of sperm transit in each segment of the epididymis is proportional to the size of the sperm reserve in that segment. Sperm transit in the caput and corpus epididymis is independent of copulatory frequency, while that of the cauda epididymis is dependent on this frequency. The rate of sperm transit in which animal has been shown to decrease from 420 mm/2 hr in the initial segment to 64 mm/ 2 hr in the distal caput and 25 mm/ 2 hr in the cauda epididymis and vas deferens (16). The mechanisms responsible for driving the contents through the lumen of the epididymis include hydrostatic pressure, muscular contractions, and the action of cilia because sperm in most species are quiescent in the epididymal lumen (16).

2.4 Maturation of spermatozoa

In mammals, spermatozoa leaving the testis do not have the ability to fertilize eggs. After exposure to the special environment during epididymal transit spermatozoa acquire the fertilizing potential. Association with the acquisition by spermatozoa of the ability of fertilizer eggs is a gain in potential for motility. This gain in motility potential is a required but not sufficient conditions for spermatozoa to become fertile. Although, in the epididymis, spermatozoa are not motile, they acquire this capacity for movement as they traverse the epididymis. A number of potential factors have been proposed as regulators or mediators. These include forward-motility protein, acidic epididymal glycoprotein and albumin, carnitine, cyclic AMP, sperm motility inhibiting factor, sperm-motility quiescence factor, immobilin, and ambient conditions. During epididymal maturation of spermatozoa there are changes in their biochemical, morphological, and physiological properties shown in the table below.

Biochemical changes	Morphological changes	Physiological changes
Phospholipids	Membranes	Cold shock (susceptibility)
Protein (structural)		
Cholesterol	Acrosome (swelling)	Permeability
Protein (enzyme)	Cytoplasm	Lectin binding
Glycoprotein		Negative charge
Calmodulin	Cytoplasmic droplet	Motility
cAMP	Migration	Metabolism
New component		Zona pellucida binding ability

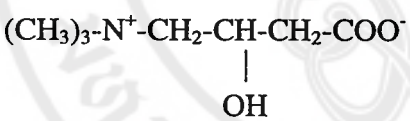
2.5 Storage of spermatozoa

The major site for storage of spermatozoa is the cauda epididymidis. The normal transit time in mammals through the cauda epididymis is in the range of 3 to 10 days, spermatozoa can be stored in this tissue for long periods of time extending beyond 30 days, in bats, spermatozoa may be stored in this tissue for many months and retain their function (1).

CARNITINE

1. Structure and biosynthesis of carnitine

Carnitine is a 3-hydroxy-4-trimethylamino butyric acid having the structure shown below:



Carnitine in the body is derived from two sources, i.e. exogenous and endogenous sources. Exogenous carnitine is derived from the diet. Animal tissues contain very much higher levels of carnitine than microorganisms or plants and the richest source of carnitine is in muscles of both vertebrates and invertebrates.

Endogenous source: N-methyl group of carnitine is derived from the methyl group of methionine. Lysine is the precursor of carnitine both in Neurosporacrana and the rat, respectively. Σ-N-Trimethyllysine stemming from lysine can be converted to γ-butyrobetaine which is further hydroxylated to form carnitine. Liver is the active site of carnitine biosynthesis. Dietary carnitine provides an important source of the compound for the body (17). Once dietary or orally administered carnitine is

absorbed, the liver will be exposed to the carnitine via the portal vein prior to systemic distribution or elimination.

1.1 Absorption and distribution

Carnitine is a dietary constituent and absorption from the gastrointestinal tract is a major route. It is a small and water soluble molecule in plasma and is readily filtered by the renal glomeruli and is actively reabsorbed by the renal tubule. Carnitine is concentrated in the tissue at much higher concentration than that in plasma.

1.2 Excretion

Carnitine in the normal animals is lost mainly by excretion in the urine. Thus more than 95% of carnitine in the glomerular filtrate is reabsorbed (18). Both free carnitine and acylcarnitines are excreted.

1.3 Function

Role of carnitine in fatty acid oxidation; carnitine serves as a shuttle for long-chain acyl residues across the inner membrane of the mitochondria. This is a prerequisite for the fatty acid oxidation providing energy to the cell. Carnitine can combine with activated long-chain fatty acid to form fatty acylcarnitine and then pass through the inner mitochondrial membrane, which is impermeable to acyl CoA and to carnitine but not to acylcarnitine. Then fatty acylcarnitine reacts with CoA to form fatty acyl CoA within the mitochondrial matrix where B-oxidation occurs.

2. Transport carnitine in the epididymis

In general experimental studies have used radiolabeled carnitine uptake as a measure of carnitine transport. The update of radioactivity by the epididymis and other

tissues had been measured following administration of L-[methyl-^{3}H] carnitine to male rats. Rapid uptake occurred in both the caput and cauda epididymis. The radioactivity was shown to be present in carnitine fraction and was located almost exclusively within the epididymal lumen (12). The transfer of carnitine from blood into the prefused, sperm-free lumen of segments of the epididymis has been examined in anesthetized rats. The entry rate showed a significant ($p < 0.001$) positive and linear correlation with the length of the perfused epididymis. In addition, carnitine transport was independent of the tonicity of the perfusing solution (9). Measurement of the concentration of carnitine in the luminal fluid along the rat epididymis indicated an abrupt increase in concentration in the caput epididymidis (19). In rats, the carnitine concentration in the cauda epididymal fluid can reach 60 mM, or 2,000 times the concentration in the blood (18). Data from micropuncture and perfusion techniques identified the caput and corpus epididymidis as more active regions than the cauda epididymidis in the transport of L-carnitine from the circulation to the epididymal lumen.

L-carnitine transport by rat renal brush border membrane vesicles was stimulated by a Na^{+} gradient. An overshoot was observed for total carnitine entry in the presence of a Na^{+} gradient. L-carnitine transport was saturable. Binding of carnitine were prevented by incubation of the cell with excess carnitine, indicating that this uptake was by a specific receptor mediator (15). The transport process was structure-specific for a quaternary nitrogen and carboxyl groups attached by a 4 to 6 carbon chain (20).

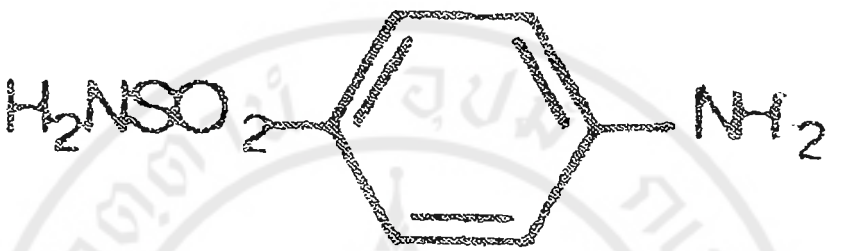
3. Role of carnitine in male reproduction

It is not known if carnitine plays any specific role in the epididymis. Recent evidence suggests that acetylcarnitine, carnitine acetyltransferase and carnitine may play an active role in the motility of spermatozoa. Carnitine is probably involved in the motility of rat spermatozoa. First there was the association of the appearance of carnitine and the capacity for motility and second there was the stimulation effect of carnitine on the motility of pre-motile spermatozoa (14). Acetyl-L-carnintine and acetyl-D-carnitine stimulate the motility of spermatozoa with low initial motility. This study suggests that carnitine may be important in the development by spermatozoa of the potential for motility and also to maintain mature spermatozoa in a quiescent state. Studies in human semen showed correlation between infertility and the level of carnitine. Those with low carnitine concentration infertility.

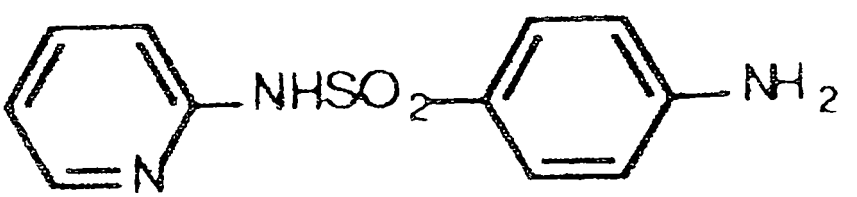
SULFONAMIDE DRUGS

1. Structure

Sulfonamides were first used as chemotherapeutic agents in 1935, in the treatment of infections. The therapeutic action of this group of compound depended on its breakdown in the body into an inactive dye and an antibacterial substance called "sulfanilamide" (p-amino-benzene sulfonamide).



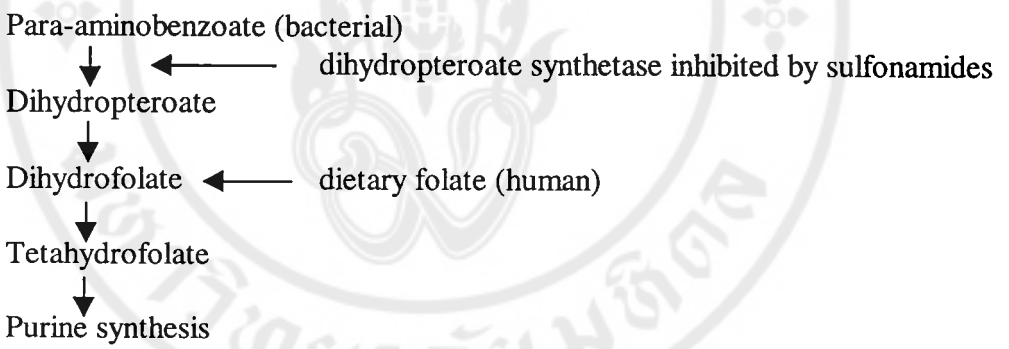
It occurs as a white, practically odorless crystalline substance, slightly bitter with sweet after taste. It soluble in hot water, hot alcohol and cold acetone. The first successful sulfonamide substitution is sulfapyridine (2 (p-aminobenzene-sulfonamide)) pyridine.



This compound is a white, crystalline, tasteless solid, soluble in water at ordinary temperature.

2. Sulfonamides as antibacterial drugs

Sulfonamides exhibit broad spectrum activity. A streptococci and pneumococci are highly susceptible. Sulfonamides act as competitive antagonists of para-aminobenzoic acid and inhibit folic acid synthesis. Folic acid derivatives are essential for purine and ultimately DNA synthesis in both humans and bacteria. Bacterial cells appear to be impermeable to folic acid and synthesize it from para-amino-benzoic acid, whereas humans absorb preformed folic acid from their diet. Sulfonamides act by inhibiting the bacterial enzyme dihydropteroate synthetase, which catalyzes the conversion of para-aminobenzoate to dihydropteroate (21).



3. Sulfonamides as antifertility agent

Sulfasalazine, a drug prescribed for the treatment of inflammatory bowel disease, has been shown to cause male infertility, which is associated with seminal abnormalities. The abnormalities include reduced sperm density and mobility and abnormal sperm morphology (22). These changes usually revert to normal about 2 months after cessation of sulfasalazine. This side effect appears to be due to the sulfapyridine component of the drug. When given by the oral route, only a small amount of sulfasalazine is absorbed in the small intestine. The major fraction is transported to the colon where it is metabolized by bacteria to yield sulfapyridine and

5-aminosalicylic acid. The former is readily absorbed by the colon. Sulfapyridine was in fact the active moiety of sulfasalazine in human (24) and in rats (23). It appears from the studies of structure-activity relationship that the pyridine ring is not essential but the presence of N^4-amino group is required for the activity. Full activity is retained after the pyridine group is replaced with hydrogen atoms or an alkyl group. Thus, the prototype of the antifertility sulfonamids is sulfanilamide (23).

CHAPTER III

MATERIALS AND METHODS

ANIMALS:

Mature male rats of the Wistar strain weighing approximately 300-400 g were obtained from the National Animal Center at Salaya Campus, Mahidol University. They were kept separately in stainless-steel hanging cages under ambient temperature and 12-hour light-dark cycle. The rats were fed with laboratory rat chow and tap water ad libitum.

TISSUE PREPARATION:

Epididymis were rapidly removed from mature Wistar rats after overdoses of ether, then transferred to an ice-cold medium containing Tyrode's medium (137 mM-NaCl, 11.9 mM-$NaHCO_3$, 5.4 mM-KCl, 0.42 mM-NaH_2PO_4, 1.8 mM-$CaCl_2$-$2H_2O$, 1.05 mM-$MgCl_2$-$6H_2O$, 5mM-D-Glucose, 20 mM-Trizmabase and 36 μM-carnitine). Each epididymis was stripped off adhering fat and the caput was separated from the distal segment. In all experiments, the outer part of the caput capsule was cut open and carefully removed. The lobes of tubule coil were gently pulled apart along natural planes of cleavage. The tubules at sites 3, according to Yeung et. al (9) were carefully uncoiled under a stereomicroscope and the luminal contents were flushed with Sudan black stained paraffin oil (Figure 2). One end of the tubule was cannulated with polyethylene tubing, the diameters of which were reduced over a flame to approximate

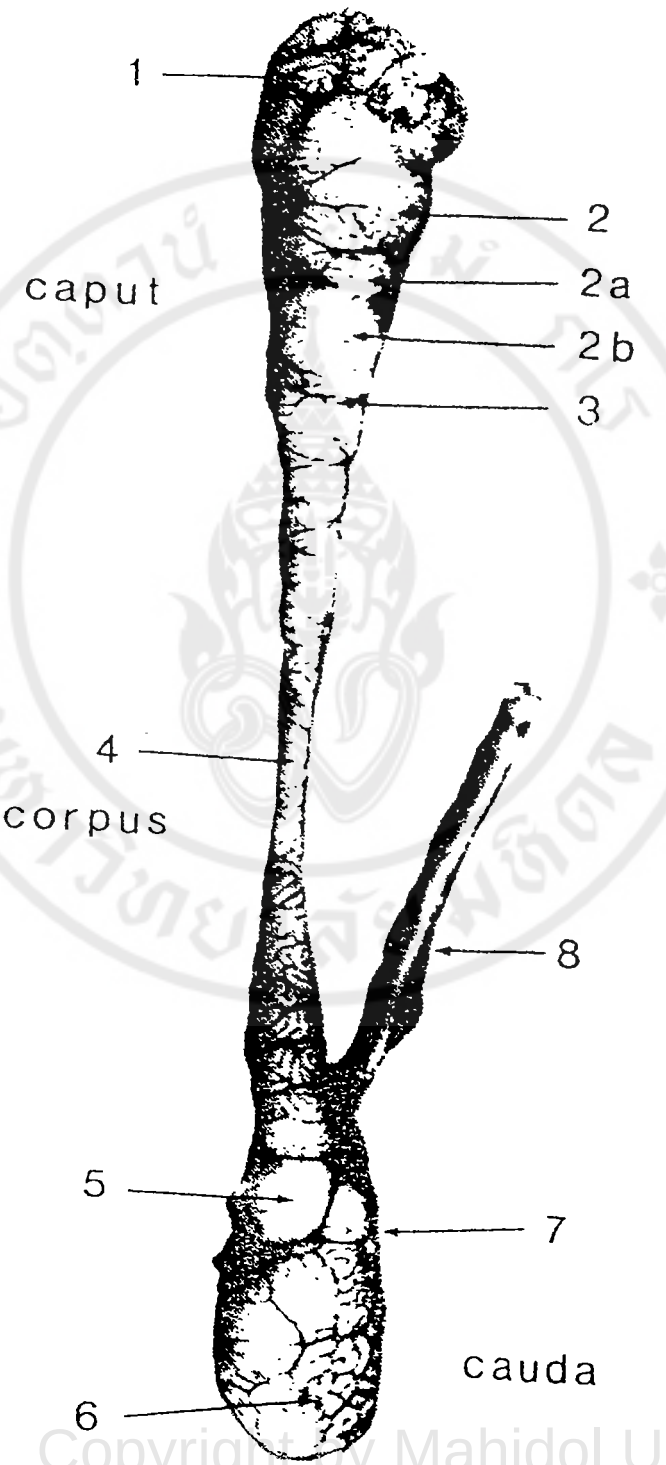


Figure 2. A rat epididymis show the site of this study; site 3: distal caput.

by the diameter of the tubule. The other end of the cannula was connected to a syringe infusion pump (Model 901A, Harvard). The tubule about 2 cm. in length was infused with Sudan black-stained oil at the rate of 0.00164 ml./min. for 1 minute. After the tubule was cleaned of its luminal content and then was no evidence of leakage along the length of the sperm-freed tubule, both ends of the tubule were tied off with silk thread (US.610). The preparation of the tubules took less than 45 minutes after removal from the animal. The sperm-freed tubule was transferred to an airate (5% CO_2 and 95% O_2) bath fluid containing 1 ml Tyrode's medium to which 2.5 µCi of L-[^{3}H] carnitine (82.0 Ci/mmole; Amersham life science) was added. The tubule was then incubated for 60 min. at 34 °C. At the end of the incubation period 20 µl incubation medium was pipetted and added into 5 ml liquid scintillation fluid. After that the tubule was removed and put on a piece of tissue paper to remove the adhering fluid then washed 3 times in Tyrode's solution containing cold carnitine. The tubule was dried on a tissue paper and the ligatures on both ends of the tubule were cut. The open-end tubule was then immersed under paraffin oil in a weighing cup of known weight. The cup containing tubule was re-weighed on an ultramicrobalance (Sartorius 4503 micro Germany). Wet weight in mg. of the tubule was obtained from the difference between the cup weight in the absent of the tubule. The tubule was blotted on a tissue paper to remove excess oil, and then transferred into a small plastic centrifuge tube containing 200 µl 3% TCA. The tubule was left for 1 night at room temperature. After extraction, 100 µl TCA solution was pipetted into a 5 ml scintillation fluid and ^{3}H was estimated in the liquid scintillation system (Beckman LS 6000 Series, U.S.A). The extracted tubule was removed from the centrifuge tube and

placed on a piece of dried filter paper, which had been weighed before. The tubule was put in an oven at 90^0C for 3 nights, or until the tubule weight was constant. Dry weight of the tubule was obtained after subtraction of the dried paper weight.

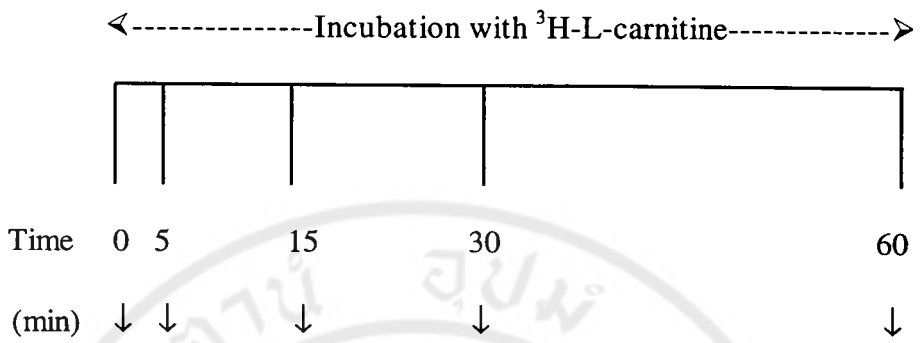
BATH FLUID:

The bath fluid for tissue preparation (Tyrode's solution) consisted of 137 mM-NaCl, 11.9 mM-$NaHCO_3$, 5.4 mM-KCl, 0.42 mM-NaH_2PO_4, 1.8 mM-$CaCl_2$-$2H_2O$, 1.05 mM-$MgCl_2$-$6H_2O$, 5mM-D-Glucode, 20 mM-Trizmabase and 36 μM-carnitine and was adjusted to pH 7.4 with HCl. The incubation medium was the bath fluid to which L-[^{3}H]carnitine (Amersham life science) at a concentration of 0.008 μCi/ml was included. In the kinetic studies medium was further modified by varying the concentration of non-radioactive L-carnitine from 36-500 μM, or cold L-carnitine was replaced by the test substance such as carnitine analogs, organic ions, and some sulfonamide. All chemicals were reagent grade and purchased from the Sigma Chemical Company St.Louis, U.S.A.

EXPERIMENTAL PROTOCOLS.

Experiment 1: Time course of carnitine uptake.

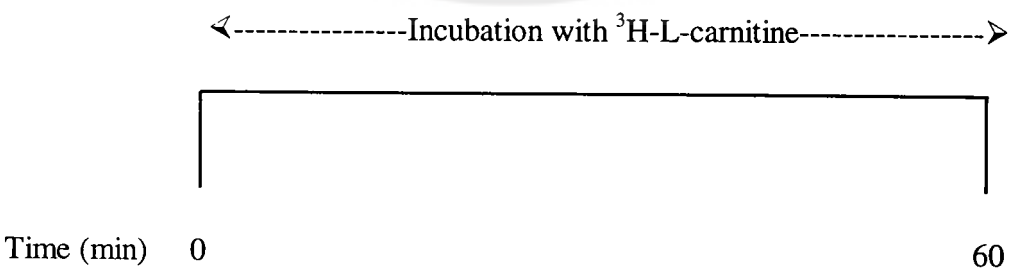
Oil-filled tubes were incubated in Tyrode's solution containing ^{3}H-L carnitine at 34 °C for 5, 15, 30 and 60 minutes (both tubes at the same time). After incubation for 5, 15, 30 and 60 minutes, pairs of tubule were removed and extracted for ^{3}H-L-carnitine as described above. The amounts of ^{3}H-L-carnitine in the tube and bathing solution were measured by liquid scintillation counter.



remove both tubes and bathing medium for measuring ^{3}H-L-carnitine.

Experiment 2: Kinetics of carnitine uptake.

Six oil-filled tubes were incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes with various concentration of carnitine at 36 μM, 59.7 μM, 95.7 μM, 154.3 μM, 271 μM and 497.5 μM. After incubation, pairs of tubule were removed and extracted for ^{3}H-L-carnitine as described above. The amounts of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.

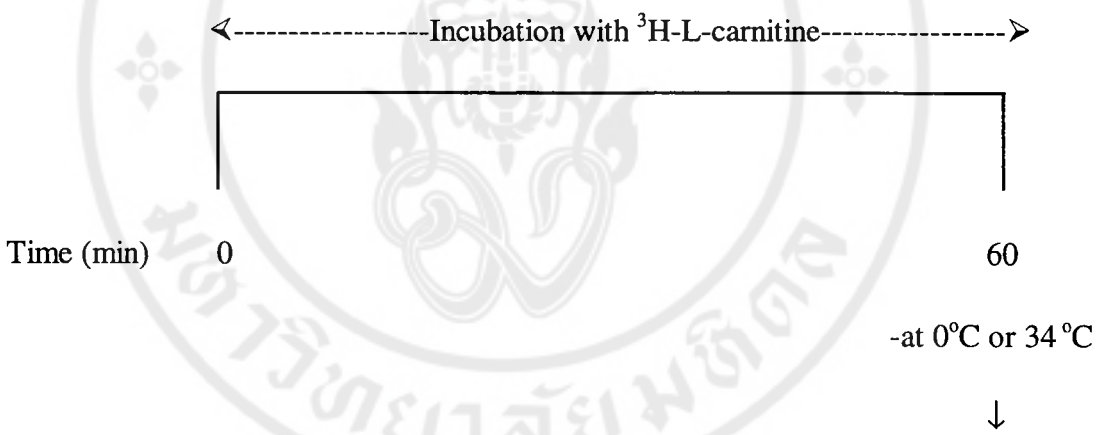


with 36 μM, 60 μM, 96 μM, 154.3 μM, 271 μM and 497.5 μM cold carnitine.

remove both tubes and bathing medium for measuring ^{3}H-L-carnitine.

Experiment 3: Effect of temperature on carnitine uptake.

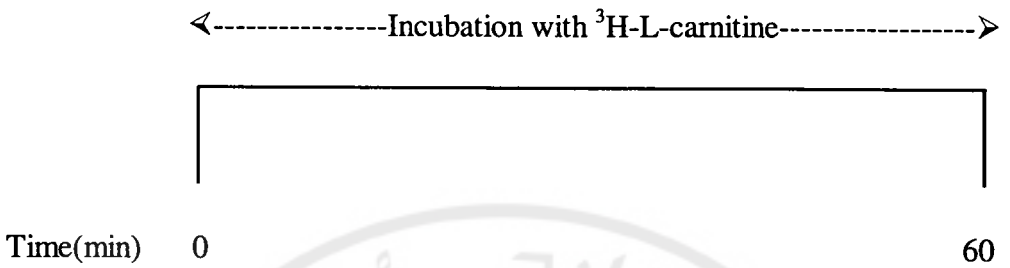
A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L- carnitine at 34°C. for 60 minutes as control, the other pair was incubated at 0°C. in Tyrode's solution containing ^{3}H-L- carnitine for 60 minutes. After incubation, all tubes were removed and extracted for ^{3}H-L- carnitine as described above. The amounts of ^{3}H-L- carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



remove both tubes and bathing medium for measuring ^{3}H-L-carnitine.

Experiment 4: Effect of sodium-free medium.

A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes as control, the other pair was incubated at 34°C for 60 minutes in modified Tyrode medium in which sodium was replaced by N-methyl-D-glucamide. After incubation for 60 minutes, all tubes were removed and extracted for ^{3}H-L-carnitine as described earlier. The amounts of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



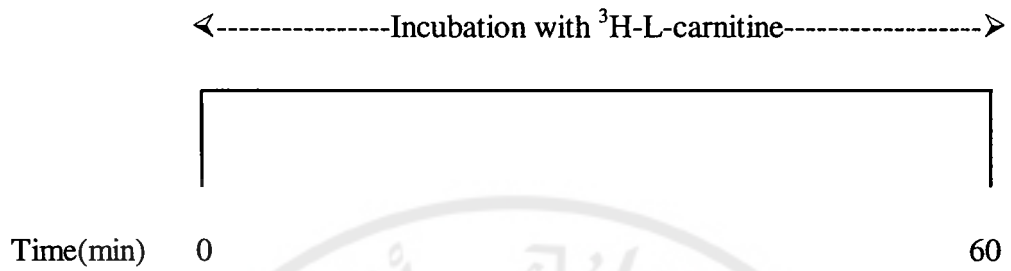
- In the presence or in the absence of Na^{+}.

↓

remove both tubes and bathing medium for measuring ^{3}H-L-carnitine.

Experiment 5: Effect of glucose-free medium.

A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes as control, the other pair was incubated at 34°C for 60 minutes in modified Tyrode medium in which glucose was replaced by manitol. After incubation for 60 minutes, all tubes were removed and extracted for ^{3}H-L-carnitine as described earlier. The amounts of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



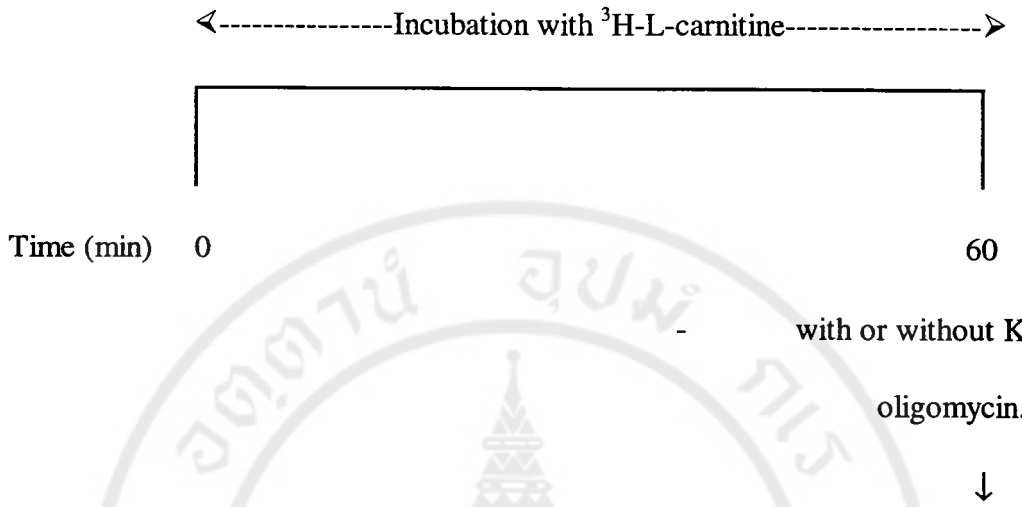
- In the presence or in the absence of glucose.

↓

remove both tubes and bathing medium for measuring ^{3}H-L-carnitine.

Experiment 6: Effects of respiratory inhibitors on carnitine uptake.

A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes as control, the second pair of tubes was incubated at 34°C for 60 minutes in the same medium in the presence of KCN (40 mM), and the third pair of tubes was similarly incubated but after adding oligomycin (30 μM) in the medium. After incubation for 60 minutes, all tubes were removed and extracted for ^{3}H-L-carnitine as descried earlier. The amount of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



Experiment 7: Effect of carnitine analogs and organic ion on carnitine uptake.

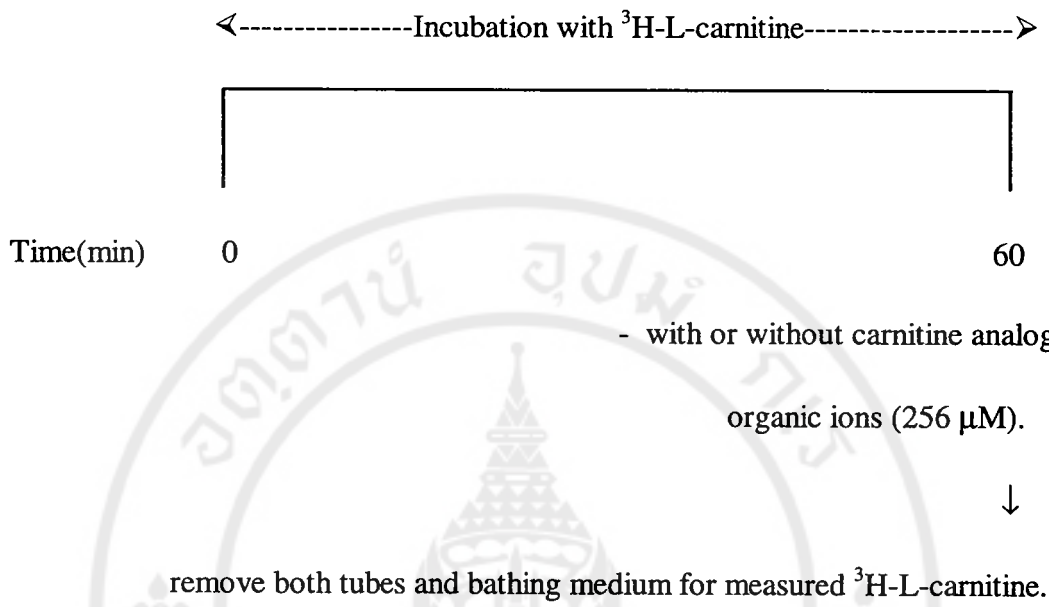
7.1 Carnitine analogs.

A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34° C for 60 minutes as control, the other pair of tubules was incubated at 34° C for 60 minutes in the presence of carnitine analogs.

7.2 Organic ions.

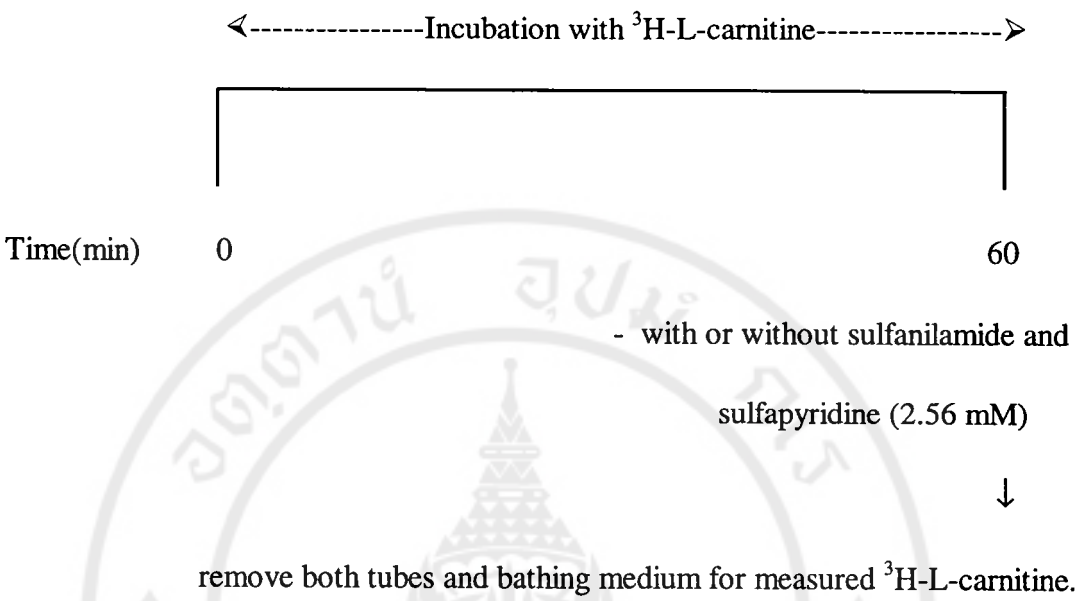
A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes as control, the other pair of tubules was incubated at 34° C for 60 minutes in the presence of organic ion.

After incubation for 60 minutes, all tubes were removed and extracted for ^{3}H-L-carnitine as described above. The amount of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



Experiment 8: Effect of some sulfonamide on carnitine uptake (Sulfapyridine, Sulfanilamide).

A pair of oil-filled tubes was incubated in Tyrode's solution containing ^{3}H-L-carnitine at 34°C for 60 minutes as control, the other pair of tubes was incubated at 34°C for 60 minutes in the presence of some sulfonamide. After incubation for 60 minutes all tubes were removed and extracted for ^{3}H-L-carnitine as described above. The amounts of ^{3}H-L-carnitine in the tube and bathing solution were measured by a liquid scintillation counter.



STATISTICAL ANALYSIS

All data were presented as means and standard errors of mean (mean±SEM). Statistical analyses were performed using SPSS program. For comparisons between different groups (in Experiment 1 and 2), Analysis of Variance (ANOVA) was performed if the data were normally distributed and had equal variance followed by Tukey Method. The unpaired t-test was used to test differences in other experiments. The critical probability for rejection of the null hypothesis was 0.05 throughout.

CHAPTER IV

RESULTS

In all experiments except the studies on the time course, the effect of temperature and the kinetics of carnitine uptake, the data were calculated in pmole of labeled carnitine per mg tissue dry weight. The data from the studies on the time course of carnitine uptake and the effect of temperature on carnitine uptake were presented in rations DPM of labeled carnitine per mg tissue to DPM of the medium since radioactivity in the medium markedly varied from experiments to experiments.

Experiment 1: Time course of carnitine uptake

In order to determine the time course of carnitine uptake, the entry of ^{3}H-carnitine into the distal caput epididymis was studied at various times after incubation. The results are shown in Table 1 and Figure 3. It is apparent that the uptake was linear up to 60 min. incubation. This incubation time was then used in the following experiments.

Experiment 2: Kinetics of carnitine uptake

To investigate whether carnitine uptake in the distal caput epididymidis is saturable, uptake of radiolabeled carnitine in the presence of various concentrations of cold carnitine at 60 min. incubation was studied. In order to illustrated the kinetic data in conventional plots the uptake data were calculated in terms of total carnitine, i.e. labeled plus cold carnitine (see Appendix XI). Carnitine uptake were then plotted

Table 1. Time course of carnitine uptake

Incubation time (min)	Carnitine uptake (DPM/mg tissue)
5	2.35 ± 0.15^{a}
15	5.44 ± 0.53^{a}
30	9.91 ± 1.81^{a}
60	19.20 ± 3.20^{b}
90	20.10 ± 2.89^{b}

Values are mean±SEM, from n = 5

Significantly differences (Oneway ANOVA and Tukey Method) form 5 min are indicated by different letters. $p<0.05$.

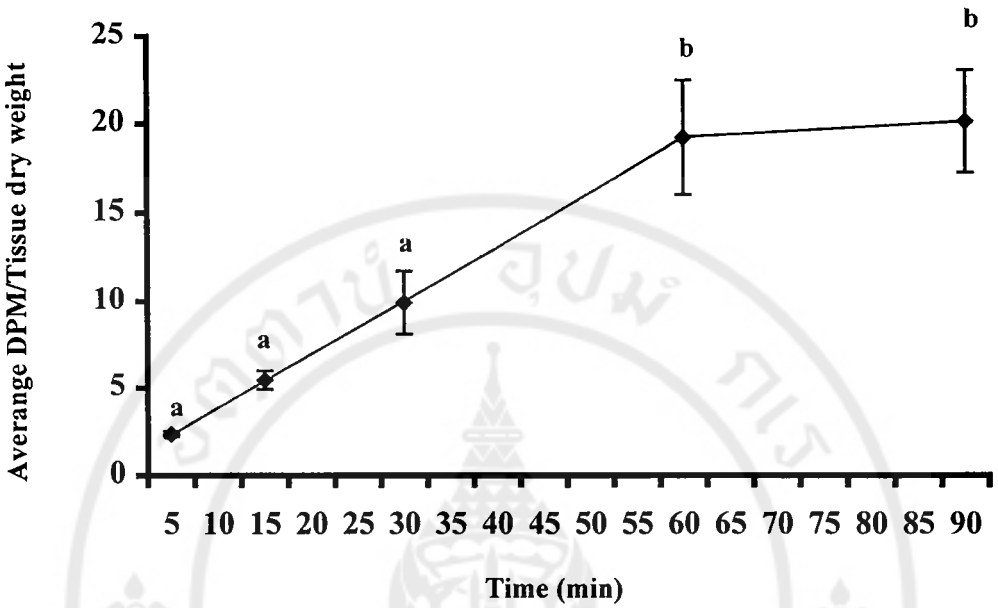


Figure 3. Entry of carnitine into the distal caput of epididymis at various time

Amount of radioactivity in the distal caput epididymides after incubation with L-[^{3}H] -carnitine at time 5, 15, 30 and 60 min. Mean±SEM, from n = 5.

Significantly differences (Oneway ANOVA and Tukey Method) from 5 min are indicated by different letters. $p<0.05$.

versus cold carnitine concentration shown in Figure 4. The results in Table 2 and Figure 4 demonstrate that the uptake was indeed saturable and it reached saturation when the concentration of carnitine was 271 μM in the external medium. This uptake exhibited saturable kinetics and followed the Michaelis-Menten equation. The calculated Km and Vmax values for carnitine were 95.06 μm, and Vmax 11.88 mmole/mg tissue, respectively (Table 2 and Figure 5).

Table 2. Kinetics of carnitine uptake system in the distal caput epididymidis

Conditions	Carnitine uptake (pmole/mg tissue)
In the presence of cold carnitine at conc.(μM)	
36	0.34 ± 0.03^{a}
59.7	0.36 ± 0.03^{a}
95.7	0.50 ± 0.08^{a}
154.3	0.69 ± 0.12^{a}
271	0.97 ± 0.09^{b}
497.5	1.08 ± 0.21^{b}

Values are mean±SEM, from n = 5.

Significantly differences (Oneway ANOVA and Tukey Method) form the uptake at 36 μM cold carnitine are indicated by different letters. $p<0.05$.

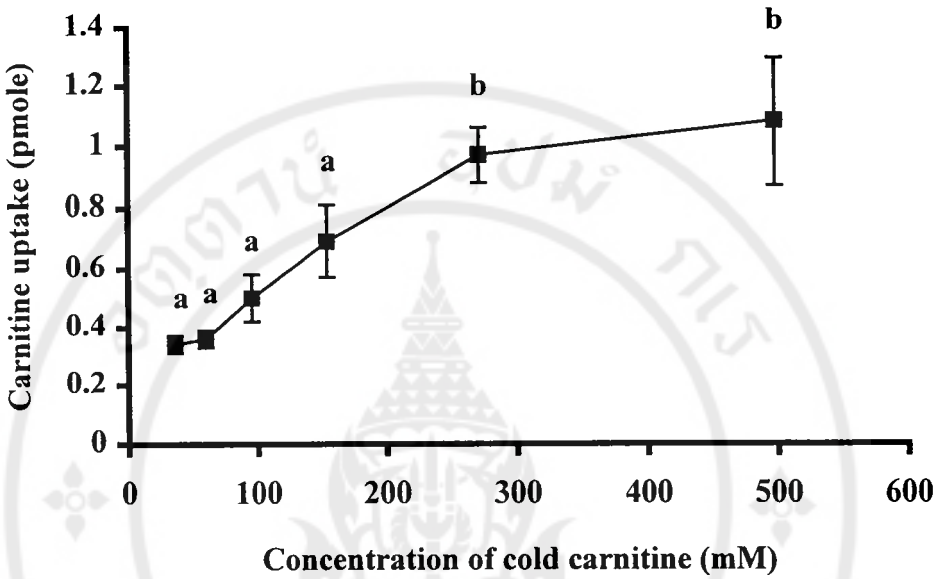


Figure 4. Effect of various concentrations of cold carnitine on carnitine uptake in the distal caput of epididymis tubule Total uptake into the distal caput epididymides after incubation with labeled carnitine in the presence of cold carnitine at 36, 59. 7, 95.7, 154.3, 271 and 497.5 μM. Mean±SEM, from n = 5. Significantly differences (Oneway ANOVA and Tukey Method) from the uptake at 36 μM cold carnitine are indicated by different letters, $p<0.05$.

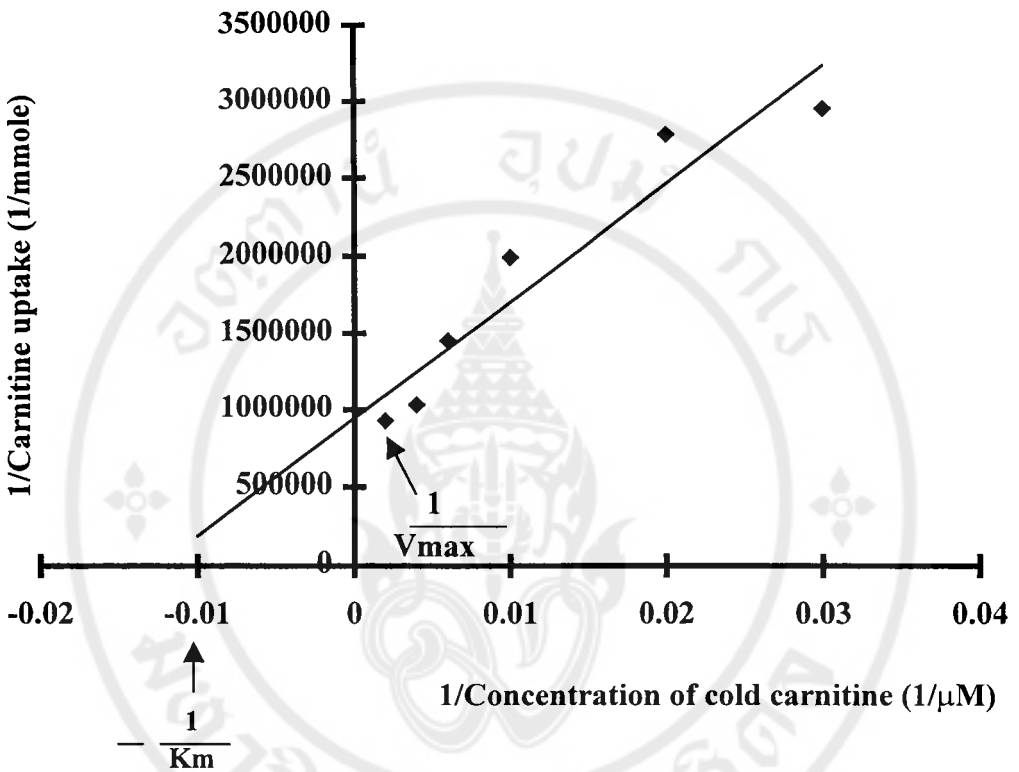


Figure 5. Kinetics of carnitine uptake in the distal caput of the rat epididymis tubule
Calculated Km and Vmax from the best fit equation, i.e.
Y = 8.4184e + 5 + 8.0025e + 7x R^2 = 0.894,
were 95.1 μM and 11.9 mM, respectively.

Experiment 3: Effect of temperature on carnitine uptake

To determine temperature activation of the carnitine transport system, is uptake at 0°C was compared to that at 34°C, which is the scrotal temperature. Reduction of incubation temperature from 34°C to 0°C markedly suppressed the uptake by 87%. This is equivalent to the activation temperature (Q_{10}) of 2.35 (Table 3 and Figure 6).

Experiment 4: Effect of sodium chloride on carnitine uptake

Since results from Experiment 2 and 3 indicate that carnitine uptake through basolateral membrane is probably carrier mediated, it is interesting to test whether this carrier require sodium ion as in other systems. When sodium in the medium was replaced by N-methyl-D-glucamide, carnitine uptake was significantly inhibited being approximately 50% of the control (Table 3 and Figure 8).

Experiment 5: Effect of glucose-free medium on carnitine uptake

To determine whether carnitine uptake requires energy from metabolism, the epididymal tubule was incubated in the medium in which glucose was replaced by manital. Surprisingly, the uptake of carnitine was not altered in the absence of glucose (Table 3 and Figure 7).

Table 3. Effects of low temperature, Na-free medium and glucose-free medium on carnitine uptake in the distal caput of the rat epididymis tubule

Conditions	Carnitine uptake (pmole/mg tissue)	
	Control	Treatment
Low temp (°C)	8.80±0.19 (DPM/mg)	1.14±0.07 (DPM/mg)* (5)
Na-free medium	0.29±0.02	0.13±0.02* (4)
Glucose-free medium	0.16±0.02	0.19±0.01 (6)

Values are mean±SEM (n = rats).

Significantly differences (unpaired t-test) between pmol^{3}H-carnitine/mg tissue dry weight (DPM/mg) of control and treated groups is indicated by an asterisks: *$p<0.05$.

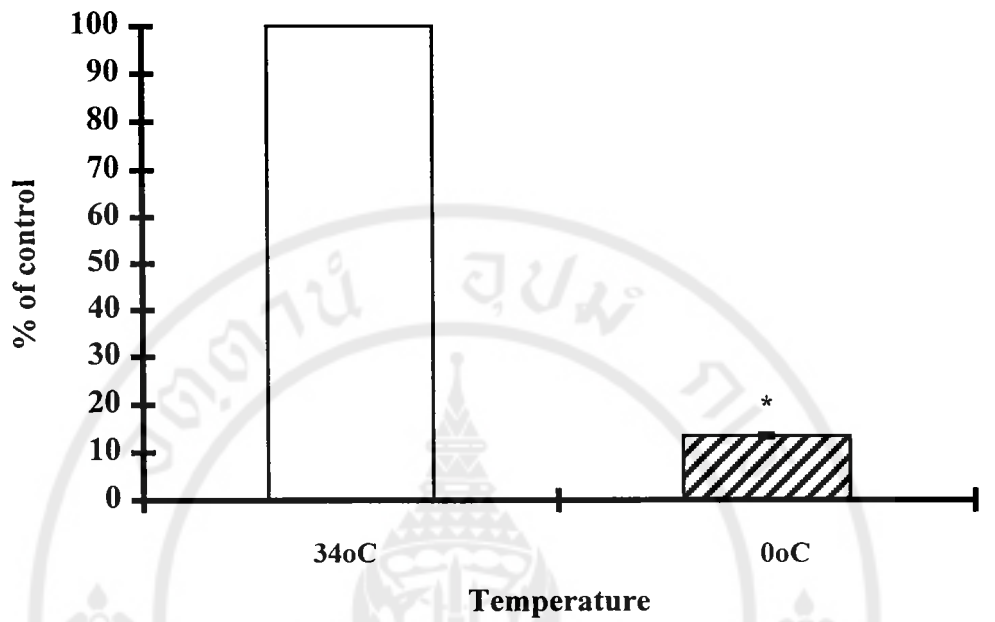


Figure 6. Effect of temperature on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM, from n = 5.
Significant difference (unpaired t-test) between DPM/mg tissue dry weight of control and temperature treated groups is indicated by an asterisk: *$p < 0.05$.

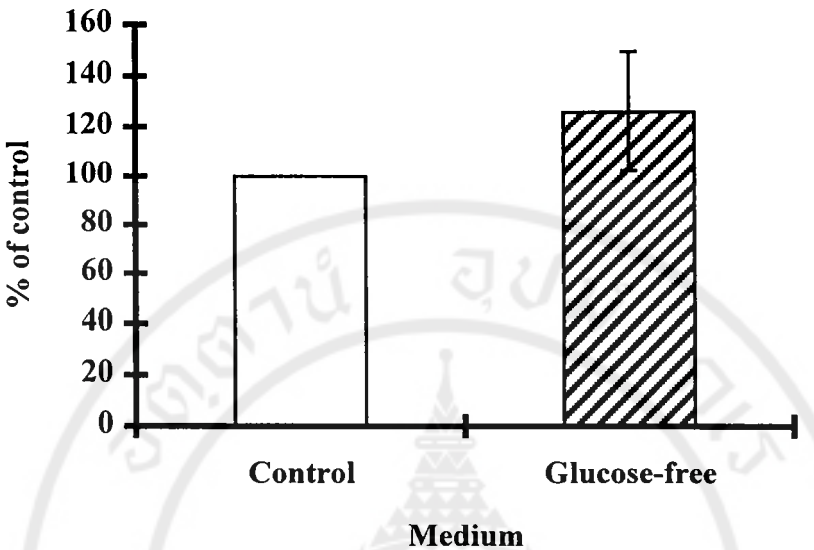


Figure 7. Effect of glucose-free medium on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM, from n = 6.

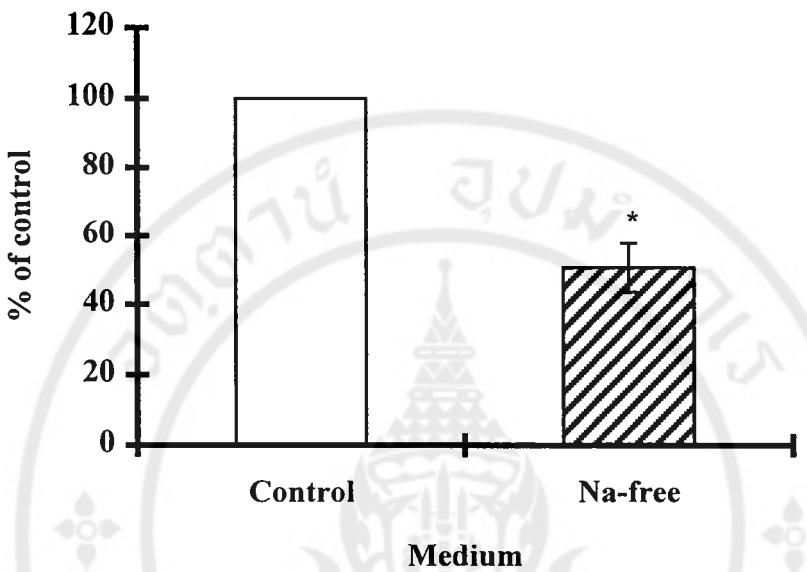


Figure 8. Effect of Na^+ on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM, from n = 4.
Significant differences (unpaired t-test) between pmole^{3}H-carnitine/mg tissue dry weight of control and Na-free treated groups are indicated by asterisks: *$p < 0.05$.

Experiment 6 : Effect of respiratory inhibition on carnitine uptake

In order to determine whether uptake of carnitine at the basolateral membrane requires energy from metabolism, potassium cyanide was added to the final concentration of 40 mM in the incubation medium. The results showed moderate (28% inhibition) but significant ($p<0.05$) suppression of the uptake (Table 4 and Figure 9).

On the other hand, when oligomycin was used it failed to inhibit the uptake even at the highest concentration, 30 μM (Table 4 and Figure 9).

Table 4. Effects of respiratory inhibition on carnitine uptake in the distal caput of the rat epididymal tubule

Compounds	Carnitine uptake (pmole/mg tissue)		% of control
	Control	Treatment	
KCN (40 μM)	0.2±0.02	0.13±0.04	71.87±11.43* (5)
Oligomycin (30 μM)	0.11±0.04	0.14±0.06	138.08±21.34 (6)

Values are mean±SEM (n = rats).
Significantly differences (unpaired t-test) between pmol^{3}H-carnitine/mg tissue dry weight of control and treated groups are indicated by an asterisks: *$p<0.05$.

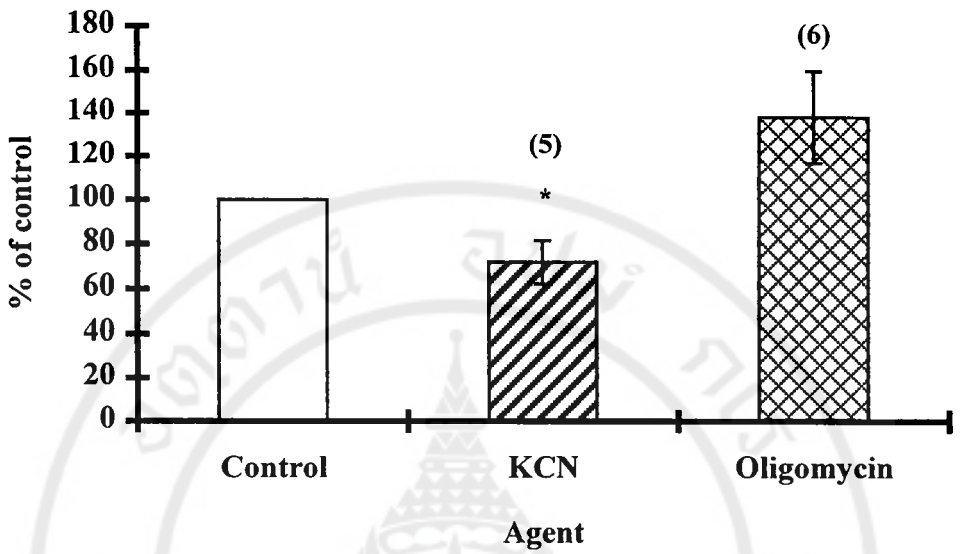


Figure 9. Effects of respiratory inhibitors on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM (n = rats).
Significant differences (unpaired t-test) between pmole^{3}H-carnitine/mg tissue dry weight of control and agent treated groups are indicated by asterisks: *$p < 0.05$.

Experiment 7: Effect of carnitine analogs and organic ions on carnitine uptake

7.1 Carnitine analogs

To determine the substrate selectively of carnitine transport compounds that have structure related to carnitine such as deoxycarnitine (γ-bytyrobetaine), acetyl-L-carnitine, DL-octanoyl carnitine, betaine, choline chloride, trimethyllysine, glycine, D-carnitine, γ-aminobytyrate (GABA) were included in the medium. Significant increases in the uptake were observed when the bathing medium contained glycine. On the other hand, deoxycarnitine (γ-bytyrobetaine), acetyl-L-carnitine, DL-octanoyl carnitine markedly reduced carnitine uptake while D-carnitine did not alter the uptake (Table 5 and Figure 10).

Table 5. Effects of carnitine analogs on carnitine uptake in the distal caput of the rat epididymal tubule

Compounds	Carnitine uptake (pmole/mg tissue)		% of control
	Control	Treatment	
D-carnitine	0.3±0.03	0.23±0.05	76.30±14.40 (7)
Deoxycarnitine	0.66±0.04	0.19±0.02	29.19±3.43* (5)
Acetyl-L-carnitine	0.53±0.06	0.09±0.02	16.62±3.49* (6)
Betaine	0.37±0.06	0.46±0.04	140.98±14.85 (6)
Choline chloride	0.39±0.08	0.44±0.07	135.99±17.63 (6)
D-L-octanoyl carnitine	0.41±0.04	0.06±0.01	12.38±2.08* (4)
Trimethyllysine	0.18±0.02	0.19±0.02	115.16±8.10 (6)
Glycine	0.15±0.02	0.20±0.03	148.12±16.39* (6)
γ-aminobutyrate	0.13±0.01	0.14±0.01	112.40±12.44 (6)

Values are mean±SEM (n = rats).
Significantly differences (unpaired t-test) between pmol^{3}H-carnitine/mg tissue dry weight of control and agent treated groups are indicated by an asterisks: *p<0.05.

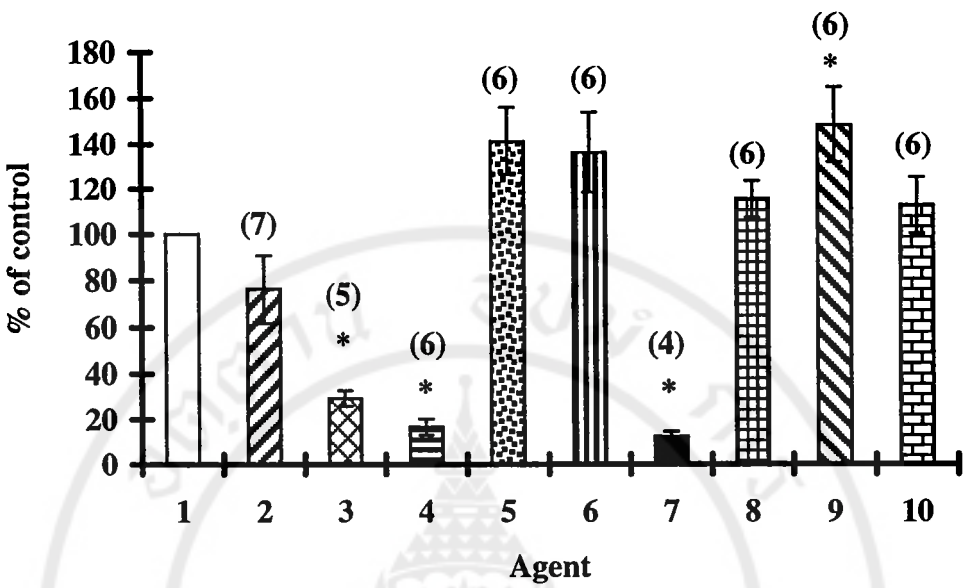


Figure 10. Effects of compounds with structure analogs to carnitine on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM (n = rats).
Significant differences (unpaired t-test) between pmole ^{3}H-carnitine/mg tissue dry weight of control and agent treated groups are indicated by asterisks: *$p < 0.05$.

# 1 = Control, 2 = D-carnitine, 3 = Deoxycarnitine,
4 = Acetyl-L-carnitine,5 = Betaine, 6 = Choline chloride,
7 = DL-Octanoyl carnitine, 8 = Trimethyllysine, 9 = Glycine,
10 = γ-aminobutyrate (GABA)

7.2 Organic ions

To test whether the carnitine transporters shown some similarity with other organic ion transporters. Organic ions that have been shown to be transported by organic cation and organic anion transporters such as tetraethylammonium, N-methylnicotinamide and cephaloridine. Unfortunately, all these compound had no significant effect on carnitine uptake. Suggesting no relation between carnitine transporters and both organic cation and anion transporters (Table 6 and Figure 11).

Table 6. Effects of organic ions on carnitine uptake in the distal caput of the rat epididymal tubule

Compounds	Carnitine uptake (pmole/mg tissue)		% of control
	Control	Treatment	
Tetraethylammonium	0.33±0.02	0.33±0.03	100.87±11.11
N-methylnicotinamide	0.15±0.03	0.14±0.03	109.92±15.90
Cephaloridine	0.14±0.01	0.11±0.01	84.46±9.33

Values are mean±SEM, from n = 6.

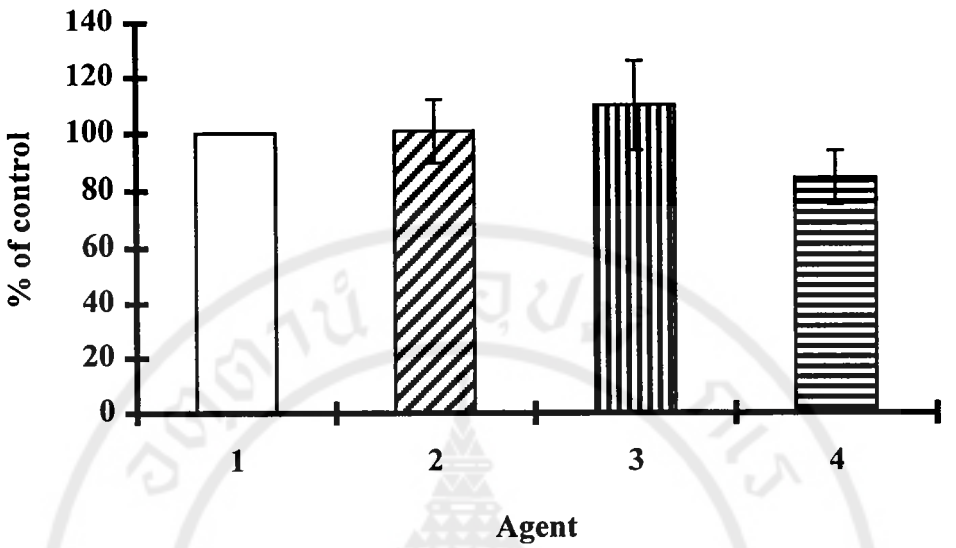


Figure 11. Effect of organic ions on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM, from n = 6.
# 1 = Control, 2 = Tetraethylammonium, 3 = N-methylnicotinamide
4 = Cephaloridine

Experiment 8 : Effect of some sulfonamides on carnitine uptake

To determine the effect of some sulfonamide such as sulfanilamide and sulfaphyridine on carntine uptake. That compound were added in the incubation medium. Both compounds, sulfanitamide and sulfapyridine not shown significant effect on carnitine uptake (Table 7 and Figure 12).

Table 7. Effects of some sulfonamides on carnitine uptake in the distal caput of the rat epididymal tubule

Compounds	Carnitine uptake (pmole/mg tissue)		% Change from control
	Control	Treatment	
Sulfanilamide	0.13±0.01	0.15±0.02	116.75±8.56
Sulfapyridine	0.09±0.01	0.08±0.01	92.27±8.48

Values are mean±SEM, from n = 6.

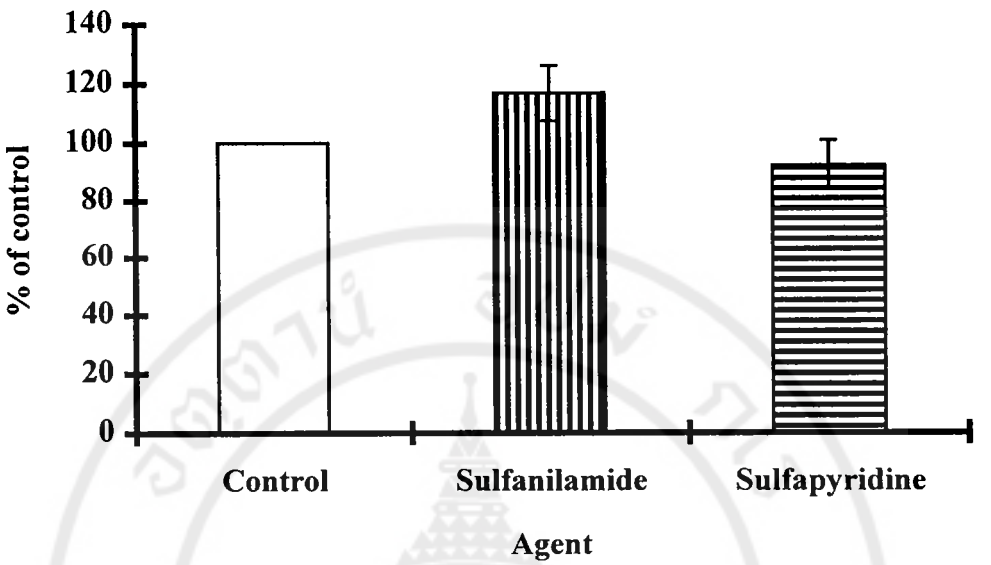


Figure 12. Effect of some sulphonamides on carnitine uptake in the distal caput of rat epididymis tubule
Values are means±SEM, from n = 6.

CHAPTER VI

DISCUSSION

1. CHARACTERISTICS OF CARNITINE TRANSPORT IN THE EPIDIDYMIS

Carnitine is ubiquitous in mammalian tissues. Its role in fatty acid oxidation is well understood. There is no doubt, therefore, that its transport mechanism has been extensively studied in various tissues. In spite of this fact, the transport process of this compound is still less understood especially in the reproductive organs.

Previous micropuncture and microperfusion studies of rat epididymis both *in vivo* and *in vitro* have shown that carnitine is transported from blood plasma and is secreted by the apical membrane into the lumen against high concentration gradient (7). It appears that the transport systems for carnitine at the basal and apical membrane of the epididymal epithelium are different (9). Although it is known from microperfusion studies that the transport activity is highest in the caput region, the characteristics of carnitine transport in this segment have not been investigated. Only those in the corpus and cauda epididymidis have been studied using microperfusion techniques (9, 25, 26). Further, *in vivo* microperfusion studies have some limitations in differentiating fluxes across a single membrane, only net transport across both basolateral and apical membranes could be evaluated. An attempt has been made to circumvent this problem by using isolated cells (27). It should be noted, however, that is such preparation cells lose its membrane polarity, and one cannot be sure which side

of the membrane is being investigated. Therefore, in the present study, an isolated segment of the caput epididymidis was used to study the properties of carnitine transport system with a particular emphasis on the net influx across basolateral membrane. Efflux of carnitine through the apical membrane is prevented by filling the lumen with water saturated paraffin oil. It should be pointed out that although this approach does not completely prevent secretion of substances into the lumen, direct observations through a microscope on the oil-filled tubule after 60 min incubation failed to visualize any fluid secretion in the tubular lumen. Thus, it may be concluded that the presence of oil column in the tubular lumen practically prevent efflux of carnitine through the apical membrane. Radio labeled carnitine contents in the tubule, therefore, represent the net influx from the medium across the basolateral membrane.

Results in the present investigation confirm previous *in vivo* and *in vitro* microperfusion studies regarding the requirement for carriers (9, 25). The evidence that support this conclusion includes: (1) kinetics study demonstrated saturation of carnitine uptake when cold carnitine concentration in the incubation medium was raised to 271 μM (Figure 4); (2) the uptake was highly temperature dependent with a temperature quotient of 2.35; and (3) carnitine uptake was specifically inhibited in the presence of its analogs. In addition, this study supported the previous investigation in the *in vitro* perfused cauda epididymidis of the rat, which suggested the requirement for energy (25). However, oligomycin failed to inhibit carnitine uptake in the present study, but not in the previous report. This is not due to insufficiency of the concentration of the inhibitor because the concentration used was 6 folds higher than that used by Cooper and his colleagues (25). This discrepancy cannot be explained at

present. It is noted worthy that carnitine uptake in the oil-filled tubules was not very sensitive to inhibition by KCN since only partial inhibition (28%) occurred in the presence of 40 mM KCN (Figure 9). This suggests that the major source of energy may be derived from non-respiratory pathway. Further studies are needed to clarify this hypothesis.

The present study demonstrated for the first time that carnitine transport in the epididymis was Na-dependent. This is not surprising since similar findings have been reported in many other tissues such as skeletal muscle strips (28), guinea-pig enterocytes (29), rat kidney brush-border-membrane vesicles (30). However, it is apparent that there is a substantial fraction of the Na-independent carnitine transport in the rat epididymis. This accounts for almost 50% of the total carnitine uptake in the caput epididymidis (Figure 8). Partial Na-dependency has also been reported in the rat kidney membrane resides (30). On the other hand, carnitine transport in the human placental choriocarcinoma cells was almost completely Na-dependent (31).

In order to determine the kinetics of carnitine uptake in the epididymis, the tubule was incubated in the medium containing various concentrations of anlabeled carnitine which, in turn, would dilute labeled carnitine in the medium. The tissue uptake of labeled carnitine showed characteristic saturation kinetics when plotted versus the concentration of labeled carnitine in the medium. Line weaver-Burk plot revealed Km of 95.06 μM and Vmax of 11.88 mmol/mg tissue. It appears that the Km in this study is slightly less than the Km for transepithelial transport of carnitine in the rat cauda epididymidis perfused *in vitro* (25). However, it is much lower than that reported for the cells isolated from rat epididymis (1 mM: (27)) or from the liver

(5 mM: (32)), and kidney (0.3 mM: (33)). The value is, on the other hand, higher than that for skeletal muscle (60 μM: (34)), human heart cells (4.8 μM: (35)), rat kidney brush-border-membrane vesicles (15-17 μM: (30)) and human placental carcinoma cells (12.3 μM: (31)).

Specificity of carnitine transport revealed by the inhibition of tubular uptake in the presence of carnitine analogs showed some similarity to that described in the *in vitro* perfused preparation (25). Thus, both tubular uptake and transport into the lumen were inhibited by acetyl-L-carnitine or γ-butyrobetaine (deoxycarnitine). In contrast, D-carnitine, which inhibited the secretion into the lumen, had no effect on the tubular uptake in the present study. The results suggest more stereospecificity for carnitine carrier on the basolateral membrane. In support of this conclusion, other compounds with structure related to carnitine such as γ-aminobutyrate, and trimethyllysine did not compete with the carnitine transporter. Interestingly, betaine choline, and glycine apparently enhanced tubular uptake. However, only glycine showed significant effect. These results are in accord with the secretion of carnitine in the rat cauda epididymidis perfused *in vivo* (9), but are at variance with those reported *in vitro* (25). Although the actual mechanism is not known at present, it may be speculated that betaine and/or choline stimulates the intracellular uptake of carnitine through the exchange system. Such exchange mechanism has previously been described in sperm (36) and human heart cells in culture (37). The latter author found that betaine increased the rate of carnitine efflux. Later Sartorelli and colleagues (38, 39) demonstrated that carnitine and acetylcarnitine were exchanged between the intra and extracellular compartments in the rat heart slice model. They also noted that the

intracellular-extracellular butyrobetaine gradient might provide the energy for carnitine influx through the exchange. It is, therefore, possible that the entry of betaine and/or choline through the carnitine exchanger or through other systems enhances the efflux of non-radiolabeled and the influxes of labeled carnitine. This hypothetical model awaits future proofs.

Compounds that have been shown to be substrates of organic cation transporters in the renal tubules such as N′-methylniconitamide and tetraethylammonium had virtually no effect on carnitine uptake in the caput epididymidis. Further, cephaloridine, an antibiotic that inhibited both organic anion and organic cation transporters in the rat proximal tubule (40) failed to inhibit uptake of carnitine in the rat caput epididymidis. These results indicate that the carnitine transporters is, perhaps, a distinct system. Indeed, recent molecular cloning of the carnitine transporter from rat kidney (41) and rat small intestine (CT_1: (42)) showed that the amino acid sequence of the carnitine transporters differed significantly from those of both organic cation and organic anion transporters. On the other hand, CT_1 is 99% identical to that of UST_2, a transporter of unknown function present in many tissues (42).

Expression studies of the carnitine transporters in *Xenopus* oocytes demonstrated the Na-dependency of carnitine transport (42). CT_1, which was clonned from rat intestine, had relatively low Km being 25.4 μM. In contrast, the transporters clonned from rat kidneys exhibited moderate Km being 149 μM (43). Comparisons of the specificity of the transporters expressed in oocytes (42, 43) and that of the isolated epididymal tubule in the present study (Table 5) revealed some similarity. For

example; actyl-L-carnitine and γ-butyrobetaine inhibited carnitine transport in all models, whereas glycine and γ-aminobutyrate had no effect on CT_1 and the epididymal tubule. There are differences in the characteristics of these transporters clonned from rat kidney, but not from rat intestine or in the rat epididymis. Carnitine transport via CT_1 was stimulated by this compound in the rat epididymis. It is not known whether the carnitine transporter in the rat epididymis is the same as CT_1 in the rat intestine, although Northern blot analysis of CT_1 demonstrated its distribution in the intestine, liver, kidney and testis (42). Unfortunately, these authors failed to isolate the testis from the epididymis (personal communications). In view of the differences in Km and substrate specificity, it is possible that carnitine transporter in the epididymis may be isoforms of CT_1.

2. EFFECT OF SULFONAMIDES ON CARNITINE TRANSPORT

Sulfasalazine, which has been used for treatment of ulcerative colitis, cause infertility in male patients (22) and rats (43). This drug is metabolized by intestinal microflora to acetylsalicylate and sulfapyridine. The latter is the active component in suppression of fertility both in man and rats (5, 24). Other sulfonamides having structures related to sulfapyridine have been shown to decrease fertility in the male rats. Their potencies, however, are less than that of the parent compound except sulfanilamide, which is as effective as sulfapyridine (44).

Because of the rapid onset of decreased fertility (3-5 weeks) and rapid recovery (1-3 weeks), and the lack of effects on the hypothalomo-pituitary axis and the rate of sperm production (5, 24) it is believed that the most likely site of action of these

antifertility sulfonamides is post testicular or at the epididymis. Attempts have been made to clarify the action of these compounds in the male rats by Pholpramool and his colleagues. They found that sulfapyridine produced decrease in sperm concentration both in the caput and the cauda epididymidis. Analyses of the sperm surface proteins showed different protein profile of the sulfapyridine treated sperm collected from the cauda epididymis. These spermatozoa had the profile resemble that of the untreated caput spermatozoa (45). The results indicate that sulfapyridine may interfere with the modifications of surface proteins of the immature spermatozoa during their transit through the epididymis. Alternatively, the sulfa drug may enhance the transit of the caput spermatozoa causing an increase in the population of the immature spermatozoa in the cauda epididymidis. The same groups of investigators later demonstrated by direct measurement of the rate of sperm transport through the rat epididymis that, indeed, sulfapyridine accelerated the transit of spermatozoa from the caput into the cauda epididymidis probably by changes in the responsiveness of the epididymis to the autonomic nervous system (46).

Sulfanilamide has also been shown to decrease fertility of male rats which was associated with decreases in the concentration of spermatozoa in the cauda epididymidis and in the electroejaculated semen (47). These authors reported that motility of both epididymal and ejaculated spermatozoa markedly decreased. Since carnitine has been implicated in the regulation of sperm motility, Lewin and his co-workers studied the effect of sulfapyridine treatment on *in vivo* uptake of radiolabeled carnitine in the rat epididymis. Unfortunately, they failed to demonstrate any effect of this sulfa drug (personal communication). However, the *in vivo* study is complicated

by the fact that three compartments are involved in the accumulation of carnitine, i.e. intracellular, luminal fluid and spermatozoa. It is not known in which compartment carnitine was accumulated in Lewin's study. The present investigation, therefore, clearly showed that both sulfapyridine and sulfanilamide had virtually no effect on the uptake of carnitine into the intracellular compartment. However, it remains to be answered whether these sulfonamides affect the secretion of carnitine into the luminal fluid, and whether the uptake into epididymal spermatozoa is interfered by these drugs.

CHAPTER VI

CONCLUSION

1. Carnitine uptake into the distal caput epididymidis of rats were measured by incubating an isolated segment of oil-filled tubule in the medium containing ^{3}H-L-carnitine.

2. The carnitine uptake was partly Na^{+} dependent and required energy from oxidative phosphorylation, not from glycolytic pathway.

3. The uptake of carnitine exhibited saturable kinetics and followed the Michaelis-Menten equation. The calculated Km and Vmax values for L-carnitine were 95.01 μM and 11.9 mmole/mg tissue-60 min, respectively.

4. The transport system showed stereospecificity for L-carnitine, and only closely related compounds such as γ-butyrobetaine, acetylcarnitine and octanoylcarnitine inhibited the system.

5. Compounds that have been shown to be transported by organic cation or organic anion transporters in many tissues such as tetraethylammonium, N′-methylnicotinamide and cephaloridine did not interfere with carnitine uptake in the epididymis.

6. Some sulfonamides such as sulfanilamide and sulfaphyridine had no effect on carnitine uptake in the distal caput epididymidis.

7. The results suggest that transport of carnitine across the basolateral membrane requires a carrier which is Na-dependent and stereospecific. The carrier is probably distinct from the organic cation or anion transporter.

REFERENCES

1. Bedford JM. Maturation, Transport and fate of spermatozoa in the epididymis. In: Hamilton DW, Greep RO, editors. Handbook of Physiology. Washington DC: American Physiological Society; 1975. p.303-17.
2. Goldman P, Pepercorn MA. Drug therapy: sulfasalazine. New Eng J Med 1995; 293(1): 20-3.
3. Pepercorn MA, Goldman P. The role of intestinal bacteria in the metabolism of saliocylazosulfapyridine. J Pharmacol Exp Ther 1972; 181(3): 555-62.
4. Cann PA, Holdsworth CD. Reverseal of male infertility on changing treatment from sulfasalazine to 5-amino-salicylic acid. Lancet 1984; 1(8386): 1119.
5. Pholpramool C, Srikhao A. Suppression of fertility by sulfasalazine and its metabolites in the male rat. J Androl Suppl 1985; 6: 50.
6. Wong PY, Lau SKD, Fu WO. Antifertility effects of some sulfonamides and related compounds and their accumulation in the epididymides of male rats. J Reprod Fert 1987; 81(1): 259-67.
7. Hinton BT, Snoswell AM, Setchell BP. The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals. J Reprod Fert 1979; 56: 105-11.
8. Radigue C, Es-Slami S, Soufir JC. Relationship of carnitine transport across the epididymis to blood carnitine and androgens in the rats. Archives of Andrology 1996; 37: 27-31.

9. Yeung CH, Cooper TG, Waites GMH. Carnitine transport into the perfused epididymis of the rat: Regional differences, stereospecificity, stimulation by choline, and the effect of other luminal factors. Biology of reproduction 1980; 23: 294-304.

10. Haper MJK. Gamete and Zygote Transport. In: Knobil E, Neill JD, editors. The Physiology of Reproduction. New York: Ravan Press; 1988. p. 103-34.

11. Setchell BP, Brooks DE. Anatomy, Vasculature, Innervation, and Fluids of the male reproductive tract. In: Knobil E, Neill JD, editors. The Physiology of Reproduction. New York: Ravan Press; 1988. p.753-836.

12. Brooks DE, Hamilton DW, Mallek AH. The uptake of L-[methyl-^{3}H] carnitine by the rat epididymis. Biochemical and Biophysical Research Communications 1973; 52(4):1354-60.

13. Krause WJ. Meiosis and Male reproductive organ. In: Schnittman ER, Jeans S, Editors. Human Histology. New York: Little, Brown and Company; 1996. p. 331-52.

14. Hinton BT, Setchell BP. Concentration and uptake of carnitine in the rat epididymis: A micropuncture study. In: McGarry JD, Frankel RA, editors. Carnitine Biosynthesis, Metabolism, and Functions. London: Academic Press; 1980. p.237-50.

15. Finaz C, Boue F, Meduri G, Leferre A. Characterization of rat epithelial epididymal cells purified on a discontinuous Percoll gradient. J. Reprod. Fert. 1991; 91: 617-25.

16. Robaire B, Hermo L. Efferent Ducts, Epididymis and Vas Deferens. Structure, Functions, and Their Regulation. In: Knobil E, Neill JD, Editors. The Physiology of Reproduction. New York: Raven Press; 1988. p.999-1080.

17. Brass EP. Carnitine transport. In Ferrari R, Di Mauro S, Sherwood G, editors. L-Carnitine and its role in medicine: From function to therapy. London: Academic Press; 1992. p.21-36.

18. Bremer J. Carnitine-metabolism and functions. Physiological reviews 1983; 63(4): 1420-80.

19. Hinton BT, Brooks DE, Dott HM, Setchell BP. Effects of carnitine and some related compounds on the motility of rat spermatozoa from the caput epididymis. J. Reprod. Fert 1981; 61: 59-64.

20. Rebouche CJ, Mack DL. Sodium gradient-stimulated transport of L-carnitine into renal brush border membrane vesicles: Kinetics, specificity, and regulation by dietary carnitine. Archieres Biochemistry and Biophysics 1984; 235(2): 393-402.

21. Kugers A, Bennett NMck, Sulfonamides. In: Kuyers A, Bennett NMck, edition. The Use of Antibiotics. Philadelppia: J.B. Lippincott, 1987. p. 1075-17.

22. Levi AJ, Fisher AM, Hughes L, Hendry WF. Male infertilits due to sulphasalazine.Lancet 1979; 2(8137): 276-8.

23. Pholpramool C, Ruchirawat S, Paovalo C, Verawatnapakul V, Lewin LM. Suppression of Male Fertility by Sulpha Drugs. In: Manchanda SK, Selvamurthy W, Kumar VM, editors. Advances in Physiological Sciences. New Delhi: SG Wasani, 1992. p. 487-93.

24. O' Morain C, Smethurst P, Dore CJ, Levi AJ. Reversible male infertility due to sulfasalazine: studies in man and rat. Gut 1984; 25(10): 1078-84.

25. Cooper TG, Gudermann TW, Yeung CH. Characteristics of the transport of carnitine into the cauda epididymidis of the rat as ascertained by luminal perfusion *in vitro*. International Journal of Andrology 1986a; 9(5): 348-58.

26. Cooper TG, Yeung CH, Weinbavcr GF. Transport of carnitine by the epididymis of the cynomolgus macague (MaCacafascicularis). J. Reprod. Fert 1986b; 77(1): 297-301.

27. James MJ, Brooks DE, Snoswell AM. Kinetics of carnitine uptake by rat epididymal cells. Androgen-dependence and lack of stereospecificity. Febs Letters 1981; 126(1): 53-6.

28. Rebouche CJ. Carnitine movement across muscle cell membranes. Biochem. Biophys. Acta 1977; 471: 145-55.

29. Gross CJ, Henderson LM, Savaiana DA. Uptake of L-Carnitine, D-carnitine and acetyl-L-carnitine by isolated guinea-pig enterocytes. Biochem. Biophys. Acta 1986; 886: 425-33.

30. Steiger B, O' Neill B, Krahenbuhl S. Characterization of L-carnitine transport by rat kidney brush-border-membrane vesicles. Biochem J 1995; 309: 643-47.

31. Prasad PD, Huang W, Ramamoorthy S, carter AL, Leibaoh FH, Ganapathy V. Sodium-dependent carnitine transport in human placental choriocarcinoma cells. Biochem. Biophys. Acta 1996; 1284: 109-17.

32. Christensen RZ, Bremer J. Active transport of butyrobetaine and carnitine into isolated liver cells. Biochem Biophys Acta 1976; 448: 562.

33. Huth PJ, Thomsen JH, Shug AL. Carnitine transport by rat kidney cortex show stimulation by dibutyryl cyclic AMP. Life Sci 1978; 28: 715.

34. Bahl J, Manian AA, Bressler R. Carnitine transport in isolated adult rat heart muscles and the effects of 7,8-dihydroxy chlorpramazine. Circulation Res 1981; 48: 378.

35. Bohmer T, Weddington SC, Hanson V. Effect of testosterone propionate on levels of carnitine and testicular androgen binding protein (ABP) in rat epididymis. Endocrinology 1977; 100: 838.

36. Calvin J, Tubbs PK. A carnitine acetylcarnitine exchange system in spermatozoa. J. Reprod. Fert. 1976; 48: 417-20.

37. Molstad P. The efflux of L-carnitine from cells in culture (CCL27). Biochem. Biophys. Acta 1980; 597: 163-73.

38. Sartorelli L, Ciman M, Siliprandi N. Carnitine transport in rat heart slices: I. The action of thiol reagents on the acetylcarnitine carnitine exchange. Ital J Biochem 1985a; 34(4): 275-81.

39. Sartorelli L, Ciman M, Montavani G, Siliprandi N. Carnitine transport in rat heart slices: II. The carnitine/deoxycarnitine antiport. Ital J Biochem 1985b; 34 (4): 282-7.

40. Ullrich KJ, Rumrich G, David C, Fritzsch G. Bisubstrates: substances that interact with both, renal contraluminal organic anion and organic cation transport systems. Pflügers Arch 1993; 425: 300-12.

41. Schömig E, Spitzenberger F, Engelhardt M, Martel F, Ording N, Gründemann. Molecular cloning and characterization of two novel transport proteins from rat kidney. FEBS Letters 1998; 425: 79-86.

42. Sekine T, Kusuhara H, Utsunomiya-Tate N, Tsuda M, Sugiyama Y, Kanni Y, et al. Molecular cloning and characterization of high-affinity carnitine transport from rat intestine. Biochemical and Biophysical Research Communication 1998; 251: 586-91.

43. Berardi S, Hagenbuch B, Carafoli E, Krahenbuhl S. Characterization of the endogenous carnitine transport and expression of a rat renal Na^+-dependent carnitine transport system in *Xenopus lavis* oocytes. Biochem J 1995; 309: 389-93.

44. Pholpramool C, Srikhao A. Antifertility effect of sulfasalazine in the male rat. Contraception 1983; 28(3): 273-9.

45. Pholpramool C, Ruchirawat S, Verawatnapakul V, Paovalo C, Lewin LM. Structural requirements of some sulphonamides that possess an antifertility activity in male rats. J Reprod Fertil 1991;92(1): 169-78.

46. Bunnag D, Chaturapanich G, Lewin LM, Pholpramool C. alterations of epididymal sperm surface proteins by some antifertility agents in the rats. Thai J Physiol Sci; 5(1): 27-39.

47. Chaturapanich G, Sujarit K, Pholpramool C. Effects of sulfapyridine on sperm transport through the rat epididymis and contractility of the epididymal duct. J Reprod Fertil 1999; 117(2): 199-205.

48. Sujarit S, Pholpramool C, Veerawatnapakul V. Effects of sulfanilamide on structure and function of epididymis and epididymal spermatozoa. J Natl Res Council Thailand 1993; 25(2): 9-25.

Appendix I . Entry of ^{3}H-carnitine (DPM/mg tissue) into the distal caput of epididymis at various incubation times.

Rat no.	Tubule no.	Carnitine uptake (DPM/mg tissue) at time (min)				
		5	15	30	60	90
1	1	2.40	4.91	11.96	37.52	9.32
	2	2.08	6.12	12.15	22.66	13.17
2	1	1.41	3.38	3.21	11.38	24.28
	2	2.83	4.30	5.59	9.93	25.49
3	1	2.16	8.06	15.42	25.99	29.55
	2	3.48	6.32	14.35	15.12	25.99
4	1	1.78	5.39	10.17	17.01	14.42
	2	3.31	5.40	5.02	14.31	22.14
5	1	2.16	5.81	9.68	23.69	19.16
	2	1.87	4.74	11.58	14.40	17.44
6	1					20.56
	2					21.57
Mean		2.35	5.44	9.91	19.20	20.10
SEM		0.15	0.53	1.81	3.21	2.89
n		5	5	5	5	6

F = 14.409
p-value = 0.000

Appendix II . Entry of ^{3}H-carnitine (pmole/mg tissue) into the distal caput of epididymis after 60 min incubation at various concentration of carnitine

Rat no.	Tubule no.	Uptake of ^{3}H-carnitine in cold carnitine (μM)					
		36	**59.7**	**95.7**	**154.3**	**271**	**497.5**
1	1	0.06	0.05	0.06	0.04	0.03	0.03
2	1	0.08	0.04	0.03	0.03	0.04	0.01
3	1	0.09	0.06	0.06	0.06	0.03	0.02
4	1	0.09	0.04	0.04	0.02	0.02	0.02
5	1	0.06	0.05	0.02	0.03	0.02	0.01
Mean		0.08	0.05	0.04	0.04	0.03	0.02
SEM		0.01	0.003	0.01	0.004	0.002	0.003
n		5	5	5	5	5	5

Appendix III Entry of total carnitine (pmole/mg tissue) into the distal caput of epididymis after 60 min incubation at various concentration of carnitine

Rat no.	Tubule no.	Total uptake (pmole/mg) in cold carnitine (μM)					
		36	**59.7**	**95.7**	**154.3**	**271**	**497.5**
1	1	0.27	0.38	0.66	0.75	0.95	1.78
2	1	0.35	0.29	0.38	0.51	1.24	0.54
3	1	0.41	0.47	0.76	1.17	1.13	1.44
4	1	0.41	0.33	0.44	0.40	0.72	1.01
5	1	0.28	0.34	0.28	0.62	0.80	0.63
Mean		0.34	0.36	0.50	0.69	0.97	1.08
SEM		0.03	0.03	0.08	0.12	0.09	0.21
n		5	5	5	5	5	5

F = 6.326
p-value = 0.001

For calcularion of total carnitine uptake see Appendix XI.

Appendix IV Effect of temperature on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (DPM/mg)		% of control
		34 °C (control)	0 °C	
1	1	8.11	1.3	16.03
	2	10.82	0.86	7.95
2	1	5.21	0.89	17.08
	2	11.33	0.96	8.47
3	1	9.2	1.3	14.13
	2	8.36	1.24	14.83
4	1	7.95	1.2	15.09
	2	9.31	1.35	14.50
5	1	8.63	0.87	10.08
	2	9.1	1.47	16.15
Mean		8.802	1.144	13.43
SEM		0.19	0.07	0.53
n		5	5	5

t = 37.56
p-value = 0.000

Appendix V Effect of Na-free medium on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Na-free	
1	1	0.39	0.1	25.64
	2	0.33	0.13	39.39
2	1	0.25	0.16	64.00
	2	0.24	0.17	70.83
3	1	0.32	0.1	31.25
	2	0.23	0.08	34.78
4	1	0.25	0.21	84.00
	2	0.34	0.1	29.41
Mean		0.29	0.13	50.67
SEM		0.02	0.02	7.14
n		4	4	4

t = 5.42
p-value = 0.002

Appendix VI Effect of glucose-free medium on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Glucose-free	
1	1	0.18	0.18	100.00
	2	0.23	0.16	69.57
2	1	0.19	0.15	78.95
	2	0.26	0.16	61.54
3	1	0.23	0.15	65.22
	2	0.11	0.16	145.45
4	1	0.17	0.19	111.76
	2	0.14	0.13	92.86
5	1	0.11	0.15	136.36
	2	0.08	0.17	212.50
6	1	0.08	0.14	175.00
	2	0.12	0.29	263.64
Mean		0.16	0.19	126.07
SEM		0.02	0.01	23.69
n		6	6	6

t = - 0.484
p-value = 0.639

Appendix VII Effect of respiratory inhibition on carnitine transport in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	KCN(40 μM)		% of control
		Control	Treatment	
1	1	0.22	0.12	54.54
	2	0.12	0.09	75.00
2	1	0.23	0.14	60.87
	2	0.22	0.08	36.36
3	1	0.19	0.21	110.53
	2	0.18	0.18	100.00
4	1	0.26	0.13	50.00
	2	0.13	0.14	107.69
5	1	0.22	0.11	50.00
	2	0.19	0.14	73.68
Mean		0.20	0.13	71.87
SEM		0.02	0.04	11.43
n		5	5	5

t = 3.328
p-value = 0.010

Rat no.	Tubule no.	Oligmycin (30 μM)		% of control
		Control	Treatment	
1	1	0.18	0.25	138.89
	2	0.15	0.28	186.67
2	1	0.13	0.12	92.31
	2	0.16	0.07	43.75
3	1	0.08	0.13	162.50
	2	0.08	0.11	137.50
4	1	0.90	0.10	11.11
	2	0.11	0.13	118.18
5	1	0.10	0.10	100.00
	2	0.08	0.10	125.00
6	1	0.07	0.16	228.57
	2	0.08	0.17	212.50
Mean		0.11	0.14	138.08
SEM		0.04	0.06	21.34
n		6	6	6

t = -1.124
p-value = 0.287

Appendix VIII Effect of carnitine analogs on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	D-carnitine	
1	1	0.23	0.29	126.09
	2	0.40	0.38	95.00
2	1	0.35	0.28	80.00
	2	0.30	0.28	93.33
3	1	0.32	0.42	131.25
	2	0.25	0.36	144.00
4	1	0.33	0.16	48.48
	2	0.40	0.18	45.00
5	1	0.38	0.34	89.47
	2	0.45	0.27	60.00
6	1	0.13	0.06	46.15
	2	0.29	0.07	24.14
7	1	0.16	0.08	50.00
	2	0.17	0.06	35.29
Mean		0.30	0.23	76.30
SEM		0.03	0.05	14.40
n		7	7	7

t = 1.127
p-value = 0.282

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Deoxycarnitine	
1	1	0.66	0.25	37.88
	2	0.70	0.19	27.14
2	1	0.75	0.28	37.33
	2	0.68	0.03	4.41
3	1	0.73	0.19	26.03
	2	0.79	0.24	30.38
4	1	0.72	0.30	41.67
	2	0.56	0.22	39.29
5	1	0.44	0.11	25.00
	2	0.57	0.13	22.81
Mean		0.66	0.19	29.19
SEM		0.04	0.02	3.43
n		5	5	5

t = 9.290
p-value = 0.000

Appendix VIII (continued)

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Acetyl-L-carnitine	
1	1	0.85	0.14	16.47
	2	0.79	0.10	12.66
2	1	0.61	0.05	8.20
	2	0.45	0.09	20.00
3	1	0.45	0.07	15.56
	2	0.44	0.07	15.91
4	1	0.63	0.09	14.29
	2	0.51	0.27	52.94
5	1	0.38	0.05	13.16
	2	0.42	0.04	9.52
6	1	0.45	0.04	8.89
	2	0.42	0.05	11.90
Mean		0.53	0.09	16.62
SEM		0.06	0.02	3.49
n		6	6	6

t = 6.693
p-value = 0.000

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Betaine	
1	1	0.66	0.46	69.70
	2	0.57	0.52	91.23
2	1	0.23	0.54	234.78
	2	0.35	0.35	100.00
3	1	0.44	0.48	109.09
	2	0.42	0.71	169.05
4	1	0.28	0.42	150.00
	2	0.30	0.48	160.00
5	1	0.34	0.28	82.35
	2	0.11	0.31	281.82
6	1	0.25	0.34	136.00
	2	0.26	0.28	107.69
Mean		0.37	0.46	140.98
SEM		0.06	0.04	14.85
n		6	6	6

t = -1.26
p-value = 0.236

Appendix VIII (continued)

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Choline chloride	
1	1	0.70	0.45	64.29
	2	0.58	0.85	146.55
2	1	0.41	0.49	119.51
	2	0.82	0.57	69.51
3	1	0.40	0.63	157.50
	2	0.30	0.54	180.00
4	1	0.29	0.19	63.33
	2	0.22	0.33	150.00
5	1	0.12	0.25	208.33
	2	0.19	0.12	63.16
6	1	0.46	0.49	106.52
	2	0.32	0.97	303.13
Mean		0.39	0.44	135.99
SEM		0.08	0.07	17.63
n		6	6	6

t = - 0.413
p-value = 0.688

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	DL-octanoyl carnitine	
1	1	0.43	0.06	13.95
	2	0.29	0.07	24.14
2	1	0.33	0.05	15.15
	2	0.31	0.04	12.90
3	1	0.61	0.07	11.48
	2	0.49	0.07	14.29
4	1	0.39	0.05	12.82
	2	0.45	0.03	6.67
Mean		0.41	0.06	12.38
SEM		0.04	0.01	2.08
n		4	4	4

t = 7.042
p-value = 0.000

Appendix VIII (continued)

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Trimethyllysine	
1	1	0.19	0.30	157.89
	2	0.21	0.22	104.76
2	1	0.10	0.12	120.00
3	1	0.12	0.25	208.33
	2	0.17	0.14	82.35
4	1	0.25	0.18	72.00
	2	0.12	0.21	175.00
5	1	0.23	0.17	73.91
	2	0.21	0.31	147.62
6	1	0.25	0.15	60.00
Mean		0.18	0.19	115.16
SEM		0.02	0.02	8.10
n		6	6	6

t = -0.325
p-value = 0.752

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Glycine	
1	1	0.15	0.28	186.67
	2	0.14	0.20	142.86
2	1	0.23	0.21	91.30
	2	0.12	0.25	208.33
3	1	0.20	0.30	150.00
	2	0.16	0.12	75.00
4	1	0.13	0.18	138.46
	2	0.12	0.37	300.00
5	1	0.12	0.12	100.00
	2	0.11	0.16	145.45
6	1	0.20	0.27	135.00
	2	0.23	0.24	104.35
Mean		0.15	0.20	148.12
SEM		0.02	0.03	16.39
n		6	6	6

t = -2.673
p-value = 0.023

Appendix VIII (continued)

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	γ-aminobutyrate	
1	1	0.14	0.25	178.57
	2	0.12	0.14	116.67
2	1	0.16	0.14	87.50
	2	0.10	0.14	140.00
3	1	0.13	0.09	69.23
	2	0.09	0.12	120.00
4	1	0.09	0.09	90.00
	2	0.09	0.11	122.22
5	1	0.32	0.25	78.13
	2	0.07	0.04	57.14
6	1	0.08	0.14	175.00
	2	0.14	0.16	114.29
Mean		0.13	0.14	112.40
SEM		0.01	0.01	12.44
n		6	6	6

t = -0.499
p-value = 0.628

Appendix IX Effect of organic ions on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Tetraethylammonium	
1	1	0.39	0.37	94.87
	2	0.34	0.39	114.71
2	1	0.31	0.27	87.10
	2	0.28	0.31	110.71
3	1	0.37	0.34	91.89
	2	0.37	0.36	97.30
4	1	0.27	0.39	144.44
	2	0.30	0.37	123.33
5	1	0.35	0.31	88.57
	2	0.28	0.42	150.00
6	1	0.44	0.20	45.45
	2	0.29	0.18	62.07
Mean		0.33	0.33	100.87
SEM		0.02	0.03	11.11
n		6	6	6

t = 0.195
p-value = 0.850

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	N-methylnicotinamide	
1	1	0.17	0.10	58.82
	2	0.11	0.15	136.36
2	1	0.38	0.24	63.16
	2	0.15	0.28	186.67
3	1	0.17	0.05	29.41
	2	0.09	0.07	77.78
4	1	0.21	0.20	92.24
	2	0.15	0.11	73.33
5	1	0.16	0.14	87.50
	2	0.06	0.14	233.33
6	1	0.12	0.11	91.67
	2	0.07	0.13	185.71
Mean		0.15	0.14	109.92
SEM		0.03	0.03	15.90
n		6	6	6

t = 0.271
p-value = 0.792

Appendix IX (continued)

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Cephaloridine	
1	1	0.10	0.11	110.00
	2	0.17	0.09	52.94
2	1	0.13	0.12	92.31
	2	0.12	0.09	75.00
3	1	0.23	0.13	56.52
	2	0.18	0.13	72.22
4	1	0.17	0.11	64.71
	2	0.11	0.09	81.82
5	1	0.14	0.07	50.00
	2	0.08	0.08	100.00
6	1	0.13	0.17	130.77
	2	0.11	0.14	127.27
Mean		0.14	0.11	84.46
SEM		0.01	0.01	9.33
n		6	6	6

t = 1.58
p-value = 0.145

Appendix X Effect of some sulfonamides on carnitine uptake in the distal caput of the rat epididymal tubule after 60 min incubation.

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Sulfanilamide(2.56 mM)	
1	1	0.17	0.13	76.47
	2	0.07	0.15	214.29
2	1	0.17	0.21	123.53
	2	0.17	0.16	94.12
3	1	0.11	0.14	127.27
	2	0.10	0.14	140.00
4	1	0.15	0.13	86.67
	2	0.11	0.14	127.27
5	1	0.07	0.07	100.00
	2	0.11	0.07	63.63
6	1	0.19	0.23	121.05
	2	0.15	0.19	126.67
Mean		0.13	0.15	116.75
SEM		0.01	0.02	9.19
n		6	6	6

t = -0.663
p-value = 0.522

Rat no.	Tubule no.	Carnitine uptake (pmole/mg)		% of control
		Control	Sulfapyridine (2.56 mM)	
1	1	0.09	0.11	122.22
	2	0.09	0.07	77.78
2	1	0.10	0.09	90.00
	2	0.11	0.05	45.45
3	1	0.05	0.06	120.00
	2	0.11	0.09	81.82
4	1	0.08	0.04	50.00
	2	0.07	0.07	100.00
5	1	0.10	0.07	70.00
	2	0.05	0.09	180.00
6	1	0.13	0.15	115.38
	2	0.11	0.06	54.55
Mean		0.09	0.08	92.27
SEM		0.01	0.01	8.48
n		6	6	6

t = 1.140
p-value = 0.281

APPENDIX XI

CALCULATION OF TOTAL CARNITINE UPTAKE IN KINETIC STUDIES

1. Concentration of unlabelled carnitine in the medium:

Three hundreds µl of Tyrode's solution (0.012 M) was added into 100 ml of Tyrode's solution. Four ml of this solution was used for the preparation of incubation medium containing labelled carnitine.

∴The conc. of unlabelled carnitine in the incubation medium;

= conc. of stock carnitine x dilution factor

or $= 0.012 \times \frac{0.3}{100}$ mmole/ml

$= 36$ µM

2. Concentration of labelled carnitine in the medium:

Five µl of ^{3}H-L-carnitine (specific activity = 82 Ci/mmole, 1 mCi/2 ml) was diluted into 4 ml of Tyrode's solution. One ml of this solution was used to incubate the epididymal tubule.

∴The conc. of labelled carnitine in the incubation medium;

$$= \frac{\text{conc. of radioactivity}}{\text{specific activity} \times 1{,}000} \times \frac{5}{1{,}000} \times \frac{1}{4} \text{ mmole/ml}$$

or $$= \frac{1}{2} \times \frac{1}{82} \times \frac{1}{1{,}000} \times \frac{5}{4{,}000} \text{ mmole/ml}$$

$= 0.0076 \ \mu M$

3. Total carnitine concentration in the medium:

= conc. of labelled carnitine + conc. of unlabelled carnitine

Since conc. of unlabelled carnitine >> Conc. of unlabelled carnitine

$\therefore$ Total carnitine conc. = Conc. of unlabelled carnitine

4. Total carnitine uptake into the tubule:

Since 1 Ci $= 2.22 \times 10^{12}$ DPM

Labelled carnitine in the tubule $= \frac{\text{DPM in tubule}}{\text{specific activity}} \times \frac{1}{2.22 \times 10^{12}}$ mmole

Total carnitine in the tubule = labelled carnitine in the tubule x correction factor

where correction factor $= \frac{\text{total carnitine in the medium}}{\text{labelled carnitine in the medium}}$

$= \frac{\text{unlabelled carnitine in the medium}}{\text{labelled carnitine in the medium}}$

BIOGRAPHY

NAME : Miss Wipaporn Phatvej

DATE OF BIRTH : 15 July 1973

PLACE OF BIRTH : Surat Thani, Thailand

INSTITUTIONS ATTENDED : Prince of Songkla University, 1992-1996: Bachelor of Science (Biology)

Mahidol University, 1996-2000: Master of Science (Toxicology)